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# Effects of contaminants on genetic patterns in aquatic organisms: a review

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**Abstract**

There is increasing awareness of the need to evaluate the effects of contaminants at the population level. Genetic techniques offer a powerful approach to assess contaminant-induced changes in populations. Yet studies to date are relatively few and not always carefully designed to maximize the utility inherent in this approach. We present a summary of contemporary genetic assessment methods and a review of published studies of genetic effects in field-exposed aquatic organisms. We discuss evaluations of genetic patterns that use genetic adaptation, allozyme variation, and molecular genetic (DNA) variation. Direct tests of genetic adaptation are very effective in establishing a concrete, and potentially deleterious population-level effect of contaminant exposure, but they are difficult to accomplish with most field-exposed organisms. Allozyme surveys are relatively simple and common, and may provide data that are suggestive of contaminant effects. However, these are rarely conclusive, primarily because few allozyme loci are variable and these few loci represent extremely small portions of the genome. Molecular genetic techniques have the potential to be very effective. But, there is a tendency to emphasize the power of the techniques, rather than the underlying causes of the molecular genetic patterns observed. The strength of the conclusions of each study varies widely, partially derived from variation in the strength of the techniques. We caution that all these approaches are greatly improved by careful experimental design that includes adequate numbers of reference and contaminated sites and sample size. In addition, careful exposure assessment is required, including site and tissue chemistry, biomarker responses, and measures of potentially deleterious effects, such as DNA damage, or reduced reproductive output or survival. 

*Keywords:* Genetic change; Natural populations; Aquatic; Contaminants

## 1. Introduction

### *1.1. Background*

The merits of evaluating genetic change in aquatic organisms as a response to contaminant exposure have been discussed for at least two decades [1,2]. Genetic approaches offer powerful tools for examining the current status of populations, inferring the history of population changes, and anticipating future population directions. Research on the effects of contaminant exposure on biological systems has historically focused on mechanisms of damage, and researchers have primarily studied test organisms exposed in laboratory settings. Recently, greater emphasis is being placed on studying resident populations to relate responses in natural populations to current

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understanding of mechanisms [3–5]. Population studies provide the necessary basis for understanding and predicting effects at higher levels of organization, including communities and landscape scales (e.g. [6,7]).

A limited number of approaches are available to study natural populations and to evaluate their status, history, and trajectory. Broadly, these include population monitoring in the field, modeling of population dynamics, and population genetic surveys. Long-term population monitoring studies continue to be preferred, where feasible, and have the potential to provide strong empirical indications of contaminant effects. Population dynamic models are increasingly applied to address contaminant effects (e.g. [8,9]), particularly in risk assessments. In an ideal setup, the advantage of a genetic approach is that it can be used to provide estimates of several life history characteristics, where possible, strengthened by periodic field surveys, that can be put into population genetic and population dynamic models to generate a broader picture, as well as predictions, regarding the population. The power of genetic data lies not only in the sophisticated techniques and vast databases available today, but in the fact that genetic information uniquely records the outcome of historical events and is the raw material that forms future populations. With the right experimental design, and a proper understanding of the data, genetic information can be an effective starting point for later targeted studies of populations, and a powerful complement to expensive and labor-intensive field surveys and population studies.

Researchers who have promoted the genetic approach have summarized and emphasized different points. Anderson et al. [4] and Shugart and Theodorakis [10] distinguish “genetic toxicology”, the study of genotoxic damage at the individual-level, from “genetic ecotoxicology”, which extends an understanding of genetic toxicological effects to the ecosystem-level through the study of populations. This concept has also been termed “eco-genotoxicology” [11] and “evolutionary toxicology” [6]. Several authors have pointed out the importance of studying population genetic changes as they may reflect phenomena that are unpredictable from the observed responses of several individuals. These categories of effects have been termed indirect effects, higher-level effects, and emergent effects (e.g. [6,12,13]). These changes may be the results of differential survivorship, health, or reproductive status that in turn, result in fitness differences among individuals or in reduced population size [6,14,15].

Frequently, discussion of genetic change has focused on potential fitness reduction resulting from local genetic adaptation [16,17] and on the predicted loss of population genetic variation from population reduction and drift [1,15]. Some have proposed an increase in mutations as a likely outcome of contaminant exposure, and have speculated on the long-term consequences. Many authors have modeled and argued convincingly that mutational load is an important risk to populations [15,18–20]. There is not, however, uniform agreement on the significance to populations of an increase in mutation rate, except possibly in small, closed populations [21]. Several authors argue that the introduction of non-lethal (nearly neutral) mutations is, or could be considered tantamount to increasing the overall genetic variability of a population [11,15]. An intermediate hypothesis proposes that mutations at loci with major adaptive effect are more threatening than those at certain quantitative trait loci with lesser effect [11,22]. Today, research can rapidly move beyond hypotheses and models. By combining field tests of molecular genetic change with a variety of field and laboratory experiments, investigators can test for mechanisms, study inter-taxonomic patterns, and consider alternate explanations for phenomena observed in the field [4,7,23,24].

The concept that genetic patterns within populations may be altered by exposure to contaminants is not novel. For decades, fisheries biologists as well as numerous researchers of agricultural pests have noted population declines followed by adaptation. Despite these observations, few studies were designed to document such changes in non-target species, or to study such phenomena more explicitly by testing for tolerance across several generations of unexposed offspring (see reviews in [23,25]). The widespread use of allozyme genetic techniques in the 1980s generated a handful of studies directed at documenting these changes in resident aquatic species. However, a large array of extremely powerful molecular genetic techniques has become available in the last decade and made

possible a wider range of approaches to address these phenomena.

Ecotoxicologists are now converging on a broader, more context-driven understanding of the effects of contamination on populations and ecosystems. It follows that a body of data regarding both population genetic patterns in contaminant-exposed populations, and variation at single loci related to detoxication and biological effect will emerge [4,15]. Working in parallel, conservation geneticists are concerned with the maintenance of population- and species-level genetic integrity as necessary components of fitness and the long-term survival of populations and species (e.g. [26]). Ultimately, the links between conservation genetics and genetic ecotoxicology will provide powerful tools to address the environmental effects of contaminants [6,15], and to understand this often-overlooked component of anthropogenic threats to the conservation of wildlife and the environment [27].

### *1.2. Approach*

Many recent papers, cited above, have discussed the importance of genetic assessment to evaluating contaminant effects in wild populations. Although aquatic toxicologists have examined aspects of genetic ecotoxicological effects in aquatic organisms for decades [14,28–31], none has attempted to assess the effectiveness of this approach by comprehensively and critically examining the literature in this field. Here, we present the results of such an assessment. We limit the scope of the review to studies of resident animal species whose primary or critical life stage or diet is aquatic, and whose exposure to contaminants occurred in its natural or naturalized habitat. We define "genetic assessment" broadly to include all methods of directly and indirectly evaluating genetic content in individuals and populations. In this review, we have attempted to include every study published to date in English, Spanish, or French, that meets these criteria. Previous authors have proffered convincing arguments on the value of the approach, along with discussions of the population genetic and evolutionary theory underlying study design and the outcomes of several familiar examples to date. Greater recognition of the strengths and weaknesses of previous investigations of field-exposed populations could improve our ability to evaluate and remediate sublethal and population-level effects of contaminant exposure.

We base our critique on the philosophy that in order to understand effects in the field, organisms must be studied in the field. However, given the limitations to this approach, namely, the lack of controlled exposures, lack of uniformity of experimental individuals and treatments, and often, small sample number or limited tissue availability, we recognize that conclusions must be drawn based on a "weight-of-evidence" criterion. Weight-of-evidence is common in human, and recently, ecological epidemiology and risk assessment, and has been accepted in these fields [32,33]. Briefly, the approach combines patterns observed in the field with other data sets that are associated with or that determine mechanisms [34]. This evidence is used to test, refute, or corroborate various aspects of a hypothesis in order to ask whether there is sufficient evidence supporting, and lack of evidence refuting it.

Based on this criterion, and the limitations of field studies, we propose, a priori, a generalized ideal study design. The highest priority is to obtain an adequate sample size to provide sufficient power to detect differences in parameter estimates. The second priority is to account for the lack of uniformity of in situ "treatments" by including several sites for each treatment type. Third, in order to convincingly assign treatment effects, the study must incorporate site or tissue chemistry, along with other appropriate measures, such as biomarker responses, to estimate contaminant exposure. Finally, the biggest, but possibly most subtle risk of field studies is overlooking confounding factors, that is alternate hypotheses that may explain observed patterns. Study design and data analysis must consider and test for effects of alternate environmental and biological explanations for the data collected (see [12]). Tables are presented that summarize the basic components of each study that examines genetic effects of contaminant exposure in field-exposed animals by testing for: genetic adaptation (Table 1), genetic change via allozyme surveys (Table 2), and genetic change via molecular genetic assessment (Table 3). Specifically, tables list: the organisms studied, the endpoints, the general class of contaminants in question, the outcome of endpoint comparisons, the exposure documentation, the outcome of alternative hypothesis testing, and the study reference.

Table 1
Tests of genetic adaptation in field-exposed animals[a]

| Species | Class of contaminants | Endpoints | Significant difference[b] | Exposure assessed[c] | Multigenerational demonstration or correlating evidence | Reference |
|---|---|---|---|---|---|---|
| Mosquitofish (*Gambusia affinis*) | Organochlorine insecticides in agricultural runoff | $F_1$ tolerance | Some insecticides$^+$ | + | 0 | Boyd and Ferguson [70] |
| Mosquitofish (*G. affinis*) | Organochlorine insecticides in agricultural runoff | $F_1$ tolerance | Toxaphene$^+$ | + | 0 | Andreasen [71] |
| Oligochaete (*Limnodrilus hoffmeisteri*) | Metal contaminated water | $F_1$ tolerance | Metal tolerance$^+$ | + | Lab tests for multigeneration resistance induction$^+$ | Klerks and Levinton [72] |
| Isopod (*Asellus aquaticus*) | Mine-tailing runoff | $F_1$ juvenile survivorship | All$^+$ | 0 | Growth$^+$, fecundity$^+$, weight$^+$ | Maltby [74] |
| Killifish (*Fundulus heteroclitus*) | Industrial runoff (PCB, PCDD, F, PAH) | $F_1$ tolerance | Survival$^+$ | + | *CYP1A1* induction$^+$ | Elskus et al. [76] |
| Mummichogs (*F. heteroclitus*) | Industrial runoff (PCB) | $F_1$ and $F_2$ tolerances | Survival$^+$ | + | EROD$^+$, $F_1$ site hybrid response$^+$ | Nacci et al. [73] |

[a] $F_1$: first generation offspring; $F_2$: second generation offspring.
[b] A significant difference was observed between treatments at all, or at specified endpoints (+).
[c] A significant difference was observed between/among sites (+); exposure was not tested (0).

Table 2
Tests of allozyme genetic variation in field-exposed animals[a]

| Species | Genetic endpoints | Type of contaminant | Significant difference[b] | Field exposure assessed[c] | Alternate tests[d] | Reference |
|---|---|---|---|---|---|---|
| Mosquito (*Culex pipiens*) | A, G, HW | Organophosphate treatments | $A^-$ | 0 | $TTD^-$, enzyme inhibition in vitro$^+$ | Pasteur and Sinègre [100] |
| Acorn barnacle (*Balanus amphitrite*) | A, G, H, HW | Industrial pollution | $A^\pm$ (some loci), $HW^+$ (some loci) | $(R)^+$ | 0 | Nevo [79] |
| Mussel (*Mytilus* sp.) | A, G, HW, H | Pollution gradient | $A^-$, $G^-$ and $H^-$, $HW^+$ (pooled loci) | $(R)^+$ | 0 | Battaglia et al. [112] |
| Gastropod (*Monodonta turbinata*) shrimp (*Palaemon elegans*) | A, G | Industrial pollution | $A^-$ | $Hg^+$ (R) some sites | Tolerance × $G^+$ (one shrimp and one gastropod) genotype at one locus | Nevo et al. [117] |
| Gastropods (*Littorina punctata*, *L. neritoides*) | A | Industrial pollution | $A^+$ (two loci) | + | Tolerance × $G^+$ | Nevo et al. [93] |
| Central stoneroller (*Campostoma anomalum*) | A, G | Uranium process runoff | $A^+$ (one locus), $G^+$ (one locus) | $(R)^+$ | Sensitivity to Cu correlated to one allele$^+$ | Gillespie and Guttman [91] |
| Mosquitofish (*G. affinis*) | A, G, H | Pesticide contamination | $G^-$, $A^-$ | + | Tolerance × $G^\pm$ | Hughes et al. [118] |
| Soldier crab (*Mictyris longicarpus*) semaphore crab (*Heloecius cordiformis*) | A, G, HW | Organophosphate runoff | $A^\pm$, $G^\pm$, $HW^+$ | 0 | Tolerance × $G^-$ | Mortimer and Hughes [95] |
| Barnacle (*B. amphitrite*) | A, G, HW | Chemical and thermal pollution | $G^+$ | $(R)^+$ | Difference in G juvenile versus adults$^+$ | Patarnello et al. [94] |
| Central mudminnow (*Umbra limi*) | A, G, H, S | Acid contamination | $G^+$ (some loci), $H^+$ (some loci) | + | Tolerance$^-$ | Kopp et al. [96] |
| Virginia river chubs (*Gila seminuda*) | A, G, HW | Rotenone-dosed streams | $A^+$ (two loci), $G^+$ | 0 | Pre- versus post-poisoned populations$^+$ | Demarais et al. [102] |
| Spotfin shiners (*Notropis spilopterus*) | A, G | Steel production plant effluent | $A^-$, $G^-$ | 0 | 0 | Gillespie and Guttman [87] |
| Mosquitofish (*G. holbrooki*) | A, G, P, H, HW | Mercury-contaminated canals | $A^+$, $P^-$ | 0 | TTD × one allele$^+$ | Heagler et al. [119] |
| Mosquitofish (*G. holbrooki*) planorbid snail (*Hellisoma trivolvis*) | A, G, HW, H | Coal power plant runoff | $A^\pm$, $G^\pm$, $HW^+$ | $(R)^+$ | Other studies$^+$, habitat$^-$ | Benton et al. [101] |
| Gastropod (*Chilina dombeyana*) | A, H, HW | Polluted estuary | $A^+$ | 0 | 0 | Bisol et al. [138] |
| Mosquitofish (*G. holbrooki*) | A, G, P, H, HW | Uranium production waste | $A^\pm$, $G^\pm$, $P^\pm$, $H^\pm$, $HW^\pm$ | $(R)^+$ | $TTD^+$ | Keklak et al. [113] |
| Central mudminnow (*U. limi*), yellow perch (*Perca flavescens*), blacknose dace (*Rhinichthys atratulus*), common shiner (*Luxilus cornutus*), creek chub (*Semotilus atromaculatus*) | A, G, P, H, HW, S | Acid deposition sites | $A^-$, $G^-$, $H^-$, $P^-$, $S^-$, $HW^-$ | $(R)^+$ some sites | 0 | Kopp et al. [97] |
| Central stonerollers (*C. anomalum*), bluntnose minnows (*P. notatus*) | A, G, H, D, HLWI | Varying water quality | $A^\pm$, $G^\pm$, $HLWI^\pm$, $H^-$ | 0 | IBI with $HLWI^+$, $ICI^-$ | Foré et al. [98,120] |
| Mosquitofish (*G. affinis*), bluntnose minnow (*P. notatus*), blackstriped topminnow (*F. notatus*) | A, G, HW, homogeneity | Mine tailing runoff | $A^+$, G pooled$^+$ | + | 0 | Roark and Brown [92] |
| Midge (*Chironomus plumosus*) | A, G, HW, F, D | Mercury gradient | – | $(R)^+$ | + | Woodward et al. [103] |
| Darter gobi (*Gogionellus boleosoma*) | P, H | PAH contaminated marsh | $P^-$, $H^-$ | $(R)^+$ | Resistance$^-$ | Klerks et al. [139] |
| Blackfly (*Simulium equinum*) | A, G | Agricultural runoff | $A^-$, $G^-$ | 0 | Esterase activity$^+$ | Parker and Callaghan [140] |
| Mayfly (*Isonychia bicolor*) | A, G, HW, G-test of independence | Mercury gradient | $A^\pm$, $G^\pm$ | 0 | TTD × $G^+$ (one locus), temporal A, G patterns$^-$ | Snyder and Hendricks [99] |
| Mummichog (*F. heteroclitus*) | A, HW, F | Bleached kraft pulp mill runoff | – | $(R)^+$ | $FA^-$ | Kirchhoff et al. [141] |
| Chub (*Leuciscus cephalus*), roach (*Rutilus rutilus*) | H, P, F, D | Hydroelectric, nuclear, and industrial plant discharge | $D^-$, $P^-$, $H^-$, $F^+$ | $(R)^+$ | $EROD^+$ (R), $DSB^+$ (R), geographic distance$^+$, river features$^+$ | Laroche et al. [142] |

[a] A: allele frequency; G: genotype frequency; H: heterozygosity; %P: percent polymorphism; HW: test of deviation from Hardy–Weinberg equilibrium; S: similarity; D: genetic distance; F: fixation indices; HLWI: heterozygous loci within individuals; TTD: time-to-death; DSB: DNA strand break analysis; ICI: invertebrate community index; IBI: index of biotic integrity; FA: fluctuating asymmetry.

[b] A significant difference was observed between treatments at all, or at specified endpoints (+); no significant difference was observed between treatments (−); results were inconsistent at specified endpoints (±).

[c] A significant difference in exposure was observed between/among sites (+); no significant difference was observed between/among sites (−); exposure was not tested (0); exposure was documented by reference to published studies performed by other investigators (R).

[d] Results of other tests or hypotheses corroborate the primary hypothesis(es) (+); results of other tests or hypotheses do not corroborate the primary hypothesis(es) (−); results were inconsistent (±); no alternate hypotheses were considered or no other tests were performed (0); alternate hypotheses or tests were documented by reference to other published studies (R).

Table 3
Tests of molecular genetic patterns in field-exposed animals[a]

| Species | Genetic endpoints | Data type | Type of contaminants | Significant difference[b] | Field exposure assessed[c] | Alternate tests[d] | Reference |
|---|---|---|---|---|---|---|---|
| Atlantic tomcod (*Microgadus tomcod*) | v-*abl* oncogene mutations, mtDNA variation | RFLP, DNA–DNA hybridization | Urban, industrial runoff | mtDNA$^{-}$ RFLP$^{-}$, mutations$^{+}$ | + | Tumors$^{+}$ | Wirgin et al. [104] |
| Atlantic tomcod (*M. tomcod*) | Alleles in *CYP1A1* gene | RFLP | Urban, industrial runoff | RFLP$^{+}$ | + | Tumors associated with the population, not allele$^{+}$ | Wirgin et al. [105] |
| Brown bullhead (*Amerus nebulosus*) | mtDNA variation | RFLP | Urban, industrial runoff | $h^{+}$ | + | 0 | Murdoch and Hebert [121] |
| Atlantic tomcod (*M. tomcod*) | Alleles in *CYP1A1* gene | Sequencing | Urban, industrial runoff | Allele difference$^{+}$ | + | Tumors associated with the population, not allele$^{+}$ | Roy et al. [106] |
| Harpacticoids (five species) | *h* | RFLP | Oil and gas production platform runoff | $h^{+}$ | + | Factored out population size effect$^{+}$ | Street and Montagna [122] and Street et al. [123] |
| Herring gulls (*Larus argentatus*) | B, novel bands | DNA fingerprints | Urban, industrial runoff | Novel bands$^{-}$ | + | Parent/offspring tested$^{+}$ | Yauk and Quinn [128] |
| Mosquitofish (*G. affinis*) | Average no. of bands/individual, BF, S, *n*, allozyme D, H, P, G | RAPD allozymes | Radionuclide-contaminated sites | Novel bands$^{-}$, overall variability$^{+}$, D$^{+}$ | + | Fecundity × G$^{\pm}$, CIB$^{\pm}$ selection coefficient × CIB$^{\pm}$ | Theodorakis and Shugart [107] |
| Mayfly (*Baetis tricaudatis*) | No. and frequency of haplotypes, DI, D | mtDNA sequence variation, SSCP | Metals, mine site drainage | D$^{+}$, DI$^{+}$ | + | Population density$^{+}$, stream features$^{+}$ | Beaty et al. [47] |
| Redbreast sunfish (*Lepomis auritus*) | B, *n*, D, NJ | RAPD | Radionuclide-contaminated sites mixed industrial contamination | Pooled endpoints$^{+}$, D$^{+}$, NJ clusters$^{-}$ | + | EROD$^{+}$, DSB$^{+}$ | Nadig and Adams [124] |
| Central stoneroller (*C. anomalum*) | S | RAPD | Agricultural discharge | Genetic diversity$^{-}$ | + | IBI$^{-}$, QHEI$^{-}$, BAP and NAPH metabolites$^{-}$ | Silbiger et al. [143] |
| Western mosquitofish (*G. affinis*) | B, G | RAPD, allozyme | Radionuclide runoff | B$^{\pm}$ | + | DSB$^{+}$, CIB$^{\pm}$ | Theodorakis and Shugart [109] |
| Eastern mosquitofish (*G. holbrooki*) | B, S | RAPD, southern blot | Radionuclide runoff | B$^{\pm}$, S$^{-}$ | + | Band homology$^{+}$, DSB in lab exposure$^{+}$ | Theodorakis et al. [110] |
| Brown bullhead (*A. nebulosus*) | No. of TBHs (from haplotype diversity and nucleotide diversity) | mtDNA sequence variation | Urban and industrial mixed contamination | TBH$^{-}$ | + | Alternate explanations$^{+}$ | Chen and Hebert [64] |
| Rusty crayfish (*Orconectes rusticus*) | Band-sharing fraction (f) | RAPD | Mixed agricultural, municipal, and industrial runoff | S$^{+}$ | − | IBI$^{-}$, ICI$^{-}$ | Krane et al. [125] |
| Western mosquitofish (*G. affinis*) | D | RAPD | Radionuclide runoff | D$^{+}$ | + | Percent survival × CIBs$^{+}$, DSB$^{-}$ | Theodorakis et al. [111] |
| Copepods (*Microarthridion littorale*) | F, D | mtDNA cytochrome B sequences from gtb | PAH and metal contaminated sites | D$^{-}$, F$^{+}$ | + | Survival$^{-}$, reproductive output$^{-}$ | Kovatch et al. [144] |
| Bay mussel (*Mytilus galloprovincialis*), acorn barnacle (*B. glandula*) | Diversity, rarefaction, S | RAPD | Hydroelectric, nuclear, and industrial plant discharge | Diversity$^{+}$, rarefaction$^{+}$, S$^{+}$ | + | Alternative explanations$^{-}$ | Ma et al. [126] |

[a] mtDNA: mitochondrial DNA; A: allele frequency; G: genotype frequency; *h*: haplotype diversity; D: genetic distance; S: similarity; F: fixation indices; B: bandsharing index; BF: band frequencies; *n*: nucleon diversity; DI: diversity index; RFLP: restriction fragment length polymorphism; RAPD: randomly amplified polymorphic DNA; AFLP: amplified fragment length polymorphism; DSB: DNA strand break analysis; ICI: invertebrate community index; IBI: index of biotic integrity; CIB: contaminant indicative bands.

[b] A significant difference was observed between treatments at all, or at specified endpoints (−); no significant difference was observed between treatments (−); results were inconsistent at specified endpoints (±).

[c] A significant difference in exposure was observed between/among sites (+); no significant difference was observed between/among sites (−); exposure was not tested (0); exposure was documented by reference to published studies performed by other investigators (R).

[d] Results of other tests or hypotheses corroborate the primary hypothesis(es) (+); results of other tests or hypotheses do not corroborate the primary hypothesis(es) (−); results were inconsistent (±); no alternate hypotheses were considered or no other tests were performed (0); alternate hypotheses or tests were documented by reference to other published studies (R).

## 2. Mechanisms of genetic change in populations

### *2.1. Genetic change as a genotoxic syndrome*

Changes in genetic patterns, including altered genotype frequencies and reduction in genetic variation in populations, have been recognized as a type of genotoxic syndrome. Mechanisms have been explored to link exposure to genotoxic contaminants with genetic consequences [4]. This syndrome may result from exposure to all classes of contaminants, not only genotoxic substances. Both direct genetic changes (i.e. mutation) and changes in population genetic patterns have been demonstrated to occur in a variety of laboratory studies. New applications of molecular techniques permit more thorough testing of the hypothesis that change in population genetic patterns represents damage and, is thus, an important genotoxic syndrome in natural populations.

### *2.2. Shifts in population genetic patterns*

Population-level shifts in genetic patterns are genetic changes at single or multiple loci that occur as a result of selection on specific alleles [35–39], or selection for multi-locus genotypes. Alternatively, they may occur as a consequence of contaminant-induced or stochastic changes in population structure, such as mortality in selected life stages, reductions in population size or changes in breeding periods [3,23,40]. Stochastic changes may also cause alterations in the degree of inbreeding, alterations in the level of gene flow, changes in age- or age-class structure, or induction of a bottleneck effect. Contaminant exposure can induce any of these changes one at a time, or in combination.

The extensive use of allozyme genetic techniques in past decades has exposed the high levels of genetic variation in natural populations, the widespread variance in amount of variation among species, and the correlation of genetic variation with environmental factors [1,41–43]. In particular, these latter observations have provided evidence in support of selectionist views of genetic variation. Allozyme loci were developed and widely used as neutral markers. Proponents of this idea contended that although the proteins examined were known to be relevant to basic physiological functions, variants were unlikely to be selectively different. Empirical observations have led to this position being challenged by various authors through the decades, and a resolution has not been achieved.

Consequently, discussions of genetic patterns observed in natural populations have overwhelmingly posed the question “how much variation reflects selection and how much reflects neutral processes?” [1,14,44–46]. This unresolved question affects the interpretation of population genetic patterns as they relate to contaminant exposure. Rapid selection against a completely susceptible genotype, for example, would quickly alter the genotype frequency of a population [18]. Population genetic change would be enhanced, in this case, by dramatic reduction in population size and subsequent genetic drift. Beaty et al. [47] have proposed an alternate scenario in which adaptation within an exposed population may itself create barriers to gene flow if unexposed populations are subjected to opposing selection. Slow selection could produce the same shifts in gene and genotype frequencies, confounded or mitigated by other population or contaminant processes, such as pulses in acute contaminant exposure or high migration rates. Further, evidence in the literature demonstrates that there can be a direct fitness cost to the evolution of resistance (selection) [15,16]. Collateral genetic changes may also occur that affect life-history traits directly. For example, resistant fish have been shown to be slow-growing and late-maturing [38,48]. It should be noted, however, that observed life-history differences in adapted populations could be related to the mechanism(s) of adaptation per se, rather than simply negative collateral consequences.

Scenarios such as these underscore the importance of obtaining a thorough understanding of both the life history and local biology of the organism in question when undertaking studies of population genetic effects. Regardless of the mechanism(s) involved, shifts in genotype frequency have the potential, through pleiotropy or linkage, to detrimentally affect life-history characteristics related to other, non-resistance-related loci [38] or to alter genetic variability [22,49]. Thus, potentially, these shifts may alter population viability and fitness (e.g. [50]). Molecular genetic or allozyme variation at unrelated coding loci, or at so-called neutral loci, would also be expected to reflect selection because of the hitchhiking effect, if

their position on the genome is proximate to adaptive loci [51]. Hitchhiking describes the process by which DNA sequences linked to selected genotypes will increase in frequency along with the adaptive genetic information. The increase in frequency of these unrelated but linked genes would tend to reduce overall genetic diversity [52].

Alternatively, chronic exposure to contaminants may cause physiological stress that leads to shortened life span, lower fecundity, or other life history alterations unrelated to the specific genotype of an individual. These life-history alterations may affect population genetic patterns in several predictable ways. Reduced survival or fecundity could lead to an overall reduction in genetic diversity, called a bottleneck effect [53]. Reduced population density may lead to altered migration patterns, altered breeding systems, or other unforeseen changes in the behavior of populations. These changes will also affect patterns of population genetic structure. Individuals with reduced genetic variability, particularly, low heterozygosity, are said to be inbred. They may suffer from inbreeding depression making them more susceptible to disease, the expression of deleterious recessives, and the effects of mutational load [54,55].

### *2.3. Direct genetic damage*

Genetic (DNA) alterations can occur as a consequence of exposure to genotoxic or mutagenic compounds. Mutations can occur in single genetic loci, or at many locations on the genome. Sites or regions especially prone to mutations, sometimes called mutation hot spots, result in specific base changes, or base changes clustered in certain areas of the genome [56,57]. For example, point mutations as a result of exposure to *N*-ethyl-*N*-nitrosurea or aflatoxin are known to occur within codons in important functional genes, such as the *p53* tumor suppressor gene [58,59]. Mutations of the *p53* gene, in turn, are linked to high incidence of cancer in exposed individuals, and these mutations are considered highly deleterious or lethal. Mutations in oncogenes have also been examined in field studies with bivalve molluscs, and these changes have been correlated with contaminant exposure and altered age class-structure [60]. Similarly, Roy et al. [61] exposed pink salmon (*Oncorhyncus gorbuscha*) embryos to crude oil and demonstrated mutations in the K-*ras* oncogene. Deleterious mutations, such as these, may cause local population reductions affecting random individuals, or increased mortality only in mutation-prone individuals. Either could result in shifts in population genetic patterns by bottleneck or selection effects.

Non-specific mutations that are not by themselves extremely deleterious or lethal can accumulate in the genome and potentially persist within a population. These "very slightly deleterious" mutations behave as neutral mutations because their selection coefficient is less than their deleterious effect [18,21]. These mutations are collectively called mutational load, and may eventually result in reduced fitness and reduced population size in some populations [62,63]. Mutational load is predicted to be enhanced in populations with low heterozygosity caused by either drift or inbreeding [54] and to be a significant risk to all populations [18,19]. Data have also supported the model that new neutral or nearly neutral mutations spread much more rapidly in small populations because of the effects of genetic drift, than they do in large populations [64]. These are examples of how direct genetic change resulting from contaminant exposure (increased mutational load) might interact with indirect genetic change (stochastic population effects). Bickham et al. [2] provide an excellent summary of the models underlying these hypotheses.

An alternative viewpoint regarding mutations has been put forth. If contaminants are mutagenic, increased mutation at multiple locations on the genome may eventually lead to detectable accumulations of genetic variation. The effects of this alteration could be deleterious (mutational load), as previously described, or, if non-lethal, have the potential to provide new, advantageous alleles to the populations [11,15]. Mutation is the source of new variation in biological systems [65], but it is not known how the accelerated effects of mutagenic compounds affects genetic variation on an evolutionary scale.

## 3. Genetic adaptation

### *3.1. Technique overview*

Genetic adaptation is the change in gene or genotype frequency in a population such that the population

is, on average, better suited for survival in the face of specific risks. Increased fitness of the adaptive alleles or the adaptive co-variation of gene complexes results in shifts in genotype frequencies with respect to those alleles as well as any genes that may be linked to them [38,66]. Adaptive genes confer a genetically-based resistance by coding for enhanced molecular, cellular, physiological or behavioral mechanisms for avoiding damage. Tolerance is a state of decreased responsiveness to a toxic effect as a result of prior exposure. Tolerance is alternately called resistance or acclimation, and arises by many possible mechanisms, including modification of gene expression or of structural components of existing genes, as well as, in some cases, by genetic adaptation. It is not always inherited through a specific allele or set of alleles [39]. Thus, tolerance is not synonymous with genetic adaptation, and would not, by itself, change the genetic structure of a population. Non-genetically-based tolerance is usually a response induced by chronic exposure of an individual over a period of time, or by exposure to maternal body burdens or to short-term mechanisms stimulated in the mother (e.g. [23]). The genetic basis for resistance can be tested directly with experiments in which populations are exposed to contaminants. Those that are not resistant are eliminated, tolerant individuals are bred, and $F_1$ and $F_2$ offspring are tested for resistance. If tolerance persists or increases in $F_1$ and $F_2$ generations, then the response is said to be genetic. Several other combinations of experiments and assays have been proposed as substitutes for direct breeding experiments, but results of such experiments are less conclusive than those obtained by direct breeding.

Genetic adaptation may also be inferred from molecular genetic variation associated with adaptively significant functional genes. The association is strengthened if coupled with direct physiological tests [12]. Alternately, allele fixation at multiple exposed sites may indicate their adaptive nature, or that of linked loci. Allozyme genotypes have been shown to correlate with individual tolerance induced in the laboratory in previously unexposed animals ([67,68]; see [33] for review), which has been used as evidence that such tolerance has a genetic basis. Observation of these patterns is inconclusive, but it is intriguing and worthy of further investigation.

### 3.2. Breeding experiments

Direct evidence that resistance has a genetic basis can best be demonstrated by examining the persistence of resistance in multiple generations subsequent to the exposed generations. Six field tests of genetic adaptation to contaminants in aquatic environments were found in the literature (Table 1).

Mosquitofish (*Gambusia affinis*) from two drainage ditches known to receive agricultural chemicals were demonstrated to be resistant to selected insecticides [69]. In a follow-up study, resistant fish were transferred to a clean pond and allowed to breed [70]. Offspring were tested for resistance to seven insecticides. Exposed and reference fish showed very different resistance levels for strobane and chlordane. Authors concluded that fish had been previously genetically adapted to related insecticides in the agricultural drains.

Andreasen [71] compared western mosquitofish (*G. affinis*) collected from agricultural drainage ditches with reference samples. The 96-h $LC_{50}$ for toxaphene in exposed fish was found to be more than 10 times that in reference fish. $F_1$ generation offspring were raised to adulthood and tested for tolerance to toxaphene. The $LC_{50}$ of exposed $F_1$ was 40 times greater than that in all reference $F_1$. This result confirms that resistance persisted at least one generation. $F_2$ offspring were not tested and no other test was performed to rule out maternal effects that may have contributed to tolerance in $F_1$ fish.

Klerks and Levinton [72] demonstrated resistance in a benthic oligochaete (*Limnodrilus hoffmeisteri*) to sediments contaminated with nickel and cadmium. Worms and sediment were collected from Foundry Cove, a battery factory wastewater site, and worms were found to be significantly more resistant to metal-rich sediment than those from a reference site in laboratory tolerance tests. Foundry Cove worms were cultured in control sediments, and their offspring were later tested for tolerance to a solution containing cadmium, nickel and cobalt in the same proportions found in Foundry Cove sediments. No significant difference was found between tolerance of field collected worms and that of their offspring. This result suggests a genetic component to resistance but does not rule out maternal effects. A selection experiment was set up with reference site survivors selected by

exposure to contaminated sediment over three generations. Control (non-selected experimental) lines were randomly subsampled so that each generation mimicked effects of inbreeding from the selection process. Overall, after three generations of selection, reference site worms showed a response equivalent to 66% of the response difference observed between Foundry Cove and reference populations in the field-adaption experiment. This latter experiment confirmed that adaptation to metals in these worms could be genetic and evolve quickly.

Nacci et al. [73] compared mummichogs (*Fundulus heteroclitus*) from heavily PCB-contaminated sites to those collected from reference sites for ethoxyresorufin-*o*-deethylase (EROD) response and survival. First and second generation offspring from field-collected fish showed a much lower EROD response than reference embryos, and significantly higher survival. Persistence of these responses for two generations is strong evidence for genetic adaptation. Authors further ruled out maternal effects by testing enzyme responses in hybrid offspring from bi-directional crosses between contaminated and reference parents. Hybrid lines showed EROD responses equal to each other and intermediate between contaminated and reference fish responses.

Using a different approach, Maltby [74] examined life-history measures in a freshwater isopod (*Asellus aquaticus*) to test the predictions of the Sibly and Calow model of genetic adaptation to contaminants. This model predicts that both the age-specific survivorship (S) of juveniles and the juvenile growth rate (G) would be reduced in the face of contaminant stress. In addition, the model predicts that populations subject to contaminants should show reduced fecundity but larger offspring; this variation in reproductive effort was expected to be genetic [75]. To test this, Maltby collected isopods from an upstream, reference location and a downstream contaminated portion, located below an effluent outfall of an old coal mine. Survivorship of juveniles ($S_j$), overall S, and G were significantly lower downstream than upstream. Females from the downstream site showed a significantly lower reproductive effort and significantly larger offspring than those upstream as predicted by the model. The genetic component of the changes was confirmed by finding consistent results in subsequent generations of isopods cultured in clean water in the laboratory for two years. The results of this study are interesting, and suggestive, given that the life-history shifts are in keeping with a life-history model of adaptive mechanisms. Nevertheless, the link between these traits and contaminant exposure, rather than pre-existing genetic or other ecologically-induced conditions, was not conclusively drawn.

Elskus et al. [76] collected killifish (*F. heteroclitus*) from PCB-contaminated areas and depurated them for 7.5 months. They showed an 80% reduction in body burdens of PCBs in these adults. Two related enzyme responses, cytochrome $P_{450}$1A1 (CYP1A1), and EROD were measured in males from contaminated and reference sites in response to test exposures after depuration. Reference fish showed high enzyme responses relative to controls, but fish from contaminated sites showed no CYP1A1 response at any dose. $F_1$ offspring were tested for CYP1A1 responses in multiple tissues. Offspring of reference individuals showed dose responses in four of five tissues at two ages, whereas no contaminated larvae showed enzyme responses. Even though $F_2$ offspring were not tested, these results are suggestive of a genetic adaptive response because of extensive depuration of parents. However, induced maternal responses could have affected offspring even in the absence of body burdens in the mothers. Furthermore, no fitness consequences of enzyme responses or lack thereof were demonstrated or are known to exist, making the link between the trait and the likelihood of a selective advantage speculative.

### 3.3. Summary

It is often assumed that tolerance observed in the field has a genetic basis, most likely because genetically-based resistance is often induced in laboratory organisms [68,77]. However, rarely in field studies has a genetic basis for tolerance been confirmed. Several of the few tests of genetic adaptation following field exposure that were found in the literature were effective in demonstrating the genetic component of resistance. It seems that because of the great difficulties of the approach, breeding experiments are rarely performed. This underscores the lack of data that validate assumptions about the genetic basis for resistance made in other studies. More field tests of genetic adaptation to contaminant exposures

would help to determine whether this phenomenon is common. The tests, however, are limited to species which can be bred in the laboratory, have short generation times, and which can be tested for toxicity [23]. Validation of principle through these tests, therefore, should be performed, where possible on testable species. Carefully planned experiments could elucidate, or at least implicate, the possible similar actions of contaminants on other species which are less amenable to laboratory experimentation.

## 4. Genetic patterns

### *4.1. Technique overview*

The two major categories of methods to assess genetic patterns directly are allozyme electrophoresis and DNA or molecular genetic techniques. Allozymes are enzymes (proteins) with different electrophoretic mobilities, encoded by different alleles (nucleotide variants) of single genetic loci [78]. Bands, visible upon staining, have differential electrophoretic mobilities and are used to designate different alleles. This technique is limited by the availability of sufficient and suitable tissues and by its very small, and finite representation of total genetic variation. In addition, since allozymes are proteins, alterations at the DNA-level that do not result in an amino acid substitution are not detected [79]. Finally, in some cases, bands scored as the same allele may actually represent multiple alleles. Thus, allozyme genetic data are very limited in scope, and generally an underestimate of the actual the DNA-level variation.

Molecular techniques allow evaluation of molecular-level (DNA and RNA) variation in populations. Most techniques use the polymerase chain reaction (PCR) to amplify short nucleotide sequences from small amounts of tissue. With DNA techniques, one can examine neutral markers [80], or patterns at coding loci (regions that are important to functional differences in important proteins or gene expression). An often-overlooked concept is that presumed neutral markers often screen the entire genome. A potentially important proportion of the loci scored will be coding, or closely linked to coding loci. Because each technique may use a different principle to sample the genome, the combination of information from the use of several techniques increases the power of the data, and, thus, may increase understanding of and confidence in results [2]. Several basic methods of generating data on DNA variation are mentioned below. Many other methods are available, but most rely on the same principles on which these techniques are based.

Restriction fragment length polymorphisms (RFLPs) are generated when single or multiple restriction enzymes are combined with mitochondrial or portions of nuclear DNA and allowed to cut. Restriction enzymes are also used in multi-locus DNA fingerprinting and restriction mapping (see [81]). The randomly amplified polymorphic DNA (RAPD) [82] and amplified fragment length polymorphism (AFLP) [83] techniques use several anonymous primers, created in different ways, that amplify, wherever there is a match throughout the DNA molecule. A large number of random fragments of DNA are amplified, each considered to be different loci (see [84], for a comparison of these methods). The principle of primer specificity is used to amplify many other kinds of loci, including microsatellites, introns, and targeted regions or genes for direct sequencing. DNA sequence variation in all amplified loci is inferred from differential band mobility during electrophoresis. Alternatively, PCR products may be directly sequenced. This is the most precise method, fraught with the fewest assumptions, but a large amount of labor and cost is required to examine very small portions of the genome [80].

Molecular genetic techniques also have the potential to detect new mutations directly ("mutation" is used here to connote heritable changes in DNA base sequences that are the result of direct alterations of DNA by xenobiotic chemicals). Point mutations can be detected by analysis of sequence deviations among individuals. Finally, band variants indicate mutations in restriction sites [85] or in primer recognition sequences [86].

The biggest difficulty in this approach is discriminating among potential sources of genetic change. In addition to contaminant-induced genetic change in one or several directions, genetic differences can result from many other, unrelated, variables [12]. Understanding the ways in which data are generated and the assumptions underlying the study design is critical to evaluating the approach in genetic ecotoxicology and to selecting the best genetic markers for genetic analysis [2].

### 4.2. Genetic data analysis

Molecular genetic data fall into one of three categories: sequence data, co-dominant allele data, or dominant allele data. Sequence data is the most informative, being both an absolute reflection of DNA patterns, and genealogical (i.e. reflecting historical lineage relationships). Co-dominant markers, in which both alleles at a locus can be scored (e.g. microsatellites, allozymes), are somewhat less informative. This is because, although DNA variation can be seen by different band mobilities, bands have greater potential for homoplasy (alleles that appear identical but have evolved from different ancestors) than do sequence data, and the data are not genealogical. Dominant loci allow only scoring of a band (locus) as being present or absent (e.g. AFLP, RAPD, DNA fingerprinting). These marker types are generally the least powerful because much information is lost when one does not know the nature of the absent allele (e.g. its size, whether it amplified). On the other hand, dominant allele techniques often generate many more data points (loci) than the others.

Dominant and co-dominant markers generate allele scores (presence or absence, or sizes, respectively) and a composite of alleles, called a genotype. Allele and genotype frequencies at individual loci are quantified (e.g. [87]) and used to estimate additional parameters based on population genetic models [88]. For example, the Hardy–Weinberg equilibrium model predicts certain genotype frequencies will be present in a population given specific starting allele frequencies, assuming, among other things, that no differential selection is acting on particular alleles or genotypes. Deviation from Hardy–Weinberg has been used as evidence that selection, such as might be caused by contaminants, is acting on a population (e.g. [89]). Given the many kinds of selection, however, that may each affect genetic patterns differently, investigators are cautioned against drawing strong conclusions from these simple deviations [65].

Estimates of population genetic parameters commonly calculated from genetic data provide information about several predictions described in the previous section. For example, allele frequency, allele number, and *F*-statistics (fixation indices), describe the genetic structure of populations. Mean heterozygosity ($\hat{H}$) and percent polymorphism (%P) are examples of estimators of overall genetic variability. As there are many mathematical models that vary in their assumptions and in the data required to use them, it is important to understand the details underlying the use of any estimator or statistical test to best interpret the data. Finally, each of these parameter estimations are merely descriptors, and by themselves, do not indicate anything about the potential effects of contaminant exposure on population genetic patterns. In order to address that question, experiments must be designed to look for genetic change. Because it is rarely possible to examine population genetic patterns before and after exposure, replicate exposed and reference populations are usually compared [12].

### 4.3. Alterations in population structure: selection or adaptation

Population genetic structure is defined as the pattern by which populations are subdivided into local breeding groups. It takes into account their sizes, the number of breeding members, the amount of gene flow, and related factors [90]. Investigators infer population structure from a variety of parameter estimates, for example, fixation indices and genetic distance values. A number of allozyme studies have demonstrated alterations in population structure (Table 2). For example, Gillespie and Guttman [91] examined allozyme variation at three polymorphic loci in the central stoneroller (*Campostoma anomalum*) from a site associated with uranium processing, Paddy's Run, and an uncontaminated stream in Ohio. Allele frequencies at one locus and two associated genotype frequencies were significantly lower in the pooled contaminated samples than at the reference site. Allele and genotype frequencies at one of the four loci examined by Gillespie and Guttman [87] in spotfin shiners (*Notropis spilopterus*) differed significantly among the locations compared along Dick's Creek, Ohio. Genotype and allele frequencies varied significantly between Willow Creek, Cherokee County, Kansas, subject to mine-tailing runoff, and a reference creek, for three species examined, bluntnose minnow (*Pimephales notatus*), mosquitofish (*G. affinis*), and blackstriped topminnow (*F. notatus*) [92]. Such differences were also seen in a number of other studies [93–99]. These data alone, however, were not sufficient evidence of contaminant effects because population structure differences

may reflect population subdivision caused by other factors.

There have been a number of interesting studies that provided additional data or comparisons as part of the data interpretation step. These show how examination of various alternative hypotheses can strengthen any conclusions drawn from the link between genetic patterns and contaminant exposure. Pasteur and Sinègre [100] examined allele and genotype frequencies at several esterase loci in larval mosquitoes (*Culex pipiens*) from 12 Dursban-contaminated locations. Dursban-exposed populations exhibited an allele at one locus that was not present in any previous study of esterase variation, providing strong evidence of allele fixation correlated with resistance. Similarly, Benton et al. [101] found near-fixation of alleles in planorbid snails (*Helisoma trivolvis*) and the eastern mosquitofish (*G. holbrooki*), contaminated with coal-fired power-plant runoff, a pattern that differed significantly from allele frequencies at the reference location. Demarais et al. [102] measured genetic changes associated with rotenone application in Virginia river chubs (*Gila seminuda*). Allele frequencies at two loci differed significantly among populations after, but not before poisoning. These authors concluded that genetic change was not the result of selection by rotenone, because the post-exposure allele frequencies did not resemble each other. They reasoned that if exposure to rotenone had caused the shift in allele frequencies, then the resultant allele frequencies should have been the same; thus, allele frequency shifts must have been caused by some other factor. Patarnello et al. [94] compared juvenile with adult barnacles (*Balanus amphitrite*) taken from three locations in the lagoon of Venice, Italy. They found that allele and genotype frequencies did not differ among juvenile populations, but did differ significantly among adults of the most contaminated location and the other two locations, supporting the hypothesis that post-settlement selection on barnacles occurred in this location. Finally, Woodward et al. [103] transect-sampled larval midges (*Chironomus plumosus*) within sample sites and along a sediment mercury gradient in Clear lake, California. *F*-statistics were calculated on within-sample data in order to be able to factor out patterns of natural population subdivision when making between-site comparisons. After factoring out within-population structure, no significant effects of contaminants were found in this study. The authors considered all likely causes of the patterns they observed and found more likely explanations than selection by contaminants. Efforts, such as these, to consider the data carefully in light of potential alternative explanations for significantly different genetic structure among sample populations have resulted in more critical analyses of contaminant effects.

Using molecular genetic methods (Table 3), Wirgin et al. [104] compared Atlantic tomcod (*Microgadus tomcod*) from the contaminated Hudson river, New York, to tomcod from two reference locations in Maine. Earlier studies found liver tumors in 90% of 2-year-old and 50% of 1-year-old tomcod from the Hudson river, and in fewer than 5% of tomcod from Maine. Restriction site polymorphisms and RFLPs were measured in nuclear DNA in the c-*abl* oncogene. Allele and genotype frequencies at one of the three oncogene alleles were significantly different in Hudson river from Maine fish. Composite genotypes of the polymorphic domains showed that one genotype was found in 40% of the fish at the Hudson river location and only in a single fish from Maine. In a parallel survey, mtDNA length variants between Hudson river and Maine tomcod were not significantly different from which the authors inferred that they were derived from the same stock. The mtDNA is maternally inherited, and thus, does not recombine. However, it evolves much more quickly than nuclear DNA. Thus, by comparing variation at the nuclear locus, the c-*abl* oncogene, with the mitochondrial locus, authors concluded that the accumulation of polymorphisms in the c-*abl* oncogene and the establishment of frequency differences between populations must have occurred recently. It was proposed that this difference might be attributed to contaminant levels found in the Hudson river. The comparison of two molecular methods permitted more extensive consideration of the primary and alternative hypotheses.

Wirgin et al. [105] later compared genetic polymorphisms at the cytochrome $P_{450}1A$ (*CYP1A*) locus of the same fish. RFLPs were detected in the *CYP1A* gene and the corresponding messenger RNA (mRNA) in 10% of the fish from the Hudson river but not from Maine. The polymorphism was detected using five different restriction enzymes, evidence that it did not represent merely a single-base substitution.

The authors found a relatively small percentage of tomcod overall with the polymorphism; this was considered evidence that the deviant *CYP1A* form did not represent a second *CYP1A* gene locus but a true genetic polymorphism at one locus associated only with Hudson river tomcod. Roy et al. [106] further characterized these genetic polymorphisms. The authors sequenced the alleles and found that the variant allele had a 606 bp deletion in the 7th exon of the untranslated region of the cDNA. This variant was not found in further sampling from the same two Maine rivers sampled by Wirgin et al. [105] and in three other rivers with lesser degrees of contamination than the Hudson river. The authors considered the possibility that stochastic processes unrelated to xenobiotic exposure caused the elevated frequency of the *CYP1A* variant allele in Hudson river fish. However, when found, the variant allele was only observed in the heterozygous state. The absence of homozygotes for the allele was considered to be evidence that selection against the allele was operating in the Hudson river population. Further evidence that the variant allele was not a pseudogene included the observations that it was transcribed, its sequence was identical to common alleles, and DNA concentrations in common and variant alleles in a heterozygote were equimolar. No functional explanation was offered for the relationship between this deletion in an untranslated region and neoplasias. The authors proposed several further lines of investigation to pursue this. It should be noted, however, that given the sample sizes tested in reference populations ($n = 24$–48), the power would have been very low to detect a genotype present in only 10% of individuals.

In a series of papers, Theodorakis, Shugart, and others, examined molecular genetic change using RAPDs, allozyme variation, and a genotoxicity assay, DNA strand break analysis, to assess the effects of radionuclide exposure on mosquitofish (*G. affinis* and *G. holbrooki*) in ponds in Tennessee. Western mosquitofish, *G. affinis*, from two contaminated and two reference sites were examined using 12 allozyme loci and 15 polymorphic RAPD primers [107]. Allozyme $\hat{H}$ and %P, and RAPD dissimilarity index and nucleon diversity values were found to be higher at contaminated than at reference sites. Genetic distance values calculated from both the RAPD data and from allozyme data at one of eight polymorphic loci, the nucleoside phosphorylase (NP) locus, indicated that contaminated populations are more similar to each other than to any of the reference sites. Each of these measures indicated that contaminated populations were genetically divergent from reference populations. However, it is important to note that most genetic distance measures tend to show less genetic distance between populations with higher within-population genetic diversity, so this latter result may be a mathematical artifact (e.g. [108]). These authors also used three contaminant-indicative measures to test for correlation with a fitness parameter, fecundity. The number of heterozygotes at the NP locus was greater than 10 times the number at other loci. Several specific RAPD bands and a high average number of bands were only found in exposed populations. NP-heterozygotes, some of the so-called contaminant-indicative bands (CIBs), and the average number of RAPD bands, all correlated positively to fecundity in these fish. However, genetic patterns were not consistent across all or most of the loci examined in this study.

Theodorakis and Shugart [109] then correlated these CIBs with incidence of DNA strand breaks induced in these fish in laboratory exposures. The analysis showed some association between CIBs and reduced incidence of strand breaks, implying a possible selective advantage correlated with the possession of CIBs. In order to test the hypothesis that the CIBs were related to adaptation to radionuclide exposure, authors surveyed for the presence of these bands in another mosquitofish species (*G. holbrooki*) from a separate radionuclide-contaminated drainage [110]. They found a greater frequency of three of the CIBs in fish from the contaminated sites. They followed this survey with a Southern blot analysis of these bands and those identified in *G. affinis*, and found them to be homologous. Authors inferred from this result that these CIBs were very likely to be selectively advantageous loci within the two species of mosquitofish. In a final study to date, Theodorakis et al. [111] caged western mosquitofish caught in reference sites in a radionuclide-contaminated pond and measured survival, RAPD genotype, and DNA strand breaks. They found a higher frequency of CIBs in survivors and fewer DNA strand breaks associated with fish possessing the CIBs. Although each step in this series produced partial or weak correlations, overall the trends are suggestive of contaminant effects on genetic patterns. Authors point out design weaknesses

and propose that the next step is to identify the CIBs at the molecular-level.

### 4.4. Genetic variability: bottleneck

Significant alterations in overall genetic variability were associated with contaminant exposure in some of the previously described allozyme studies, and several others (Tables 1 and 2). For example, significant deviations from Hardy–Weinberg expectations were caused by heterozygote deficiencies at contaminated locations and not at reference locations in Battaglia et al. [112] and Benton et al. [101], although these deviations were not necessarily consistent for all contaminated locations tested. Keklak et al. [113] found nearly twice the %P and $\hat{H}$ values at the reference region as at the contaminated region. Patarnello et al. [94] showed significantly higher proportions of multi-homozygotes at a contaminated site than at a reference site. These results are expected by a simple bottleneck model in which populations are reduced by contaminant stress.

Roark and Brown [92] found a significantly higher proportion of heterozygous individuals at the contaminated site, which is counter to the predictions of a bottleneck model, or to a model of selection on specific alleles at the loci examined. There are, however, several possible explanations for this observation. Selection for specific alleles at other, presumed adaptive, loci could result in gene frequency alterations at tested allozyme loci because of linkage or pleiotropy. Alternatively, heterosis, or a heterozygote advantage [65], could provide a selective advantage for these individuals. Selection against susceptible or for robust individuals at the contaminated site might result in populations with high $\hat{H}$ and %P. Few studies have actually tested these kinds of hypotheses in the field, although some have done so in the laboratory. Genetic variability was correlated to tolerance in the laboratory by Kopp et al. [96] who found significantly higher numbers of heterozygous loci per individual (HLWI) in the most tolerant fish. This supports the theoretical link between heterozygosity and fitness (e.g. [114,115]). The approach of calculating a number of genetic indices and comparing results to independent tests of fitness or other biological differences has also been used, for example to address conservation questions using genetics (e.g. [116]).

Several field studies couple measures of genetic variability with limited tolerance testing. Nevo et al. [117] compared allozyme genotype frequencies at a contaminated site in the Israeli Mediterranean to samples pooled from seven reference locations. At the contaminated site, they found significantly lower frequencies of two allozyme alleles shown in a laboratory study to be associated with mercury sensitivity in the gastropods sampled (*Littorina punctata* and *L. neritoides*). Gillespie and Guttman [91] similarly found significant reduction in certain alleles and genotypes in central stonerollers exposed to uranium; these alleles and genotypes correlated with increased sensitivity to copper sulfate, demonstrated in the laboratory. Pasteur and Sinègre [100], Hughes et al. [118], Heagler et al. [119], Benton et al. [101], and Snyder and Hendricks [99] documented similar patterns of allele or genotype frequency that correlate with demonstrated sensitivity.

Foré et al. [98,120] correlated allozyme variation with ecological indices of habitat quality and found significant correlation between the mean proportion of HLWI and extremely low- or high-site rankings using the Index of Biotic Integrity. The prediction of a simple selection hypothesis would be supported by correlation between HLWI and a high ranking if selection for adaptive allozyme alleles resulted in low overall genetic variability. However, superior adaptive qualities associated with high, as discussed above, could explain the association of HLWI with low-site rankings. This inconsistency makes the results of this study inconclusive.

Murdoch and Hebert [121] measured mtDNA sequence variation in exposed brown bullhead (*Ameiurus nebulosus*). Sequence variation, as measured with RFLPs generated from 16 different restriction enzymes, was examined in fish from 9 sites subject to a range of sediment contamination. Composite genotypes, called haplotypes, were generated from the resulting mtDNA fragment patterns. The four populations with the lowest within-population haplotype diversity estimates ($h$) were from highly contaminated locations. Populations from one of the five highly polluted sites showed an intermediate diversity estimate. In pairwise comparisons, populations from contaminated sites showed lower haplotype diversity than any of the reference sites. Populations

from contaminated sites were dominated by one haplotype found in 50–100% of the individuals, whereas the most frequent haplotype in reference sites were present in 15–44% of the individuals. A similar comparison of mtDNA haplotype diversity in marine harpacticoids was made between animals collected within 50 m and greater than 3 km of offshore oil and gas production platforms in the Gulf of Mexico. Three pairs of near and far platform populations were compared at four enzyme loci. Populations sampled near platforms showed lower haplotype diversity (*h*) than those sampled far from them [122]. In a later paper, Street et al. [123] tested statistically for possible confounding effects of population size on the levels of genetic variation and demonstrated that the difference in *h* remained after factoring out the effect of population size. Beaty et al. [47] collected mayflies, *Baetis tricaudatus*, from several sites at different distances downstream from a mine site, in the Arkansas river. They identified sequence variants in a mtDNA region using single stranded conformational polymorphisms (SSCPs), and sequenced each allele. Haplotypes from contaminated populations had reduced genetic variation (Shannon diversity measure, and Nei's genetic distance). The authors tested for possible confounding effects of geographic features and found no correlation. The hypothesis of an association of reduced genetic variation with contaminant exposure, therefore, was supported in each study examining mtDNA variation, although the studies were not designed to discriminate between genetic drift and selection as a cause. The trend in population genetic work, however, is away from using mitochondrial markers except for generating specific kinds of data (e.g. matrilineal, non-recombinant). Nuclear loci have been identified that show levels of variation comparable or higher than the high levels that made mtDNA markers so popular. In addition, because of the recombination in nuclear genes, each nuclear locus used is considered to be independent, whereas, the mtDNA genome is a single locus.

Three studies used the RAPD technique to assess nuclear DNA variability in populations exposed to contaminants [124–126]. Nadig et al. [124] compared several measures of variability in redbreast sunfish, *Lepomis auritus*, from four mercury-contaminated sites and two reference streams. A subset of primers indicated reduced genetic variation at, or increased similarity of, contaminated sites when site types were pooled. None of the indices reflected the gradient of contamination present at the contaminated sites. These suggestions of correlation between reduced genetic variability and exposure are strengthened in this study, however, by correlation with the biomarker and tissue chemistry data that confirm fish exposure, and the data generated in a related study that demonstrated DNA damage in exposed fish. This example shows the importance of providing correlative data that potentially link genetic patterns to damage from contaminants. However, although the reliability of the RAPD technique can be strengthened by rigorous standards and controls, newer marker types are currently coming into favor that share some significant advantages with RAPDs, but that have been shown to be more easily rendered repeatable (see [84,127]), and thus, that could provide stronger results in similar studies.

### *4.5. Genetic variability: mutation*

Using molecular genetic techniques, Yauk and Quinn [128] screened for mutations in herring gulls (*Larus argentatus*). DNA fingerprints from adult and juvenile herring gulls were sampled from several contaminated and reference sites in the Great Lakes and elsewhere in Canada. Samples from adults and the fledgling gulls from the same nests permitted authors to use average band-sharing indices from reference locations as estimates of the normal accumulation of new bands in offspring in one generation. Novel bands, defined as "bands arising from length-change mutations", were counted in offspring, and used to calculate mutation rates of the DNA. Juvenile gulls from the contaminated Hamilton Harbour showed a significantly higher rate of DNA mutation than gulls from the three reference locations. In a follow-up study, Yauk et al. [129] sampled nine populations distributed across a more clearly delineated range of contaminant exposure levels, inferred from their distances from steel mills. Again, they found significantly higher mutation rates in chicks near mills than in chicks from "rural" nests. Investigators also addressed two alternative hypotheses, allele identity differences among populations, and possible effects of parental age differences among sites. They found no significant difference in allele identities among sites,

corroborating the hypothesis that new alleles arose via mutation. Although they could not age all older birds used in their experiments, they did show that in several cases of known older parents, mutation rates were consistent with those of other individuals in their populations.

Chen and Hebert [64] sampled several hundred brown bullhead, *A. nebulosus*, collected from four contaminated and three reference sites in the lower Great Lakes. They evaluated two regions of the mtDNA genome for SSCPs, and sequenced several representatives of each allele. Using a calculation of nucleotide diversity, they found no difference in levels of genetic variability between the contaminated and reference sites. They also built trees using the neighbor-joining method to evaluate relatedness among the sequences. Terminal branch haplotypes (TBHs), that is haplotypes that are recent mutational derivatives, were identified using two criteria: these haplotypes had to show only a single nucleotide difference from another haplotype in its population, and they had to be derived from a recent mutational event as indicated by the phylogenetic analysis. Although their data did not show any correlation between TBH and contaminant exposure, the approach is a rigorous one that could be applied to any molecular genetic study that generates genealogical data, such as sequence data. One of the challenges of the genetic approach to assessing contaminant effects is discerning among the hypotheses of genetic change [12]. Both of these studies applied methods designed specifically to assess new mutations and reduce the potential of confounding the data interpretation with other possible patterns of genetic change.

### *4.6. Summary*

Overall, many allozyme and molecular genetic studies on field-exposed animals were successful in demonstrating significant differences between contaminated and reference locations in at least a subset of measures. Inconsistencies and lack of conclusive results were most commonly associated with a lack of site chemistry and insufficient knowledge about the life history of the animals studied, and the low power of the data based on low or unequal sample sizes, or low marker variability or specificity. Attempts to demonstrate the same patterns of genetic change across several species subject to similar exposures (e.g. [97,101,126]) failed to show consistent results. This is understandable in light of the evidence that species vary considerably in their overall genetic variability, their tendency towards gene flow, their exposure, and their resistance to contaminants (e.g. [79]). In discussions, authors most commonly speculated that inadequate consideration of each of these factors had resulted in comparisons that did not cleanly test predictions of the hypothesis of selection by contaminants.

There are a relatively large number of field studies using a variety of techniques, and although many are inconclusive, the examples here show that most of the general predictions of genetic change mechanisms (as described in Section 2) may be tested with these techniques. Molecular techniques can target specific genetic loci that may be related to contaminant metabolism or effects. However, because "single-locus information [may be] capricious and provide no direct information on selection" [15], it requires significantly more work to fully understand the results (e.g. [106]). Non-specific techniques, such as RFLP or RAPD surveys, provide tests of the predictions but show some of the same limitations as allozyme data. Specifically, the link between patterns and cause are not mechanistically demonstrated, and the data generated provide little opportunity to follow up with a more locus-specific investigation. As with all field studies, it is harder to control experimental conditions than in laboratory settings. Assumptions must be made about past conditions, life-history features of the animals, exposure, and other potential causes of genetic change. This underscores the importance of correlating genetic comparisons with rigorous site chemistry and, where possible, with tests of alternative hypotheses or demonstration of direct effects of contaminants. As described, the most convincing demonstrations included consideration of alternate validation, such as correlative measures of tolerance, or measures of reproductive success. Future studies must also include more rigorous consideration of experimental design, with several exposed and reference populations needed to avoid some of the pitfalls of "natural" experiments. Additional possibilities might include measures of exposure, or biomarker responses to verify effects of contaminants (see [12]).

## 5. Conclusions

### *5.1. Synopsis and recommendations*

Biologists have discussed the relationships between reduced levels of heterozygosity or shifts in genotype frequencies and fitness for decades. The relationship between fitness and overall population genetic patterns is still debated in the theoretical literature, and consensus has not been reached. Measures of genetic patterns provide data from which several genetic parameters may be calculated, including many indices of genetic variability, heterozygosity, gene and allele frequencies, but they do not provide direct links to genotoxic syndromes unless they are explicitly linked to contaminant exposure. For this reason, genetic studies should be performed in conjunction with adequate exposure validation, and other, more direct, measures of damage that would provide supportive evidence of genetic change (e.g. [111,116,123]).

Each of the approaches that have been used to study the genetic effects of contaminants in field-exposed animals, as reviewed here, has been successful at demonstrating that in principle, genetic change can occur. Studies combining the skills and knowledge of toxicologists with those of population geneticists have become increasingly important to contemporary work in genetic ecotoxicology. This review has attempted to lay out the reasoning behind each approach, followed by descriptions of a subset of the studies to provide examples of specific study designs. Acceptance that long-term genetic effects could affect fitness and may be consequences of chronic contaminant exposure for populations in the wild is becoming widespread among toxicologists. Some of the studies reviewed to date provide compelling evidence of how this may be occurring. Others are more suggestive and have served, perhaps, to inspire other workers to embark on such studies. Below, we reiterate the main recommendations regarding each approach described.

Genetic adaptation has been demonstrated in several occasions. When properly designed, tests for adaptation are very effective in showing a concrete population-level effect of contaminant exposure that has been shown in numerous other cases to reduce average population fitness. Direct tests for adaptation are difficult to accomplish with most field-exposed organisms because they require rearing of multiple generations. A common shortcoming of the studies to date is that researchers often measure resistance and then attribute the results to genetic adaptation. The distinction is important if one is to make inferences about long-term effects on populations through the mechanism of genetic change. Some studies have included other, indirect assessments to substitute for breeding experiments. These will not replace breeding experiments in their ability to draw strong conclusions, but they may strengthen the conclusions where such experiments are not feasible.

Protein and molecular genetic surveys permit nearly direct visualization of raw genetic variation. There are tradeoffs in choosing any of the available techniques. Allozyme surveys have revealed some patterns related to contaminant effects (see [33]). The observation that at least four or five loci show patterns consistent with selective effects of contaminants, at least of metals, is intriguing. However, even strongly correlative results can be frustrating because the technique limitations prohibit further genetic examination of the loci. This limitation is lifted when using DNA techniques, since there are many ways to “locate” a molecular marker on the genome, and thus, to potentially track down the linked variation that may be relevant to contaminant exposure.

Molecular genetic techniques have proven very effective at examining large amounts of genetic information. The plethora of available techniques and the relative ease of exploring multiple approaches has attracted many researchers to generating large amounts of genetic data. However, even large amounts of data, if poorly understood or overstated, are not better than small amounts of well-understood data, underscoring the importance of careful marker selection, study design, and diligent data analysis. Many neutral techniques provide a random sub-sample of the genome, rather than targeting any particular area. For this reason, a negative result does not necessarily mean that any of the hypothesized mechanisms are not occurring, only that the investigator has been unable to identify them using the markers employed. In the examples mentioned in this review, we note important variation in the strength of the conclusions. This variation partly reflects variation in the strength of the markers or selected techniques. However, as we have stated, it is partially explained by study-design weaknesses.

Finally, a generalization regarding the effectiveness of study designs used to date is warranted. At least among published results, studies that have good replication, exposure confirmation, and consideration of multiple hypotheses are much less likely to conclude that population genetic change has occurred as a result of contaminant exposure than those using less rigorous tactics. This observation may be a reminder that on first review, we commonly find what we are looking for. But, more careful observation into the data may reveal inconclusive findings. Germano [130] has thoroughly and thoughtfully summarized the difficulties of predictive ecological field studies. He demonstrated how reliance on dichotomous classical null hypothesis testing is prone to erroneous conclusions about complex systems. He advocates a suite of experimental design and data analysis considerations to improve inference, including the use of Bayesian approaches. Bayesian statistics incorporate prior knowledge about outcome probabilities (what he calls 'base rates') that forces investigators to consider the organism in its ecological context, thus, automatically considering many alternative hypotheses.

### *5.2. Selection of genetic markers*

At this early stage in our understanding of the effects of contaminant exposure on genetic patterns in field-exposed populations, we are not able to predict, a priori, which of the possible outcomes is most likely (i.e. bottleneck, selection, mutation). Some authors have suggested that bottlenecks are the most likely outcome of chronic contaminant exposure in natural populations (e.g. [2]). It is certainly the pattern that is easiest to test for using any highly variable, neutral marker, and that has been observed most frequently so far. However, these facts are confounding, and methods must be sought to test for a variety of possible effects and more confidently attribute patterns to their probable causes [12].

Tests for selection are problematic. Even if we have reason to anticipate that selection is occurring, we do not know which loci to investigate for evidence. An ideal demonstration of selection at the molecular-level would be the identification of a polymorphism in a coding region the function of which is directly relevant to the mechanisms of adaptation, and in which variants could be demonstrated to show distinct fitness differences. Finding such a polymorphism with little advance knowledge would be laborious, and perhaps impossible. For this reason, some investigators have chosen "neutral" screening approaches, such as AFLPs or microsatellites. They reason that these markers are likely to reflect patterns of bottleneck effects, if they are present, and to reveal mutations. Also, by their relatively rare, but finite probability of linkage to or situation within relevant coding regions, they may uncover polymorphisms related to adaptation. Other investigators have access to some information regarding specific contaminant defense mechanisms or fitness differences within exposed populations, and some have used this information to target certain genes or gene groups looking for polymorphisms (e.g. [105]). This approach has been called the "candidate gene" approach.

Given technology and information available at this stage, we propose than an ideal approach would be to combine a neutral marker with another marker type that is more likely to find coding-region polymorphisms. If no candidate genes are obvious, then there are few marker-types in the latter category. Some possible screening approaches for finding adaptive polymorphisms follow. Certain allozyme loci, for which there is a reasonable body of data to suggest unspecified adaptive significance, may be worth screening. If polymorphic patterns are suggestive, techniques of DNA investigation at those loci may be warranted. A suite of microsatellite loci that have been previously mapped in the vicinity of one or several candidate genes thought to be involved in detoxication processes or repair processes, for example, could reveal, through linkage, polymorphisms at coding regions using less intensive screening than would be required if all of the genes were sequenced directly. This approach was used by Kohn et al. [131] with some success. A third possibility, that will become easier and less expensive as techniques become more common, is screening cDNA (complementary or copy DNA, which is the DNA made using RNA as a template, thus, not containing all intervening sequences found in the original DNA) libraries using chip arrays for gene expression. Where variation in expression is found, investigators can examine the genes related to that gene expression for polymorphisms. The cDNA chips can screen hundreds of processed genes at a time, and are

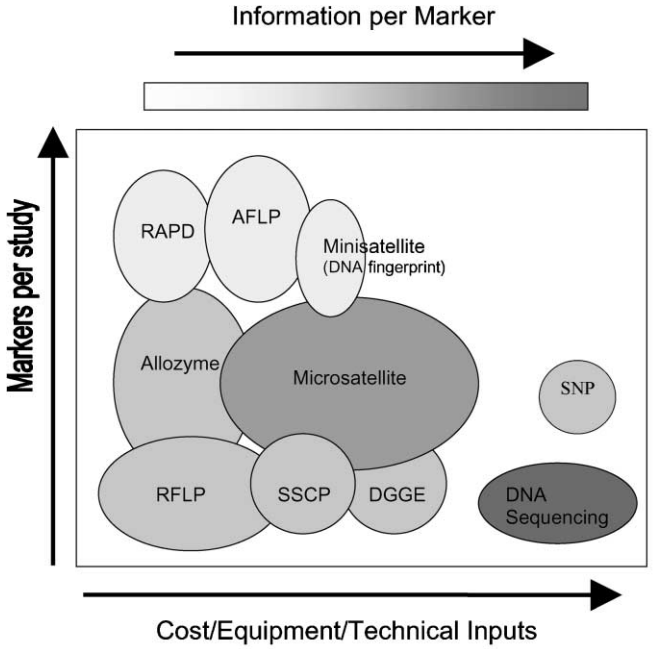


Fig. 1. Conceptual illustration of the relative strengths and weaknesses of different molecular marker strategies. Markers are compared in relation to information per marker, the number of markers (loci) per typical study, and costs per study in terms of capital outlay and technical expertise. The figure is not based on quantitative data and is presented for illustration purposes only.

currently available for many taxa (e.g. *Caenorhabditis elegans*, zebrafish, *Drosophila*, mice, *Arabidopsis*, agricultural plants). They have the potential to work with related species and will rapidly become cheaper and easier to construct for specific taxa of interest.

We present a rough schematic for its heuristic value (Fig. 1) that compares several marker categories and methods by the amount of data points generated, by the cost (in effort and money), and by the information content (i.e. the power to discriminate among hypotheses). This chart indicates, for example, that given current method availability, a combination of targeted allozyme screening and AFLP screening would require a moderate amount of development and money, in exchange for a moderately high probability of finding contaminant-indicative patterns if they are present. Of course, these datasets should be combined with good exposure documentation, careful experimental design, and markers of effect, where possible.

### *5.3. Future directions*

Tests for increased mutations that may occur by exposure to contaminants have been few, and have generally been separate from tests for bottleneck or selection effects. Although testing for these effects may require additional field measurements, such as additional test sites, or samples, or samples from known families, several authors have made it clear that the potentially deleterious effects of mutations, bottlenecks, and selection are not inseparable, and may be synergistic [2,12,15]. Integrating tests for new mutations into study design and marker selection will help genetic ecotoxicologists to come to an understanding of which, if any, of the mechanisms of genetic change may be the biggest threat to natural populations.

New techniques as well as new data generated from other disciplines are likely to benefit future interest and research in mutation analysis. For example, large scale mutation analysis, in which larger regions of the genome than are typically evaluated in

population genetic surveys, and large cell populations are screened for unique distributions of point mutations, is called mutational spectrometry [57,132]. This approach is most often used on homogeneous cells from cell culture and requires comparison to previously obtained "baseline" DNA sequences in order to calculate distributions of mutations. Thus, it is not yet well-adapted for analyses in field applications. Methods will undoubtedly be modified in the future using PCR technology to make it possible to detect mutational spectra at the population-level.

The advent of genomics brings a vast database of new information to the study of molecular genetic variation. In addition, it has accelerated the arrival of new technical enhancements to increase throughput for molecular genetic approaches. In general, genomics is the study of genes, their functions, and their interactions. Data are published daily on the genomics of several well-studied organisms, humans, yeast, nematodes, and *Eschirichia coli*, for example. We anticipate that data gathered on population genetic patterns and single-locus variation in multiple organisms will benefit from the rapidly expanding understanding of genes, gene functions, and genetic variation at coding loci in related organisms. Inter-taxonomic and inter-individual gene differences and levels of genetic variation will be better understood with the help of bioinformatics, which is the use of computational and mathematical techniques to address biological problems. The field of bioinformatics promises to sort and assemble the huge databases of sequence and functional information now available for multiple species, and derive an empirically-based set of generalizations about gene variation and function. This information will provide a context for understanding the relationships among locus variation, marker positions relative to other markers, gene functions in relation to other genes, and cross-taxa similarities. These advancements will help genetic ecotoxicologists to understand the genetic data gathered regarding wild populations, and to better infer the relationships between genotype variation and potential fitness consequences.

Several techniques that are beginning to be explored in genetic toxicology have come out of the genomics race. One example, microchip arrays, can now be assembled based on known gene sequences. These arrays are then probed with genomic DNA to look for single nucleotide polymorphisms (SNPs), or, with messenger RNA to determine levels of gene expression from particular loci. While this approach permits high-throughput screening for single base changes, and could eventually elucidate subtle mechanisms for adaptation, carcinogenesis, or other processes that affect population survival, it is still extremely limited for scientists wishing to study organisms other than those for which large-scale genomic efforts are underway. Many other methods for identifying and then screening for SNPs are reported regularly in the literature. This approach is likely to become very useful in this field as we understand more about what genes may be involved in genetic resistance to chronic exposure to multiple contaminants in wild populations.

In addition to new molecular genetic techniques currently under development, novel analytical methods may contribute more to the field than is currently realized. Many authors have proposed unusual, and sophisticated statistical analyses that will permit us to take advantage of the information differential in multiple-marker studies. For example, the use of genealogical data provides unique opportunities for investigation of selection effects [133], more powerful than inference from non-genealogical genetic patterns. Alternatively, some have proposed methods for deriving genealogical data (or "pedigrees") from certain types of co-dominant markers (e.g. [134]). Lemaire et al. [135] discuss a marker comparison approach to investigating selection effects. Otto [136] proposes a method to distinguish among selection types, using certain kinds of data. Finally, Madsen et al. [137] used a dataset with a known history and compared the power of different marker types to detect it, ultimately predicting that non-neutral loci will reflect the degree of population fluctuation and isolation better than neutral loci. Although these statistical approaches may be emerging from the vast and well-funded field of human genomics, and thus, may not always be designed directly for analysis of genetic ecotoxicological data, it is highly recommended that investigators in this field stay abreast of these developments and take advantage of their strengths.

We believe that genetic analyses performed to date provide some ability to infer the history and status of, and risk to natural populations exposed to contaminants, as predicted by many researchers to date. The

predictions of bottleneck effects and selection were borne out in several studies using a variety of means of assessing genetic change. In addition to these commonly perceived risks, mutation has been put forth by some as an important risk to populations, and was demonstrated in one notable study [129]. It is clear to us that population-level effects occur at ambient contaminant concentrations. Thus, the risk of chronic sublethal exposure to populations is real, and a deeper understanding should be pursued by ecotoxicologists, and considered by conservation biologists. It is our hope that this review has provided some concrete suggestions regarding study design that will strengthen the field in the future.

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