○ ○

The science of environmental toxicology: Concepts and definitions

2.1 The development of environmental toxicology

2.1.1 An historical perspective on the science of environmental toxicology

In Chapter 1, we discussed some of the historical, social, and economic background that accompanied the development of environmental toxicology. Here, we address some of the scientific and technical aspects of the subject.

The connection between community living and human health problems has been recognised for many centuries, long before any cause-effect relationship was clearly understood. It was probably in the earliest centres of human civilisation that the first pollution control laws were enacted. Fully 2,500 years ago, Athens had a law requiring refuse disposal outside the city boundaries. Today, the disposal of "unwanted" products of humans, including sewage, domestic refuse, and industrial waste continues to occupy a large proportion of the resources of human communities, and many of the attendant problems continue to challenge environmental scientists, public health professionals, and engineers.

The development of methods for determining the impact of man on the environment has advanced on several different fronts. When assessing damage to an ecosystem, we are chiefly concerned with the route(s) of exposure of biota to toxic agents and the degree of effect. The latter will depend on the inherent toxicity of the chemical(s), their availability to sensitive segments of the ecosystem, and their persistence in the biosphere.

Toxic chemicals enter the environment through a great variety of human activities including the mining, smelting, refining, manufacture, use, and disposal of products. Natural products of organisms themselves may reach levels toxic to portions of the ecosystem (Figure 2.1).

2.1.2 An evolutionary perspective on environmental toxicology

When considering the impact on the environment of toxic chemicals and other agents developed by humans, differentiating between the anthropogenic component and products of natural biological and geological processes is not always easy.

Perceived Relationships of Various
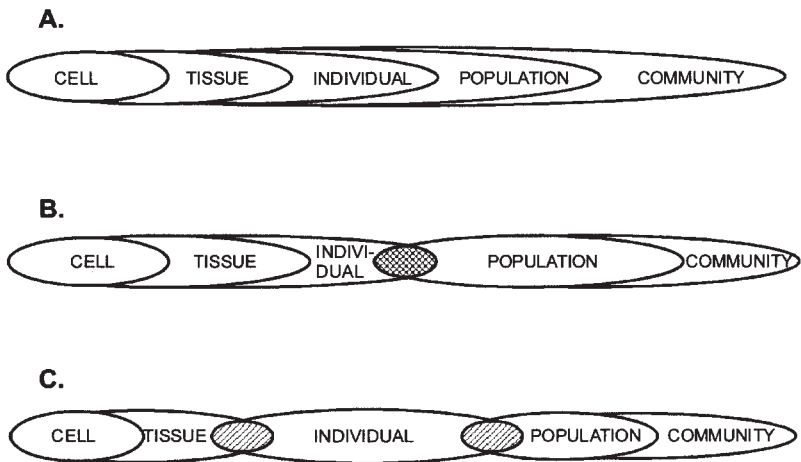Categories of Biological Organization

A.

CELL | TISSUE | INDIVIDUAL | POPULATION | COMMUNITY

B.

CELL | TISSUE | INDIVI-DUAL | POPULATION | COMMUNITY

C.

CELL | TISSUE | INDIVIDUAL | POPULATION | COMMUNITY

Figure 2.1 A conceptual scheme describing the relationship between toxic chemical effects at different levels of biological organization. Shaded areas represent areas of uncertainty. For example, difficulties may exist in relating changes seen at the subcellular or physiological level to the survivability of an individual organism. Further difficulties may exist in extrapolating toxic effects on an individual (usually in laboratory assays) to effects seen at the population or community level.

An appreciation of how organisms have evolved both before and after human influence is important in understanding not only the extent of human impact but also the mechanisms through which organisms regulate their chemical environment. In this context, the metabolism of toxic chemicals may be seen as an extension of homeostatic mechanisms that maintain normal physiological processes. Where such mechanisms result in the elimination of a toxic chemical from its site of influence or the formation of a nontoxic by-product, we refer to the process as detoxification.

Most normal physiological functions of living organisms rely on a relatively few elements that are ubiquitous in the biosphere. Life probably originated in a strongly reducing atmosphere containing simple gaseous compounds of carbon, nitrogen, oxygen, and hydrogen. Although some bacteria retain an obligatory anaerobic metabolism, oxygen has become a constant and stable component (ca. 21%) of the Earth's atmosphere. Initially, a significant proportion of the oxygen in the atmosphere was probably formed by the photolytic effect of solar ultraviolet energy on water in the upper atmosphere, although photosynthesis is now by far the largest source of atmospheric oxygen.

Every year 146 billion tons of carbon combine with 234 billion tons of water to release over 400 billion tons of oxygen. Almost all of this oxygen is used by organisms to oxidise organic food molecules to carbon dioxide. The unique prop-

erty of carbon atoms to combine with each other and a variety of other atoms creates the potential for vast numbers of organic chemicals, several million of which have been identified by chemists. Hundreds of thousands of synthesised organic chemicals have been added to the list, although the manufacture of organic chemicals only started at the beginning of the nineteenth century. Organic chemistry took a quantum leap forward in 1858 with the development of valency theory through independent publications by Couper and by Kekulé (Leicester, 1956). In 1865 Kekulé first described the structure of the benzene ring, and by 1880 over 10,000 organic chemicals were in production. It may be argued, then, that in fewer than 200 years humans have unleashed on the Earth a huge array of chemicals to which biota have had little time to adapt. Certainly none would argue that humans continue to have a dramatic effect on their environment. However, the adaptation of organisms to foreign chemicals long predates human influence.

Humans have contributed to toxic organic pollution ever since they deposited uneaten food and untreated excreta from themselves and their domestic animals within their operational living area and that of their neighbours. Early use of fire for heat and cooking would have made a modest contribution to airborne pollution, and probably within the last 10,000 years primitive smelting began. Many of these activities amounted to nothing more than rearranging and concentrating chemicals that were already part of the biosphere.

Organisms have adapted to many chemical and physical changes in their environment over several hundred million years and have evolved mechanisms to regulate and protect themselves from chemicals that may damage them. One legacy of life having evolved in a marine environment is that body fluids of even terrestrial and freshwater animals resemble salt water in several respects. Further, it is not surprising that the major ionic constituents of seawater (chloride, sodium, potassium, calcium, and magnesium) perform a variety of vital functions including maintenance of skeletal and cellular integrity, water balance, and neuromuscular function. Several enzymes require calcium and magnesium as cofactors, and many more are supported by a number of trace elements such as copper, zinc, and iron, which may be an integral part of the enzyme molecule or may act as electron receptors. For a physiologically useful element such as copper, the "window" between the amount required for normal physiological function and that which is toxic may be quite small (see Section 6.1 and Figure 6.1). Therefore, we can reasonably assume that regulatory mechanisms exist for physiologically useful trace metals just as they do for major electrolytes. However, the substrate concentrations involved and their scale of operation are much smaller. Consequently, these regulatory mechanisms are more difficult to characterise than the transepithelial pumps responsible for major ionic regulation.

When we refer to detoxification mechanisms, we may, therefore, be speaking about regulatory mechanisms that may serve a detoxifying function if the substrate (toxicant) has reached a potentially harmful level. Specific metal-binding proteins such as metallothioneins (see also Sections 4.3.2, 4.4.2, 6.2.6, and 6.6.2) probably

both regulate and detoxify nutrient metals such as zinc and copper, yet apparently perform a solely detoxifying function for metals that play no physiological role such as mercury. Whether or not organisms were exposed to potentially toxic concentrations of these metals prior to human mining and smelting activity is a matter of speculation. There are, however, several instances of plant and animal populations existing in environments naturally enriched with trace metals. Grasses growing in the Zambian copper belt represent a well-known example. Therefore, concluding that some members of a population were preadapted for some degree of metal detoxification when elevated metal levels were introduced into the environment seems reasonable. Following man's influence, there are many examples of rapid development of metal-tolerant races of organisms through selection of detoxification and/or metal exclusion mechanisms (Mulvey and Diamond, 1991).

An even stronger case can be made for preadaptation to the metabolism and detoxification of toxic organic compounds. The following chapters contain several examples of relatively nonspecific mechanisms capable of handling a broad spectrum of both endogenous and exogenous organic compounds. The latter may include many of the novel compounds and natural compounds currently released from anthropogenic sources. Novel or newly synthesised compounds are commonly given the name xenobiotics (from the Greek word *xenos* meaning stranger). With some notable exceptions, newly synthesised compounds are often sufficiently similar to natural products that they mimic their toxic action and share aspects of their metabolism. Where waste products from human activity are similar or identical to naturally occurring chemicals, it is important from a regulatory standpoint to differentiate between contaminant and background levels.

2.2   Assessment of toxicity

To assess the potentially detrimental effects of a chemical (or other agent) on biota, it is necessary to establish a reproducible quantitative relationship between chemical exposure and some measure of damage to the organism or group of organisms under investigation.

Most current environmental toxicological data come from controlled laboratory assays and generally involve single chemicals and very small populations of test organisms. Even in a standard toxicity bioassay, an attempt is made to simulate what would happen in a large population by observing perhaps only 30 (10 organisms × 3 replicates) per exposure or treatment. Hypotheses concerning the mechanism of toxic action of a specific chemical or group of chemicals are typically tested at the cellular or subcellular level on the basis of which investigators often make assumptions about how a particular biochemical or physiological change may affect the overall fitness of the organism.

In linking the whole organism response to that of a population or community, we are also making assumptions about how toxic responses of individuals may be

reflected at higher levels of biological organisation. Such an approach is embodied in the "nested" scheme shown in Figure 2.1A where cellular effects imply effects at all levels of biological organisation. Clearly, this represents an enormous oversimplification. Two particular areas of uncertainty are represented by the shaded areas in Figures 2.1B and 2.1C. For example, a suborganismal response such as an alteration in enzyme activity or immune response may represent a healthy reaction to a stress; therefore, finding a quantitative relationship between such a response and the "fitness" of an organism may be difficult (see discussion of biochemical markers in Section 4.4). Extrapolation from the individual to the population or community (Figure 2.1C) presents a different set of problems. For example, the disruptive effects of humans on the ecosystem must be viewed within the context of natural physical and chemical variations within the environment, such as climatalogical effects. In addition, organisms may become adapted to manmade pollution in the same way that they may adapt to numerous other environmental variables such as temperature, salinity, and food and oxygen availability.

A fundamental aspect of toxicological investigation is the relationship between the amount of chemical exposure and the degree of toxic response. This dose-response relationship is usually explored using a toxicity bioassay, the principal features of which follow.

2.2.1   The dose-response

The relationship between chemical exposure and toxicity is fundamental to toxicological investigation. Typically, the relationship is characterised from the relationship between two variables: dose and response. A dose is a measure of the amount of chemical taken up or ingested by the organism and may be quantified or estimated in a number of different ways (Table 2.1). From Table 2.1, we can see that dose may be broadly or narrowly defined. In toxicity bioassays involving terrestrial vertebrates, dose may be precisely circumscribed by injecting an exact amount of chemical into the organism. In several environmental toxicological studies, however, dose is implied from a measure of chemical concentration in the exposure medium (e.g., air, water, or sediment) and a knowledge of total exposure time. Together, these two parameters provide a measure of exposure rather than dose, and, even though the term dose-response is commonly used to describe laboratory-based bioassays, what we are often determining is an exposure-response relationship. The term exposure has great relevance to a field setting, where estimates of ambient chemical concentration and exposure time provide the basis for risk estimation when coupled with data from laboratory and other controlled exposures.

Toxicity bioassays are performed by exposing a representative population of organisms to a range of chemical concentrations and recording responses, or end-points, over a period of time. Responses may be an all-or-none (quantal) phenomena such as mortality, or they may be graded effects such as growth or reproductive performance (fecundity). An end-point may be any quantifiable response that can

Table 2.1. Concept of toxic dose: Ways by which toxic chemical dose[a] is measured or estimated in different applications

A measured amount of chemical may be injected into the tissue/extracellular space of the test organism (e.g., premarketing test for therapeutic agent).
Chemical may be administered as part of a controlled dietary ration (e.g., therapeutic agent/food additive/pesticide testing).
Chemical may be administered as a one-time dose in the form of a skin patch for cutaneous testing (e.g., testing therapeutic, cosmetic agents).
Ingested chemical may be analysed at target tissue or in surrogate tissue/excretory product having predetermined relationship with receptor site/tissue: blood, urine, hair (e.g., therapeutic agent/pesticide testing, industrial exposure studies).
In small organisms, whole body burden may be measured, although important information on tissue distribution is lost (e.g., skeletal chemical composition may be very different from soft tissues and may be misleading if included).
Dose may be inferred from the product of exposure concentration and time. This estimation is important in many macrophytic plant assays where the toxicant is a gas that may not bioaccumulate (see also ppm hr). It is common in aquatic toxicity testing.
Lethal body burden (critical body residue) is measured at the time of death. It may be related to a laboratory bioassay or be a result of intoxication in the field.

[a] See Section 8.2.2 for a discussion of ionizing radiation dose.

be related to chemical dose or exposure and may include changes in enzyme activity, tissue chemistry, pathology, or even behavioural changes.

The population response may be viewed at any one test concentration over time or at any prescribed time (t) over the concentration range. In either case, a quantal response such as mortality usually follows a normal distribution. For example, if percent mortality at time (t) is plotted against toxicant concentration on an arithmetic scale, a sigmoid curve results. In Figure 2.2, this is superimposed on a mortality frequency plot. The Gaussian curve essentially reflects a normally distributed population response with early mortalities among more sensitive individuals and prolonged survival of the most resistant organisms.

For statistical reasons we are most interested in the centre of the response curve, specifically the point at which the mortality of the test population is 50%. If a vertical line is drawn through this point, it intersects the abscissa at a concentration termed the LC (Lethal Concentration)$_{50}$. This is the toxicant concentration, which kills 50% of the test population at time (t). As such, it represents an estimate of the concentration that would cause 50% mortality of the infinitely large population from which the test population is taken. Such an estimate is strengthened by the use of larger test populations and appropriate replication of the assay. Preferably, such an assay should be conducted at least in triplicate, although it is recognised that large-scale replication presents logistical problems. In Figure 2.2, the mortality frequency curve follows the typical bell-shaped (Gaussian) distribu-
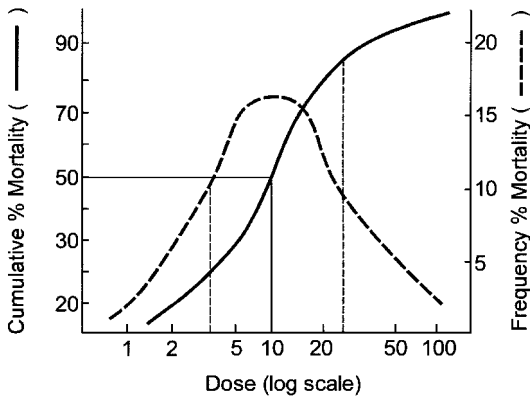
Figure 2.2 Relationship among cumulative percentage mortality, frequency percent mortality, and dose.

tion describing the overall population response. The LC_{50} represents the mean response of the population. In a normally distributed test population, 68.3% of the population is included within ±1 standard deviation (SD) about the mean (or median). This is the region of the curve between 16 and 84% (Figure 2.2). The mean ±2SD includes 95.5% of the test population, and the mean ±3SD, 99.7% of the test population. Various mathematical devices have been used to straighten out the response curve so that this central portion of the curve can be examined in a standardised manner. The most commonly used transformation is the probit transformation where percentage mortality is plotted on a probability scale on the *y*-axis versus log chemical concentration on the *x*-axis (hence the logarithmic range of chemical concentrations used for the test). The plot is based on the normal equivalent deviate or NED (equivalent to the standard deviation about the mean), initially proposed by Gaddum (1933) and then modified to the probit unit by Bliss (1934a, 1934b) by adding 5 to avoid negative numbers (Figure 2.3). The typical plot encompasses 6 probit units and results in a mortality curve that theoretically never passes through 0 or 100% mortality.

In addition to determining the LC_{50}, it is also desirable to obtain values for the 95% confidence limits and the slope of the line. Methods for calculating these parameters are described by Litchfield and Wilcoxon (1949) and Finney (1971). Probably the first and most commonly used method for determining the LC_{50} was the probit transformation for the analysis of quantal assay data, which, as we have seen, generates a sigmoid curve when plotted against the log of concentration. Empirical studies, however, suggested that probit transformation was not always the optimal method to use, and Finney (1971) showed that the method could be laborious and time-consuming. Subsequently, Gelber et al. (1985) proposed the logistic function (Hamilton et al., 1977), the angular arc-sine transformation of the percent mortalities, and a combination of the moving average interpolation with the latter as competitors to the probit transformation. Not until the 1970s did a
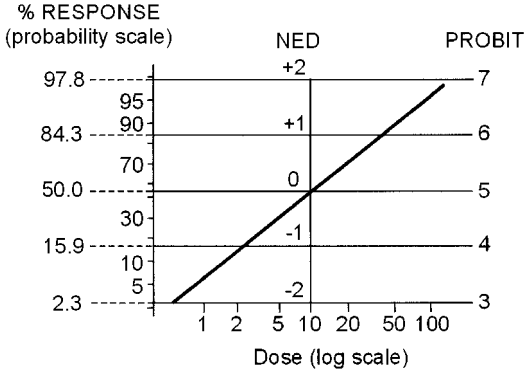
% RESPONSE
(probability scale)

Figure 2.3 The development of probits from the log probability dose response relationship. NED ≡ normal equivalent deviations (numerically equivalent to standard deviations).
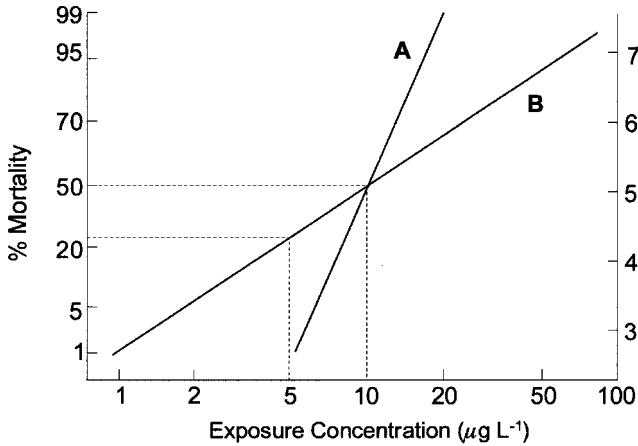
Figure 2.4 Dose response for two chemicals A and B having the same LC_{50} but different slope functions.

nonparametric technique, the trimmed Spearman-Karber method, come into widespread usage. The method produced fail-safe LC_{50} estimates but was not applicable in its original form if the bioassay yielded test concentrations with no partial kills. The method could not be used to generate confidence limits.

Calculated slopes can provide useful additional information on the mode of toxic action. Figure 2.4 shows probit-transformed response curves for two chemicals A and B. In the case shown, the LC_{50}s are identical, although the response for A exhibits a much steeper slope than that for B. The steep slope may signify a high rate of absorption of chemical A and indicates a rapid increase in response over a relatively narrow concentration range. By contrast, the flat response curve for B suggests a slower absorption or, perhaps, a higher excretion or detoxification rate.
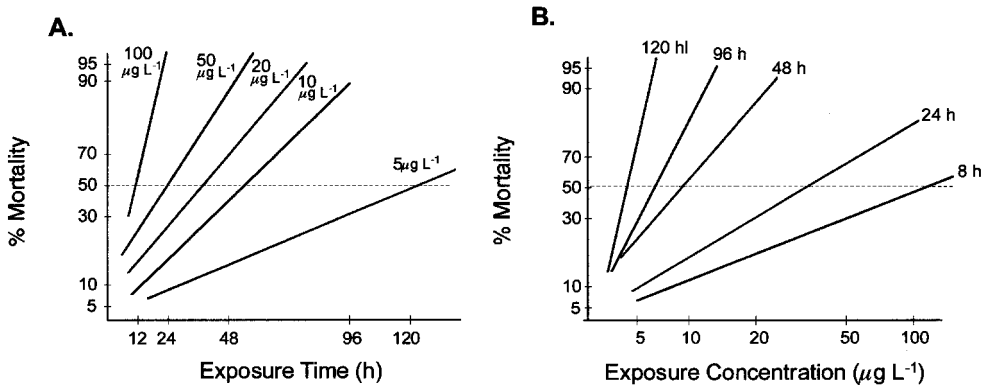
Figure 2.5 Two different expressions of the same dose response data. (A) Mortality at different toxicant concentrations is recorded over time. (B) Mortality at set time intervals is recorded for different toxicant concentrations.

Notwithstanding the fact that LC_{50}s for A and B are both $10\,\mu g\,L^{-1}$, the steeper slope for A might, at first sight, suggest a greater degree of toxicity associated with this chemical. However, in environmental toxicology, we are increasingly concerned with toxic effects of low concentrations of chemicals (i.e., those less than the LC_{50}). In the lower left portion of the graph, we note that, at concentrations half of the LC_{50} ($5\,\mu g\,L^{-1}$), chemical A kills less than 1% of the test population whereas the mortality associated with chemical B is still as high as 20%.

All the foregoing methods for calculating LC_{50} tend to agree very well, but there is much less agreement at the upper and lower ends of the curve (e.g., LC_{10} and LC_{90}). A fundamental weakness of the LC_{50} is that, in focusing on the statistically more robust centre of the curve, where confidence limits are relatively narrow compared with the mean, potentially useful information is lost at the two ends of the curve. This is particularly true at the lower end, where we are interested in the lowest concentration of a chemical that elicits a toxic effect. This is known as the threshold concentration.

Information from acute toxicity tests can be expressed in one of two basic ways. Cumulative mortality may be plotted over (log) time for different chemical concentrations. Alternatively, log concentration is used as the *x*-axis, and plots are made of differential mortality at different time intervals. In Figure 2.5, the same data have been plotted both ways. Time-based plots, favoured particularly by European laboratories, occasionally illustrate instances where toxicity apparently ceases after an initial die-off (Figure 2.6). The point of inflection may occur anywhere along the curve and indicates that the chemical is only toxic to a part of the test population at that concentration. In some rare data sets, mortality may proceed at a different rate following an inflection point. An example is the split probit plot (Figure 2.7), which is probably indicative of a change in mode of toxic action by the chemical. The concentration-based plot (Figure 2.5B) illustrates how the LC_{50}
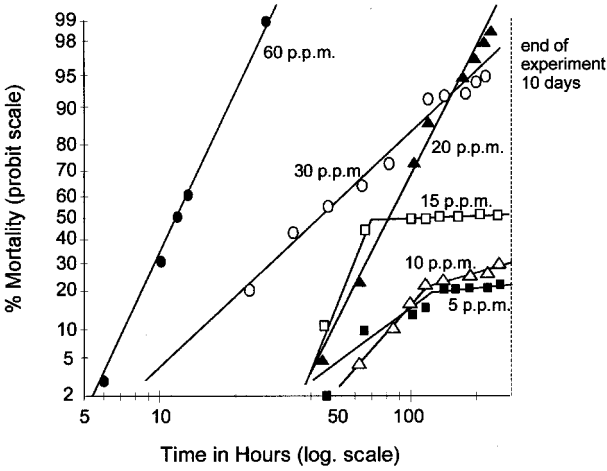
Figure 2.6 Fluoride toxicity to brown trout (*Salmo trutta*) fry showing curtailment of mortality at lower F⁻ concentrations. From Wright (1977).
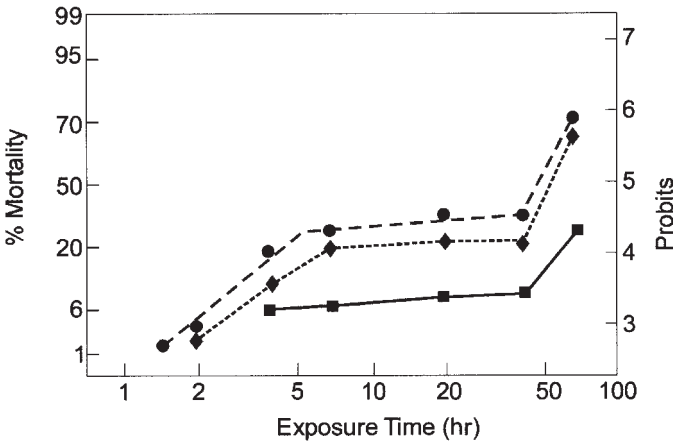

Figure 2.7 The split probit response, which is probably related to different toxic actions varying in intensity.

becomes progressively smaller at longer exposure time, although the incremental decrease in LC_{50} diminishes as the time of the test is extended. When viewed in this way, it becomes clear that there is a point where longer exposure does not lead to any further change in LC_{50}. This concept is illustrated in Figure 2.8, where toxicant concentration is plotted against the time to reach 50% mortality. This time is referred to as the median lethal time. The concentration at which the curves become asymptotic to the time axis is referred to as the threshold, incipient LC_{50}, or incipient lethal level.
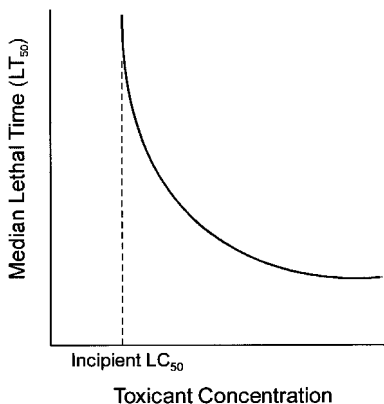
Figure 2.8 Relationship between median lethal time and dose, showing derivation of incipient LC_{50}.

2.2.2 The acute toxicity bioassay

The most commonly used test is the so-called acute toxicity bioassay wherein cumulative mortality is recorded over a 48- or 96-hr period. Sprague (1973) has advocated more frequent observations at the beginning of the test, although, typically, mortalities are recorded at 24-hr intervals. The time course for such an assay has been arbitrarily chosen for the convenience of the investigator and has been accepted as one of a number of "standard practices" designed to assist comparability of data between chemicals, between organisms, and between investigators. If no background information is available on the probable toxicity of the chemical to be tested, an initial "range-finding" test is performed using a wide range of concentrations, which may cover several orders of magnitude. As a result of preliminary data, this test is reduced to a more narrow, logarithmic, range of concentrations. Usually, at least four chemical concentrations plus a (chemical-free) control are included in the definitive test.

In an aquatic assay, organisms may remain in the same medium throughout the test, in which case it is termed static. Flow-through tests are performed using metering devices (dosers) designed to deliver an appropriate range of chemical concentrations on a once-through basis. The flow-through test better maintains the integrity of the chemical concentrations where uptake, degradation, evaporation, and adsorption to container surfaces may lead to the depletion of the test chemical in the exposure medium and prevents build-up of waste products from the test organisms. A compromise is the static-renewal test, where some or all of the test medium is replenished periodically.

2.2.3 Subacute (chronic) toxicity assays

A variety of approaches has been used to investigate subacute toxicity. This term is used here to describe toxic effects at concentrations less than the acute LC_{50}.

Mortality may still be used as an end-point but may involve longer exposures than 96 hr. In this regard, the term *chronic toxicity* is often used. However, in many texts, the term *chronic* is conventionally reserved for a group of sublethal bioassays involving graded end-points such as level of biochemical activity, fecundity, or growth.

Although the sensitivity of a bioassay may be augmented by lengthening the exposure time, the use of mortality as an end-point remains a somewhat crude measure of toxicity. Considerable improvements in sensitivity may be achieved by employing the most susceptible stages of the life-cycle (e.g., embryos and larvae). However, the most sensitive bioassays have usually involved long-term (chronic) exposures and a variety of subtle end-points. In the scientific community, a good deal of debate over the selection of these end-points has occurred, particularly in terms of how they relate to mortality. Several dose-response studies deal with mechanistic aspects of toxic action where the linkage between the end-point and the health of the organism is not fully established (see Sections 4.4.1 and 4.4.2). Nevertheless, most sublethal assays involve parameters clearly linked to productivity such as growth and reproduction. Although the terms acute and chronic have been used in association with lethal and sublethal end-points, respectively, the time-based distinction between these two categories of test has become blurred. Chronic assays, which were originally designed to cover the complete life-cycle of the organism, or a substantial proportion of it, have been largely replaced by shorter tests, sometimes no more than a few weeks in duration.

For example, for some species of fish, so-called egg-to-egg tests might last more than a year, and have been superseded by early life stage tests, which go from the egg or embryo through to the larval or juvenile stage. Such tests may include multiple end-points such as the delay of onset and/or completion of hatch, percentage abnormal embryos, delays in swimming/feeding activity, abnormal swimming behaviour, abnormal or retarded metamorphosis to juveniles, and reduction in growth rate.

In the last two decades, increased efforts have been made to expand sublethal tests to a broad spectrum of organisms from a variety of aquatic and terrestrial habitats. Some of these assays are summarised in Table 2.2. In the aquatic environment, invertebrate assays figure prominently. One reason for this emphasis is the relatively short life-cycle of mysids such as *Mysidopsis bahia* and ostracods such as *Daphnia magna*. Another reason is the improvement in conditioning and spawning techniques facilitating the availability of gametes for much of the year. This availability has enabled the development of fertilisation tests for sea urchins and bivalve molluscs as well as single gamete (sperm) assays.

In keeping with the objective of characterising threshold levels of toxicity as far as possible, a primary objective of the sublethal (chronic) bioassay is the determination of what is called the maximum acceptable toxic concentration (MATC). The MATC has been defined as the geometric mean of two other values: the no observed effect concentration (NOEC) and the lowest observed effect

concentration (LOEC). The NOEC is the highest concentration having a response not significantly different from the control, and the LOEC is the lowest concentration that shows significant difference from the control. Sometimes these concentrations are referred to as the no observed effect level (NOEL) and the lowest observed effect level (LOEL). In most cases, it is possible to calculate a median effective concentration or EC_{50}. This is analogous to the LC_{50} and is the chemical concentration causing a sublethal response in 50% of the test population. In each case, the significance (or otherwise) of the response is measured by comparison with the mean control value but is counted in a quantal way (e.g., an organism is either scored as normal or abnormal).

Another useful way of expressing graded sublethal results is in terms of percentage inhibition concentration (IC_p). For example, a chemical concentration causing a 30% inhibition of hatch or growth rate (relate to controls) is referred to as the IC_{30}. The IC_p has the advantage of flexibility insofar as degrees of response can be compared.

2.2.4 The relationship between acute and chronic toxicity

Because of the relative ease and economy with which acute toxicity data can be obtained, this type of information is much more commonly and widely available than chronic data. Although the gap is being closed with more abbreviated "chronic" assays, such as the early life stage test in the aquatic environment and a variety of screening tests for mutagenicity and teratogenicity, a great deal of attention is still given to determining the relationship between short-term and long-term toxic effects. The acute-to-chronic ratio (ACR), the ratio of acute LC_{50} to a measure of chronic toxicity (e.g., MATC),

$$ACR = \frac{Acute\ LC_{50}}{MATC}$$

has been used to extrapolate between different species and different chemicals where chronic toxicity data are unavailable.

Thus, an acute LC_{50} divided by an ACR determined for a similar species will provide an estimate of chronic toxicity. A high value for this ratio indicates that the chronic toxicity of a chemical may not be satisfactorily determined from its acute toxicity and may indicate two quite different modes of toxic action in both the short term and the long term. Kenaga (1982) found acute-to-chronic toxicity ratios varying from 1 to over 18,000, although for 93% of the chemicals surveyed the mean ratio was about 25. The ACR represents a somewhat crude means of estimating chronic toxicity in the absence of more appropriate data, but it probably has its place in environmental toxicology as long as acute toxicity tests form the basis of toxicological assessment. The aim of this procedure is to provide a regulatory tool for affording maximum protection for the largest number of species without spending precious resources performing toxicity tests on very large numbers of organisms.

Table 2.2. Bioassays in common use in environmental toxicology

Test species	Test/conditions	End-point
	Aquatic – Freshwater	
Teleosts		
Fathead minnows (*Pimephales promelas*)	48/96 h acute bioassay	Mortality, % hatch, swim-up time, % normal larvae, growth
	21–32 d early life stage (20–25°C)	
	Modified as sediment-related test in United States	
Bluegill (*Lepomis machrochirus*)	48/96 h acute bioassay	Mortality, % hatch, swim-up time, % normal larvae, growth
	28 d early life stage (28°C)	
Brook trout (*Salvelinus fontinalis*)	48/96 h acute bioassay	Mortality, % hatch, swim-up time, % normal larvae, growth
	28 d early life stage (28°C)	
Rainbow trout (*Oncorhynchus mykiss*)	48/96 h acute bioassay	Mortality, % hatch, swim-up time, % normal larvae, growth
	30–60 d early life stage	
Lake trout (*Salvelinus namaykush*)	60–72 d early life stage	% hatch, swim-up time, % normal larvae, mortality, growth
Amphibians (*Xenopus laevis Rana* spp.)	96 h embryonic exposure (FETAX test)	Malformation of head, gut, skeleton (teratogenicity)
	Sediment – Freshwater/marine	
Amphipod		
Hyallella azteca	Freshwater	
	8 parts water: 1 part sediment	
	10 d assay	Mortality, growth
	28 d assay	Mortality, growth
	Brackish water (5–20‰)	
Leptocheirus plumulosus	8 parts water: 1 part sediment	
	10 d assay	Mortality, growth
	28 d assay	Mortality, growth
	Marine (>25‰)	
Ampelisca abdita	8 parts water: 1 part sediment	
	10 d assay	Mortality, growth
	28 d assay	Mortality, growth
Polychaetes	Marine	
Neanthea arenaceodentata	8 parts water: 1 part sediment	
	96 h assay (20°C)	Mortality, growth
Crustaceans – Ostracods		
Ceriodaphnia dubia	48 h acute bioassay	Mortality, mean no. and survival of larvae from 3 broods
	10–14 d fecundity per surviving female (20°C)	
Daphnia magna,	48 h acute bioassay	Mortality, mean no. and survival of larvae from 3 broods
D. pulex	7–10 d fecundity per surviving female (20°C)	Survival and brood size of F0. No. of F1 generation
	21–28 d life-cycle test	
Microalgae		
Selenastrum capricornutum	96 h biomass production from concentrated innoculum	Cell counts, dry weight, chlorophyll a, ^{14}C assimilation

Saline water

Species	Test	Endpoint
Teleosts		
Sheepshead minnows (*Cyprinodon variegatus*)	48/96h acute bioassay	Larval mortality
	7d growth assay	Larval growth
	30d life-cycle test (25–30°C)	Hatch, embryo/larval
		Development, growth, mortality
Mummichogs (*Fundulus heteroclitus*)	48/96h acute bioassay (20–25°C)	Larval mortality
Turbot (*Scophthalmus maximus*)	48h acute bioassay (15°C)	Yolk sac, larval mortality
Crustaceans		
Grass shrimp (*Palaemonetes pugio*)	48/96h acute bioassay (20°C)	Larval mortality
Gulf shrimp (*Mysidopsis bahia*)	7d fecundity test (27°C)	No. of eggs per female
	28d life-cycle test (27°C)	Growth, survival of F0, no. of F1 per female productive day
Acartia tonsa (copepod)	96h acute bioassay (20°C)	Adult mortality
	14d life-cycle test (starting with young naupliar larvae) 20°C	Mortality of F0, no. and survival of F1 generation
	Brackish water conditions (5–15‰)	
Eurytemora affinis (copepod)	96h acute bioassay (20–25°C)	Adult mortality
	10–14d life-cycle test (starting with young naupliar larvae)	Mortality of F0, no. and survival of F1 generation
Bivalve molluscs		
Pacific oyster (*Crassostrea gigas*)	48h embryo/larval assay	Survival to the shelled (D-hinge) stage
	Exposure begins with fertilized eggs (20°C)	
Echinoderms		
Atlantic purple sea urchin (*Arbacia punctulata*)	Short-term (10–20min) sperm cell exposure (10–15°C)	Fertilisation of unexposed eggs

Sediments/aquatic

Species	Test	Endpoint
Dipteran larvae		
Chironomus tentans	fresh – brackish water 10d assay 28d partial life-cycle (from 2nd/3rd instar)	Larval mortality
		Adult emergence from pupa

Terrestrial

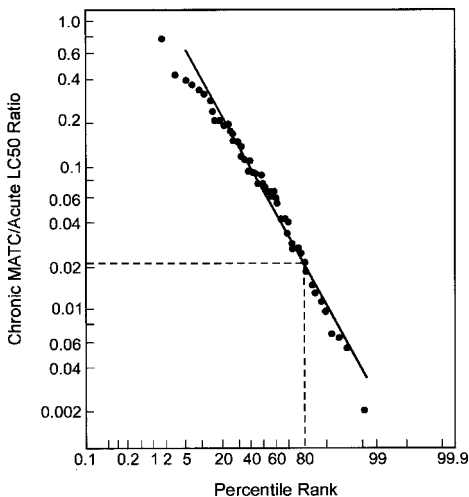| Species	Test	Endpoint
Birds		
Mallard (*Anas platyrhynchos*)	Acute oral; single dose followed by >14d observations	Mortality (necropsy); feeding behaviour, other signs of intoxication; LD_{50} normalised to body weight
Northern bobwhite (*Colinus virginianus*) various avian species	Dietary LC_{50} (toxicant mixed with daily diet); c.10d–10 weeks before egg laying	Mortality, no. of eggs, thickness of egg shell, hatching
Mammals		
Rat (*Rattus norvegicus*)	Acute oral; single dose followed by >14d observations	Mortality (necropsy); feeding behaviour, other signs of intoxication; LD_{50} normalised to body weight
Rats and wild rodent (e.g., voles, *Microtus* spp.)	14–30d LC_{50} (toxicant mixed with daily diet)	Mortality, breeding success

Figure 2.9 Probability plot relating application factors (MATC/Acute LC$_{50}$) to relative chemical toxicity. Data computed from the most sensitive couplet of three species, *Daphnia magna*, *Oncorhyncus mykiss*, and *Pimephales promelas* (Giesy and Graney, 1989).

One drawback of this approach is the variability in ACR seen between different species exposed to the same chemical. This drawback is due, in part, to the different mode of toxic action on dissimilar taxonomic groups. In many instances, a compromise is reached by pooling data from a small group of sensitive species. In the state of Michigan, for example, toxicity data from three species have been used as the basis of a probability plot relating MATC : acute LC$_{50}$ ratios, or application factors (AF) to relative chemical toxicity characterised in terms of percentile rank (Figure 2.9). In this figure, data points are computed from the most sensitive couplet of three species: *Daphnia magna*, rainbow trout (*Oncorhynchus mykiss*), and fathead minnows (*Pimephales promelas*). The objective is to determine an AF that can be satisfactorily used to protect a predetermined percentage (in this case 80%) of species through regulatory action. From Figure 2.9, an AF of 0.022 applied to acute toxic data would estimate a protective chronic toxicity value for 80% of compounds used to compile the data set. A similar approach is used to protect 80% of a broad species range. An advantage of the method is that it can define the degree of certainty and probability of protection desired when estimating safe or chronic, no-effect concentrations from acute toxicity data.

Mayer and co-workers (Mayer et al., 1994; Lee et al., 1995) have used regression techniques to extrapolate time, concentration, and mortality data from acute toxicity bioassays to estimate long-term effects. Computer software available from the U.S. Environmental Protection Agency laboratory in Gulf Breeze, Florida, essentially uses a three-dimensional approach to arrive at a no-effect concentration (NEC), which is operationally defined as the LC$_{0.01}$ from a probit curve at t_∞. Oper-
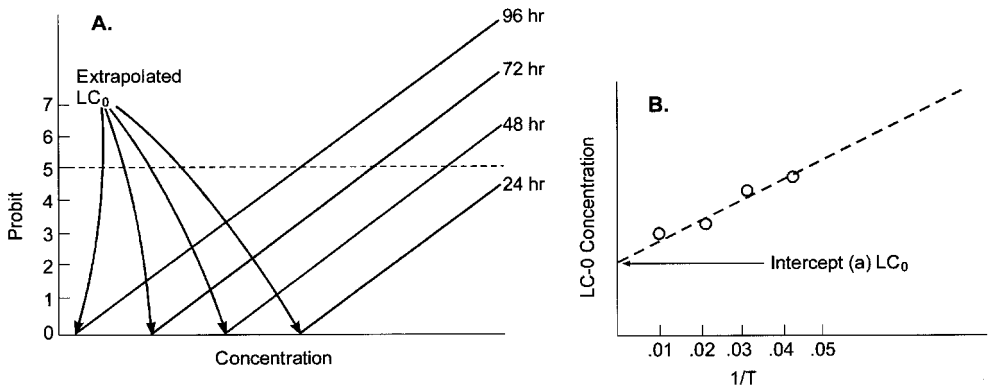
Figure 2.10 Derivation of no-effect concentration (A) by regression of dose response data at different times to determine LC_0 ($\equiv LC_{0.01}$) and (B) plot of LC_0 vs. $1/T$ to determine LC_0 @ t_∞ ($\equiv y$ NEC) (Giesy and Graney, 1989).

ationally, the program performs two separate regressions: initially determining the LC_0 at each time interval (Figure 2.10A) and then plotting these LC_0 values against reciprocal time to extrapolate to a hypothetical infinite exposure time (Figure 2.10B). NEC values derived from acute toxicity data for several fish species have been shown to correlate highly with corresponding MATCs from more time-consuming chronic bioassays (Mayer et al., 1994).

A useful variant of the acute-to-chronic ratio is the chronicity index (Hayes, 1975), which uses data obtained from dose response assays using vertebrates such as mammals and birds. Here, the chemical dose is accurately known, whether given intravenously or inserted into the stomach with a food bolus, and is commonly measured in milligrams per kilogram. Acute toxicity may be measured following a single, 1-day dose or may be assessed as a result of repeated doses up to 90 days. In both cases, the toxic end-point is expressed as the rate of dose (i.e., $mg\,kg^{-1}\,day^{-1}$). The chronicity index is the ratio of acute to chronic toxicity data expressed in terms of rate and is designed to detect cumulative toxic effects. Lack of cumulative toxicity is signified by a chronicity index of 1.

When toxic exposure is transient, difficulties may sometimes occur in characterising dose response in the environment in a satisfactory manner. Such a situation may arise in the case of a spill, when highly toxic concentrations may exist locally for a short period of time before they are dissipated. Exposure times and concentration may vary, and it is useful to have a flexible means of expressing toxicity. A typical example is an oil spill where the term parts per million ($mg\,L^{-1}$) hour has been used to characterise total petroleum hydrocarbon toxicity. Such a concept assumes that a toxic "dose" may be approximated by the product of concentration and time of exposure, as shown in Figure 2.11. Any number of rectangles may be subtended by the curve, each representing the same degree of toxicity (i.e., short exposure to high toxicant concentration or long exposure to low chemical levels). This approach may be applied to a variety of dose-response
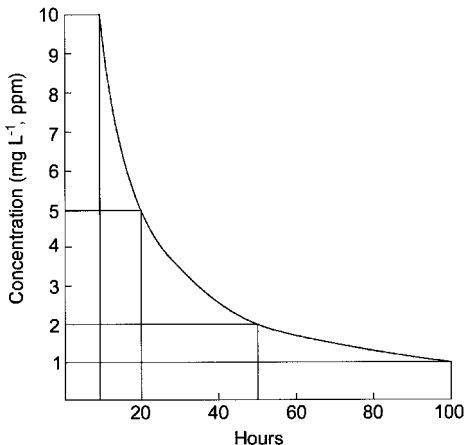
Figure 2.11 Relationship between toxic chemical concentration and exposure time. Subtended rectangles hypothetically have same toxicity value, although boundary conditions apply (see Section 2.2.4).

relationships, although its utility is clearly limited at either end of the curve. For example, at the high concentration/short exposure time end of the curve, toxicity will become limited by the kinetics of transepithelial transport of the chemicals or saturation kinetics at the receptor site (see Section 2.3.6), and at the low concentration/long exposure time end the boundary will be represented by the toxic threshold.

2.2.5   Statistical considerations

Statistics play two principal roles in environmental toxicology. One is in the process of hypothesis testing (i.e., determining the difference between two populations with some predetermined level of confidence). The other can be defined as predictive in nature (e.g., determining the outcome of chemical-biological interaction) and often involves the influence of one or more independent variables on one or more measures of effect [dependent variable(s)].

Statistical methods associated with bioassays are concerned with differences among a few very narrowly defined test populations [e.g., those exposed to different chemical concentrations (treatments) and differences between treatments and controls]. They are also concerned with the relationship between test populations and the larger population from which they were taken. Measurement of treatment/treatment and treatment/control differences requires calculations that take into account the variance associated with the observed end-points (e.g., mortality). Differences between or among treatments can be measured with varying degrees of precision, depending on the numbers of organisms and replicates used. Increases in both of these parameters will decrease the variance associated with end-point measurement and will increase the precision with which treatment differences can be determined. Precision is characterised in this case in terms of a confidence limit.

For example, most tests of population (treatment) differences allow a 5% error. This difference means that there is one chance in 20 of reaching the wrong conclusion based on the data obtained. Even though such an error can be lessened through increased replication of each treatment, there are clearly practical limits to the numbers of replicates that can be handled in an assay. For treatment populations to reflect accurately the overall population from which they were taken, care must be taken to avoid inappropriate clumping of treatments either within a laboratory assay or a field sampling program. This is called pseudoreplication. There are many different examples of this. One example is the selection of organisms for chemical body burden analysis from only one of the replicates. Likewise, replication of tissue sample analyses from a single individual is not true replication within the context of a bioassay. A good overview of pseudoreplication is provided by Hurlbert (1984).

For both lethal and sublethal data, control values need to be taken into account. Where control mortality is low, simple subtraction of control values from other treatments will suffice. However, organisms that spawn large numbers of offspring [e.g., *Arbacia* (sea urchin); *Crassostrea* (oyster); *Morone saxatilis* (striped bass)] often sustain large mortalities of embryos and larvae, even under control conditions, perhaps 30% over a 96-h period. In such cases, adjustment of a treatment value relative to a control is made using the formula of Abbott (1925):

$$\text{Adjusted \% response} = \frac{\text{Test \% response} - \text{Control \% response}}{100 - \text{Control \% response}} \times 100$$

For a 70% observed test response and a 30% control response, the adjusted response becomes

$$\frac{70 - 30}{100 - 30} \times 100 = 57\%$$

Often, sublethal (chronic) data are not normally distributed (as determined by a Chi-Square or Shapiro-Wilks test) or homogeneous (e.g., Bartlett's test). In such cases, results may be analysed using a nonparametric procedure such as Steel's multiple rank comparison test (for equal size replicates) or Wilcoxon's rank sum test (unequal replicates). Normally distributed data can be analysed using Dunnett's test. In certain instances, nonnormally distributed data may be transformed to a normal distribution using a mathematical device that has the effect of lessening differences between data points. One such transformation is the arcsine transformation, which still has a role where statistical treatment demands normal distribution of data. One example is multiple regression analysis, which may be appropriate where more than one variable is being tested. For example, if toxicity due to metal exposure is being examined at different metal concentrations over time at different temperature and salinities, metal concentration, exposure time, temperature, and salinity are all regarded as independent variables and the toxic
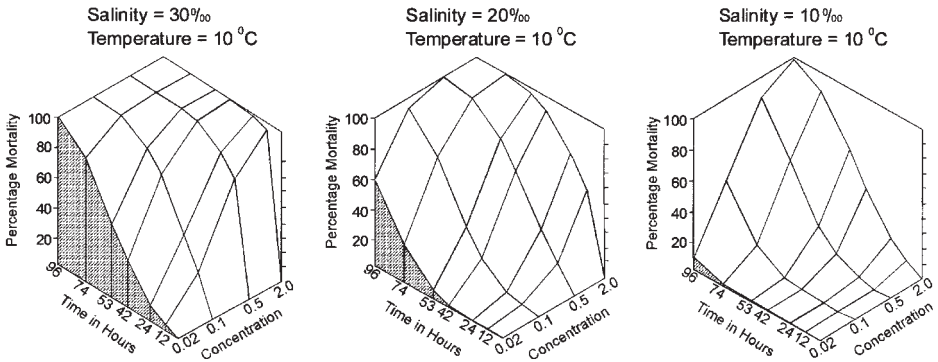
Figure 2.12 Response surfaces describing toxicity of trimethyltin to hermit crab (*Uca pugilator*) larvae at different temperature/salinity combinations. Data from Wright and Roosenburg (1982).

end-point, say, mortality, is the dependent variable. In such an instance, the regression analysis is performed on arcsine-transformed results, which are then back-transformed for presentation in a regression equation, or as response surfaces. Figure 2.12 shows response surfaces describing the mortality of hermit crab larvae (*Uca pugilator*) as a function of trimethyltin concentration and exposure time in the different salinities (Wright and Roosenburg, 1982). A comprehensive account of appropriate statistical methods and procedures is given by Newman (1995).

Statistical treatments of acute toxicity tests. Historically, the LC_{50} that forms the basis of the acute toxicity test has been determined using either the parametric, moving average, or nonparametric technique. The first is based on obtaining a known parametric form for the transformed concentration (dose) mortality relationship, whereas the second is based on numerical interpolation where a moving mortality rate is obtained by calculating the weighted sum for each concentration, and the third uses the data to generate an empirical curve that is based on the symmetry of the tolerance distribution. These techniques are discussed in greater detail in Section 2.2.1.

Statistical treatments for chronic bioassays. For many years, the use of the analysis of variance (ANOVA) F test and Dunnett's procedure has formed the basis for the statistical assessment of chronic toxicity tests. Gelber et al. (1985) outlined the rationale behind the use of these procedures and highlighted some of the potential difficulties associated with these tests.

A review of the literature reveals no clear consensus on the most appropriate statistical procedures to use with chronic toxicity data. The American Society for Testing and Materials (1992) issued a guideline for toxicity tests with *Ceriodaphnia dubia*, which stated that several methods may be suitable. Recommended procedures for reproductive data included analysis of variance, t-tests, various

multiple comparison tests, regression analysis, and determination of the test concentration associated with a specified difference from control. The latter is purported to represent a biologically "important" value. Opinions differ as to what this biologically important value should be. The U.S. Environmental Protection Agency recommends the use of an IC_{25} (i.e., toxicant concentration causing 25% reduction in reproductive output relative to controls), although such a figure must be regarded as somewhat arbitrary pending more detailed information on the reproductive strategy of the test species concerned. The ASTM (1992) guideline also described methods for detecting outlying data values and for transforming response data to obtain homogeneous variances. Nonparametric methods were recommended for heterogeneous data.

An increasing concern on the part of many toxicologists is the overuse and misuse of multiple comparison procedures in chronic toxicity tests. Although many tests are highly appropriate in, say, field situations where the toxicities of various sites are compared to a control or reference site, they are inherently poorly suited to analysing data from experiments where treatments form a progressive series (e.g., the serial dilution of a toxicant in a bioassay). A characteristic of Dunnett's test, for example, is that its relative immunity to false positives in a dose-response situation is obtained at a cost of reduced sensitivity (i.e., more false negative readings). Nevertheless, this procedure is part of a battery of tests recommended by the U.S. EPA for data analysis of chronic assays of effluent toxicity and is incorporated into several computer software packages serving this purpose.

Today, researchers are faced with the dilemma of choosing the most appropriate statistical approaches to test data from acute and chronic bioassays in light of an expanding range of statistical tests and computer programs. Software packages (such as SAS®, Toxstat®, ToxCalx, and TOXEDO) present a number of different alternative methods for the statistical analysis of data sets, and an application using SAS/AF software has been recently developed to estimate the sample size, power, and minimum detectable differences between treatments for various statistical hypotheses and tests of differences between means and proportions.

TIME RESPONSE (TIME-TO-DEATH) MODELS

An increasingly attractive alternative to the dose-response approach is the time response or time-to-death model, wherein the mortality of test organisms is followed for a period that typically extends beyond the duration of an acute bioassay. The approach is similar to that illustrated in Figure 2.5B, except that the time scale of the assay may embrace most or all of the life-cycle of the organism. As such, the assay may be defined as chronic but utilizes more information than the chronic assays described previously. Where observations are made over the complete life-cycle, the organism may grow through different stages (embryo, larva, juvenile, adult). Developmental data from different stages, together with information on mortality and reproductive performance, are collectively known as vital rates (see

Caswell, 1996) and form the basis of demographic models of population structure (Section 4.5.3).

The life table integrates all the stage-specific developmental data and can be used as the basis for a set of analyses variously called survival analysis or failure time analysis. The advantage of these methods over dose-response relationships is that they may incorporate survival times for all invididuals within each treatment group rather than select an arbitrary timed observation such as percentage killed by 96 hr. Thus, more information is utilized and can result in better estimates of mortality rates and covariates.

A critical parameter in this regard is the cumulative mortality distribution function, $F(t)$, which has a value of zero at $t = 0$ and 1 at 100% mortality. The inverse of this is the survival function, $S(t)$, which is equal to $1 - F(t)$. The hazard function, $(h)t$, describes the probability of percentage survival at time t and is mathematically related to the survival function as follows (Dixon and Newman, 1991):

$$h(t) = -d \log S(t)/dt = \frac{-1}{S(t)} \cdot \frac{dS(t)}{dt}$$

The hazard function is useful in determining whether toxicity is constant over time, or whether it accelerates or decelerates as exposure continues. As such, it may determine the choice of the model that best fits the results of a time-to-death assay. Typical regression analyses, accompanied by ANOVA, which assume normal distribution of data and a variety of different distributions have been used. For example, variants of the Weibull distribution have been used to accommodate time-to-death data that are skewed either toward a preponderance of deaths earlier in the assay or to accelerated mortality as the assay proceeds. If no specific distribution can be satisfactorily assigned to the results, a semiparametric proportional hazard model, the Cox proportional hazard model, is commonly employed. If the underlying time-to-death relationship can be satisfactorily described by a specific distribution model, results can be treated using parametric methods such as proportional hazard or accelerated failure time. The latter model can incorporate covariants such as may modify toxicity as the assay progresses. Likewise, the model can accommodate comparisons of two or more different strains of organism, which may have differential tolerance of the toxicant. Several computerized procedures are available for time-to-death data analysis. A typical example is the SAS PROC LIFEREG program, which describes the relationship between failure time (time-to-death) and covariates in the following form:

$$\ln(t_i) = f(x_i + de_i)$$

where t_i = time-to-death of individual I, x_i = vector of p predictor variables (x_{i1}, x_{i2}, . . . , x_{ip}), $f(x_i)$ = linear combination of covariates ($\beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2}, + . . . + \beta_p x_{ip}$), d = scale parameter, e_i = error term for individual I.

In this analysis, individuals that have not died by the end of the assay are taken into account through censoring. Censoring is a general term that describes a common phenomenon in time-to-death studies (i.e., organisms not reaching the end-point of interest at the time of record). A variety of statistical methods for handling such data is available in the literature (e.g., Cox and Oakes, 1984). The reader is directed to accounts of time-response models in the context of environmental toxicology by Dixon and Newman (1991) and Newman and Dixon (1996).

Generalised linear models. Many toxicologists are moving towards the generalised linear models (GLM) approach, which produces more accurate lethal concentration and effects concentration estimates and confidence intervals. Rather than using regression analysis on data that have been transformed to fit a preconceived notion of how a test population might respond, the GLM approach accurately reflects the true nature of the data no matter how they are distributed. For example, 0 and 100% mortality are true end members of the response curve instead of an expanding logarithmic scale that never reaches absolute values at either end. Nyholm et al. (1992) pointed out that the tolerance distribution that results in the sigmoid curve (Figure 2.2) is particularly inappropriate for microbial toxicity tests where the response is typically a continuous variable and is much more suited to a GLM approach. An important advantage of the GLM method is that it provides a much more accurate picture of the response probabilities associated with all levels of toxicant concentration, not just around the 50% mark. The method supplies confidence intervals not only for the LC value but also for the true toxicant concentration that would give rise to a specified response. Kerr and Meador (1996) outlined several advantages of the GLM approach and concluded empirically that any LC value below LC_{10} calculated from their data set would be statistically indistinguishable from the control. They suggested that the method may be useful in determining the lowest observed effect concentration in chronic toxicity tests and proposed that the LOEC would occur where the horizontal line from the upper 95% confidence interval for zero concentration intersects the right 95% band of the model. For their model (Figure 2.13), this occurs at $16\,mg\,L^{-1}$, which becomes the LOEC.

As already mentioned, one of the principal advantages of using a GLM approach is that it allows researchers to focus on information imparted by toxicity data over the whole concentration range without having to fit the data into a preconceived distribution model. This is particularly important at the low end of the concentration range where the focus is on the threshold of toxic effect. GLM methods are also appropriate for time-to-death data described in the preceding section.

2.2.6 Comparative bioassays

Most of the features of the toxicity bioassay described previously are incorporated into a broad range of different types of assay covering a variety of media and circumstances. Bioassays are integral parts of several regulatory programs ranging
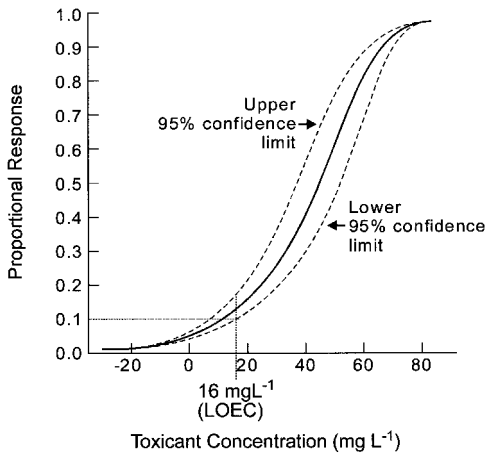
Figure 2.13 Generalised linear model approach for assessing lowest observed effect concentration (based on Kerr and Meador, 1996). The horizontal line intersecting the upper 95% confidence limit associated with zero concentration also intersects the lower confidence limit associated with the 16 mg L^{-1} (the LOEC). Note that the response curve encroaches into "negative dose" territory, not uncommon for such empirical curves. Although the LOEC coincides with the LC$_{10}$ for this data set, this may vary considerably with other empirical dose-response relationships.

from premarketing registration of new products to testing of water quality. Water quality assays have included a multitude of studies from university and government laboratories of known compounds, which have been used to produce water quality criteria. Increasingly, however, bioassays are being used to test groundwater, runoff, and effluent where the actual chemical composition of the medium may be unknown. In these circumstances, a range of "doses" is created by serial dilution of the environmental sample with clean (nontoxic) water, and toxicity is related to the degree of dilution. Assays of this nature are generally conducted in parallel with comprehensive chemical analyses of the sample, and both chemical and toxicological data are used to arrive at decisions on effluent treatment options. An extension of this approach is the toxicity identification estimate (TIE), also called toxicity reduction estimation (TRE), which involves testing and retesting an effluent before and after a series of chemical extractions and/or pH adjustments designed to isolate and identify the toxic fraction (U.S. EPA, 1991). In a review of this approach also known as whole effluent toxicity testing, Chapman (2000) makes the point that such tests identify hazard, not risk (see Chapter 10), and as such should only be regarded as the preliminary phase of a risk assessment process.

   Birds and mammals. Toxicity bioassays on birds and mammals are often important components of preregistration testing of a variety of chemicals such as agrochemicals, therapeutic agents, and food additives. In these tests, the range of chemical exposure is created by serial dilution of an injected dose or by mixing

Table 2.3. Examples of types of end-points in use for plant assays

Nonlethal
Chlorophyll-a concentration
^{14}C uptake
Cellular effect (e.g., membrane permeability)
Growth (rate of increase of weight, volume, cell density, etc.)
Rate of change in leaf biomass
Sexual reproduction
Abundance (number of individuals)
Overwintering
Population growth rate
Acute, lethal
Mortality

Modified from Swanson et al. (1991).

different amounts of chemical with food. A more precise means of delivering an oral dose is used in the LD_{50} test, where a food + toxicant bolus is inserted into the stomach of the animal. Often, such tests are conducted on the basis of a one-time dose, normalised to body weight, followed by a 14-day observation period during which animals are fed uncontaminated diets. Mortality, growth (weight), and feeding behaviour are all used as end-points in these tests. A typical example is the bob-white quail test. It is common for such assays to form part of a tiered approach to regulatory toxicity testing. For example, if no adverse effects result from a dose of $2\,mg\,kg^{-1}$ in the foregoing bob-white quail assay, no further oral tests are performed. This dose also represents the cut-off dose for the standard cutaneous assay, where the chemical product is applied in the form of a patch applied to the shaved skin of the test species, usually a rabbit. Essentially, the assay has the same design as the LD_{50} test; a one-time, weight-normalised dose followed by a 14-day observation period. Depending on the proposed use of a particular product, genotoxicity assays may also form part of the chemical preregistration procedure. Examples of these are given in the following section.

Plant assays. Photosynthetic organisms can be used in acute or chronic assays of potentially toxic substances or effluents. Acute and chronic tests have been developed using various types of aquatic or terrestrial vascular plants as well as unicellular or multicellular algae. Tests using vascular plants frequently employ nonlethal end-points, such as root elongation of seedlings, and effects on development or physiological responses such as carbon assimilation rates. Table 2.3 provides a more comprehensive list of the types of end-points that have been used in plant testing.

Recently, tissue cultures (cell suspension or callous culture) have been proposed as test systems (Reporter et al., 1991; Christopher and Bird, 1992); using tissue

cultures should avoid some of the variability that occurs and the long time periods required for whole plant tests, but may not provide such realistic results in terms of the environmental effects.

Higher plants can also be used for mutagenicity tests, which are normally done with microorganisms or higher animals. A system for the detection of gene mutations has been devised using the stamens hairs of spiderwort (*Tradescantia*). For chromosomal aberrations, the micronucleus of the spiderwort, along with root tips of bean, *Vicia faba*, have been employed. According to Grant (1994), "higher plants are now recognised as excellent indicators of cytogenic and mutagenic effects of environmental chemicals".

Unicellular plants, such as algae and blue-green bacteria, cultured in static or flow-through systems, provide population assays, since all the cells in the test are essentially identical and undifferentiated and have conveniently short generation (mitotic) times. For the most part, only a few species of algae have been employed in routine testing; of these, the green algae (Chlorophyta) *Selenastrum capricornutum* and *Chlorella vulgaris* are the most frequently cited. Parameters that can be used include cell density (direct counting or particle analysis), measurement of production through carbon fixation, or chlorophyll-*a* production (see Nyholm, 1985). The latter pigment, being common to all green plants, including the various groups of different coloured algae, is a particularly useful parameter, because it can be used in the field or laboratory, and in mixed- or single-species collections, at least in a comparative way, as a universal measure of plant production.

Some caution is needed in selecting the type of test to use when designing bioassays with algae. Although the physiological parameters such as carbon fixation rates can provide rapid results (generally hours, rather than days), measurements of growth by cell number may prove more sensitive. For *Selenastrum capricornutum*, short-term tests with endpoints such as CO_2 fixation rates or oxygen generation were less sensitive, for cadmium and simazine respectively, than population growth tests, Figure 2.14 (Versteeg, 1990). A more recent aquatic bioassay utilises clonal lines of Sago pondweed *Potamogeton pectinatus* and relies on respirometry under light and dark conditions to determine net photosynthesis rates. LC_{50} values are calculated from linear regression of log concentration and photosynthetic response expressed as a percentage of control (undosed) plants (Fleming et al., 1995).

Table 2.4 lists some of the ways in which plant responses can be used in testing and assessment. For the purposes of the present chapter, phytotoxicity will be emphasised. Table 2.5 provides information on several of the most widely used phytotoxicity tests. The application of other types of plant responses, such as their application as sentinels and monitors in environmental toxicology, are discussed in Section 4.4.3.

Plant tests often produce rapid results, and the procedures are often less expensive than, for example, rainbow trout assays, but their use should not be based simply on convenience. Selection of the type of organism and the type of test obvi-
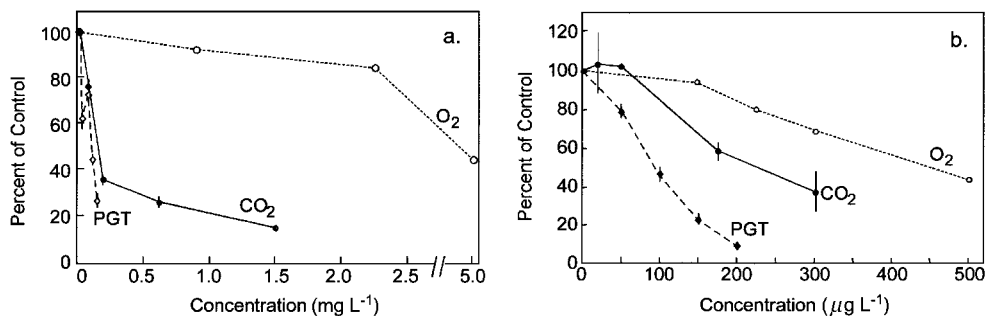
Figure 2.14 Three types of tests for the effect of (a) cadmium and (b) simazine on the green alga *Selenastrum capricornutum*. Based on Versteeg (1990). PGT = population growth tests; CO_2 = carbon fixation test; O_2 = oxygen generation test.

Table 2.4. Classes of environmental assessment analyses using plant responses

Class	Purpose	Example end-points
Biotransformation	Determine influence of plants on chemical fate of environmental pollutants	Change in chemical concentration
Food chain uptake	Establish the amounts and concentrations of toxic chemicals that enter food chains via plant uptake	Chemical concentration
Phytotoxicity	Evaluate the toxicity and hazard posed by environmental pollutants to the growth and survival of plants	Death, discolouration, reduced growth
Sentinel	Monitor the presence and concentration of toxic chemicals in the environment by observing toxicity symptoms displayed by plants	Death, discolouration, reduced growth
Surrogate	Use inexpensive, socially acceptable plant tests as a substitute for an animal or human assay	Chromosome aberrations

Source: Fletcher (1991).

ously should be geared to the questions of concern; furthermore, the relevance and potential for extrapolation to other plants as well as to the more general situation from plant responses need to be considered. This latter caution is not, of course, unique to plant assays. In all toxicity testing, the choice of organisms and type of test has to be made based upon a number of practical considerations, as well as concern for realism. In human or classical toxicology, we find the oft-repeated question of how useful rat (or guinea pig) studies are when considering the poten-

Table 2.5. Plant phytotoxicity tests use by regulatory agencies

Test	Response measured to chemical treatment	Agencies requiring limited use[a]
Enzyme assay	Enzyme activity	None
Process measurement	Magnitude of a process: photosynthesis, respiration, etc.	None
Tissue culture growth	Changes in fresh or dry weight	None
Seed germination (soil solution)	Percent of seeds that germinate	EPA (1985)[b] (50)[c] FDA (1981)[b] (25)[c] OECD
Root elongation (soil solution)	Length of root growth during fixed time period	EPA (1985)[b] (50)[c] FDA (1981)[b] (25)[c] OECD
Seedling growth (soil solution or foliar application)	Changes in height, fresh weight, and dry weight	EPA (1985)[b] (50)[c] FDA (1981)[b] (25)[c] OECD
Life-cycle	Changes in height, fresh weight, dry weight, flower number, and seed number	None

[a] EPA, Environmental Protection Agency; FDA, Food and Drug Administration; OECD, Organization for Economic Co-operation and Development.
[b] Year when the agency started requiring plant testing as a concern for potential hazard posed by chemicals to nontarget vegetation.
[c] A rough approximation of the current annual number of agency-requested data sets used for hazard assessment.
Source: Fletcher (1991).

tial toxicity of substances to humans. In many ways, the choice of test organisms in environmental toxicology is immensely more complex.

Comparative studies have been made, addressing the response to a particular chemical, among different types of plants or, more rarely, between the response of plants and higher organisms. Fletcher (1990, 1991) reviewed the literature on phytotoxicology with the objective of making comparisons among plant species and between algae and higher plants. He compared the variability related to taxonomic differences with that resulting from differences in test conditions and found large differences among taxa, above the level of genus. He concluded that taxonomic variation among plants in terms of the effects of chemicals had a greater influence than did the testing condition.

In the context of the relevance of algae as test organisms, Fletcher (1991) found that in 20% of the time, algal tests might not detect chemicals that elicit a response in vascular plants. This relative lack of sensitivity among algae may be explained in part by the fact that higher plants have more complex life-cycles than algae, and that a number of physiological phenomena in higher plants (e.g., development, leaf abscission) have no equivalent in algae. Comparing algal and higher plant tests for

21 herbicides, Garten (1990) showed, using *Selenastrum capricornutum* in 96-hr tests, that there was only a 50% chance of identifying herbicide levels that would reduce the biomass of terrestrial plants.

A few examples that compare plant responses with those of other organisms exist. Gobas et al. (1991) compared the response of guppies (*Poecilia reticulata*) with the aquatic macrophtye Eurasian water milfoil (*Myriophyllum spicatum*) to a series of chlorinated hydrocarbons. They concluded from their study that, since the toxicokinetics in both types of organisms involve passive transport and are related to the octanol-water partition coefficient, the acute lethality would be similar in plants and fish. Such generalisation would not be possible for inorganic toxicants or organics for which uptake could not be readily modelled. Normally, a more detailed consideration, based on the problem of concern, would be needed before plants could be used as surrogates for higher organisms.

Plants constitute more than 99.9% of the biomass on Earth (Peterson et al., 1996) and have intrinsic value of themselves. In the context of environmental toxicology, the eradication of plant communities can result in modified habitats and changes in trophic structure of ecosystems that may have more impact on higher trophic levels than the direct effects of a toxic substance. Thus, plants may be used in conjunction with animals in batteries of tests, in attempts to represent various trophic levels. Many regulatory agencies already require a plant and two higher trophic-level organisms to be used in screening "new" chemicals. The approach of incorporating several trophic levels in one test, as in microcosms and field enclosure tests, is even more realistic (see Section 4.7.1).

2.2.7 Sediment toxicity assays

By the late 1960s, concern over the deterioration of water quality in the Great Lakes prompted the Federal Water Quality Administration (the predecessor of the U.S. Environmental Protection Agency) to request the U.S. Army Corps of Engineers to initiate studies on the chemical characteristics of harbours around the perimeter of the Great Lakes system. Initially, harbour sediments were analysed using methods that were developed to characterise municipal and industrial wastes rather than sediments. In 1970, the Rivers and Harbors Act of 1890, which was originally written to protect access to navigable waters in the United States, was amended to include specific provisions to begin a "comprehensive evaluation of the environmental effects of dredged material" through the Dredged Material Research Program (DMRP). Under this program, the first effects-based testing approach was developed; it relies on the use of bioassays to integrate the potential effects of all the contaminants present in a complex sediment matrix.

The first published study of the potential toxicity of field-collected sediments was performed by Gannon and Beeton (1971). Experiments were specifically designed to test the effects of dredged material on freshwater benthic organisms. The amphipod *Pontoporeia affinis* was exposed in 48-h acute bioassays to five sediments collected from different sites. The tests were designed to assess sedi-

ment selectivity (behaviour) and viability (survival). Although survival data indi-cated a graded response to contaminated sediments, *P. affinis* clearly preferred a sediment particulate profile similar to their natural sediments. Over the succeed-ing 20 years or so, it became apparent that substrate preferences, salinity tolerance, and ease of culturing/availability were all-important considerations in the selection of test organisms. Candidate species for freshwater assays have included the bur-rowing mayfly *Hexagenia limbata*, the ostracod *Daphnia magna*, and the isopod *Asellus communis* (Giesy and Hoke, 1990). Early suggested species for bioassays in saline environments included the bivalves *Yoldia limatula* and *Mytilus* sp., the infaunal polychaetes *Neanthes* sp. and *Nereis* sp., and the crustaceans *Mysidopsis* sp. and *Callinectes sapidus*. Variants on the use of whole sediments have included sediment leachates and pore-water extracts/dilutions obtained through squeezing and centrifugation. Such techniques had the effect of transforming sediment toxi-city tests into standard assays that could be carried out in aquatic media, yet they suffered the disadvantage of removing many of the unique characteristics of the sediments that affected sediment chemical bioavailability.

In most current sediment toxicity assays, there has been an increasing focus on the use of small infaunal (usually amphipod) crustaceans, which have proven to be among the most sensitive phyla to sediment contamination. Several different species have been considered depending on such attributes as substrate preference, salinity tolerance, and ease of culture. Both acute and chronic toxicity have been examined, and most protocols now include a standard 10-day bioassay. Fecundity and reburial behaviour have also been considered as end-points. Commonly used species include *Ampelisca abdita* (marine), *Repoxynius abronius*, *Leptocheirus plumulosus* (marine/estuarine), and *Hyalella azteca* (brackish/freshwater).

Along with the focus on standardized infaunal bioassays has come a reaffirma-tion of the idea that measures of ecosystem integrity should include indicators at the population and community level. Consequently, ecological risk assessments for sediment-associated chemical contaminants are universally based on a "weight-of-evidence" approach consisting of what is called a sediment quality triad (Figure 2.15) (Chapman, 1986). This triad uses a combination of sediment chemistry, mac-robenthic community data, and results from single-species bioassays to assess the sediment condition. Such an approach has proven cost effective for regulatory pur-poses. However, in specific studies of sediments contaminated by potentially car-cinogenic compounds (see Section 4.4.2), histopathology of benthic organisms (fish) has often been included in the investigation.

2.3   Toxicity at the molecular level

A major concern of environmental toxicologists in recent decades has been the effect of chemicals and other agents on the normal development of organisms and their offspring. By the 1960s, it was clear that reproductive performance of many
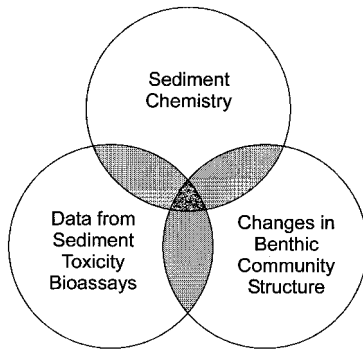
Figure 2.15 Components of a sediment quality triad (Chapman, 1986).

wild animals was being compromised by chlorinated pesticides and other similar compounds. Since then, research has elucidated the mechanistic basis of many of these phenomena and has identified major problems such as the disruption of endocrine and other chemical signals (see Section 7.5) and impairment of genetic transcription. Damage to the DNA molecule will result in abnormal somatic growth and the development of neoplasms (cancer).

Where DNA damage occurs in germinal tissue (sperm and eggs), degraded genetic material may be passed on to offspring. This, in turn, may result, directly, in decreased survival or fitness of these offspring. Changes in the genotype of succeeding generations are also likely to lead to subtle shifts in the organism's relationship to a variety of environmental parameters not necessarily toxicological in nature (see Section 13.5).

Thirty years ago, chemical carcinogenicity was largely viewed within the context of occupational exposure and was, therefore, seen principally as the purview of classical or clinical toxicology. This attitude is no longer the case. The identification of numerous surface water, groundwater, and sediment contaminants (e.g., solvents, pesticides, combustion, and chlorination products) as carcinogens has placed such studies squarely within the realm of environmental toxicology. For example, in 1998, the U.S. Environmental Protection Agency issued a report stating that 7% of coastal sediments in the United States were heavily contaminated with chemicals, many of which were carcinogenic in nature. Many studies of chronically polluted sites have focussed on neoplasm development in fish from these sites; in some instances, carcinogenic models using fish have been examined for their applicability to higher vertebrates.

Although regulations governing the handling and release of most toxic chemicals are formulated as a result of information from assays such as those described in Sections 2.2.1–2.2.7, cancer-causing agents (carcinogens) are treated differently. The concept of toxicity for carcinogens is founded on a knowledge of their mode of toxic action (i.e., mechanistic considerations). These modes shape our perception of dose-response and, in particular, toxic threshold. However, before this is
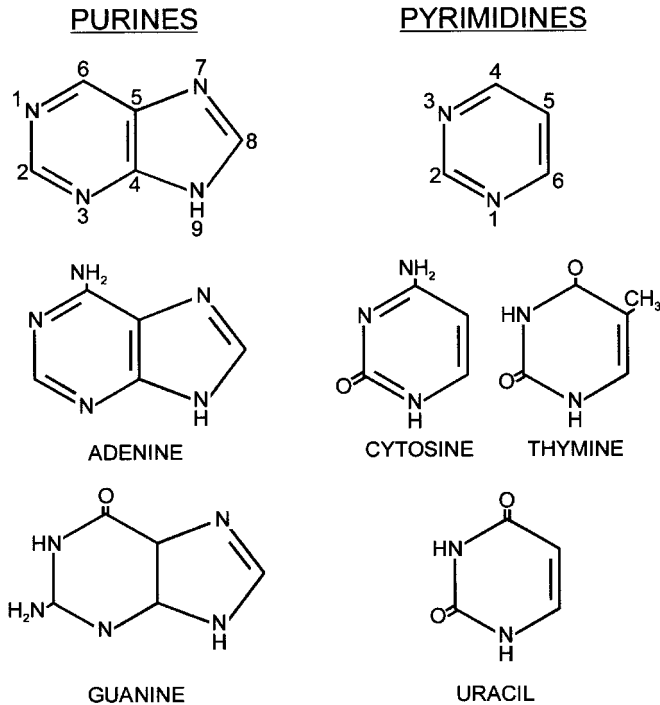
PURINES

PYRIMIDINES

Figure 2.16 General structure of purines, pyrimides, and component bases of RNA and DNA.

discussed, it is important to understand the principal factors that initiate and influence the development of cancer.

2.3.1   Carcinogenesis

DNA LESIONS

The origin of most cancers or neoplasms can be traced to damage (lesions) in the DNA molecule leading to faulty transcription of the genetic signals that regulate the normal growth of somatic tissue. If scrambled genetic information is passed to germinal tissue, chromosomes may be damaged and normal development of offspring will be compromised.

DNA contains two pyrimidine bases (cytosine and thymine) and two purine bases (guanine and adenine). Each base in combination with the monosaccharide 2-deoxy-β-ribofuranose and phosphate forms a nucleotide. Each cytosine-based nucleotide on one strand of DNA is paired with a guanine-based nucleotide on the opposing strand; each thymine-based nucleotide is paired with an adenine-based nucleotide. RNA has a similar structure except that uracil replaces thymine, and the constituent monosaccharide is β-D-ribofuranose. The general purine and pyrimidine structure is shown in Figure 2.16, together with individual bases.

Damage to DNA caused by chemicals or other agents such as UV light or radioactivity takes a variety of forms. Chemical lesions usually result from the
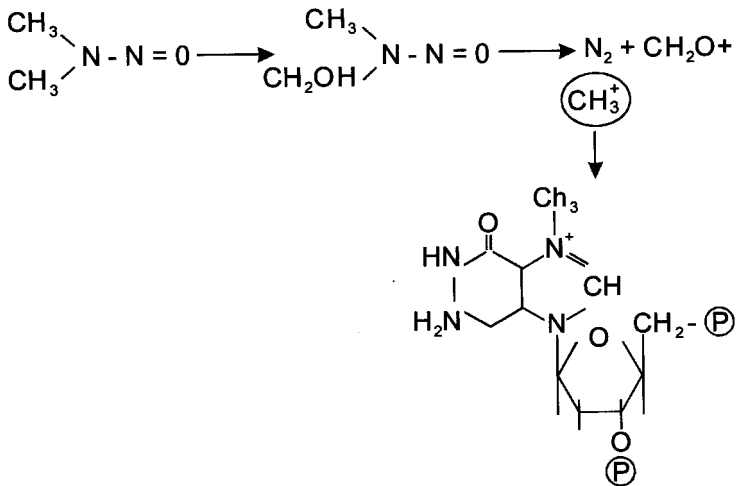
Figure 2.17 Alkylation of N-7 position of guanine by dimethylnitrosamine.

formation of covalent bonds (adducts) between a carcinogen and one of the bases. Sites of action differ depending on the carcinogen involved, and these in turn affect the type and severity of the resulting lesion.

Due to the electron-rich (nucleophilic) nature of the N-7 position of guanine, this site is particularly vulnerable and is subject to attack by alkylating agents such as dimethylnitrosamine (Figure 2.17), epoxides of aflatoxin, and benzo-a-pyrene. The latter also forms a covalent bond with the exocyclic N of guanine. Activated acetylaminofluorene bonds at the C-8 position of guanine. Some planar cyclic compounds insert themselves between the bases of the DNA without forming a covalent bond. This process is called intercalation and may result in distortion of the DNA molecule leading to interference with replication and transcription. Examples of intercalating molecules include acriflavine and 9-aminoacridine.

MUTAGENESIS

Gene mutations may be caused by breaks, omissions, and translocations of chromosome pieces (clastogenesis); uneven distribution of chromosomes following mitosis (aneuploidy); or alterations of DNA known as point mutations. Point mutations may result from base substitutions or from the addition or deletion of base pairs. Numerical changes in base pairs will alter the sequence of base triplets (codons), which form the basis of the transcription process. Such frameshift mutations may, therefore, lead to major changes in protein structure.

The progression from ingestion of a potentially carcinogenic compound to the development of a cancerous condition involves an enormous array of chemical reactions which may advance or retard neoplasia. Many of these chemical transformations are interactive, and the product may be thought of as the net result of a complex of additive, synergistic, and antagonistic events. This complexity, and

the fact that some reactions set the stage for further reactions, virtually eliminates the possibility of a single carcinogenic molecule causing a malignant neoplasia (i.e., a one-hit model where the dose response curve passes through zero). Existing multihit models, although closer to reality, must obviously represent gross simplifications of all the components that contribute to the threshold situation referred to earlier.

Some of these complicating factors are outlined next.

Multidrug resistance. Multidrug resistance (MDR) describes a phenomenon whereby cells become resistant to a variety of unrelated compounds, including xenobiotic chemicals with carcinogenic properties. The term multixenobiotic resistance has been used in some studies. MDR has been described in both prokaryotes and eukaryotes and results from the activity of a 170-kDa transmembrane protein capable of extruding chemicals from cells using an energy-dependent, saturable process. In eukaryotes and some prokaryotes, the protein has been identified as P-glycoprotein. It has a low substrate specificity, and the principal common property of potential substrates appears to be a moderate degree of specificity. p-Glycoprotein is encoded by a small family of genes that differ in number from species to species. In humans, two genes have been identified and in other mammalian species there are up to five genes. p-Glycoprotein is induced by exposure to a variety of endogenous and xenobiotic compounds including estrogen, progesterone, aflatoxin, sodium arsenite, and cadmium as well as X-ray irradiation and heat shock. p-Glycoprotein is particularly highly expressed in late tumour stages, although how this equates with its normal function is poorly understood.

Chemical activation. Some chemicals must be activated to become carcinogenic. Usually the mixed function oxidase (MFO) (cytochrome P450) system is involved. Details of this enzyme system and associated reactions are given in Chapter 7. Carcinogens are produced as by-products in the metabolism of other, relatively benign compounds. Examples of chemical activation include phase I epoxidations such as those of benzo(a)pyrene, aflatoxin B_1, and vinyl chloride (Section 7.8.3). Dichloroethane, a laboratory solvent, may be activated by phase II conjugation with glutathione to haloethyl-*S*-glutathione, an unstable triangular electrophile capable of conjugation with DNA. Activation of acetylaminofluorine (AAF) to the powerful electrophilic nitrenium ion occurs through a phase I hydroxylation and phase II formation of an unstable sulphonate intermediate.

MFO activity may result in the formation of reactive compounds capable of direct interaction with DNA or the formation of reactive oxygen intermediates (ROIs). ROIs may also result from metabolism of compounds that are not themselves carcinogenic. During normal respiration, oxygen accepts four electrons in a stepwise manner in becoming reduced to water. This progressive univalent reduction of molecular oxygen results in the production of such ROIs as the superoxide radical ($O_2^-\bullet$), hydrogen peroxide, and the hydroxyl radical (Figure 2.18). The
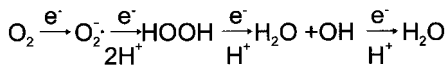
$$O_2 \xrightarrow[]{e^-} O_2^{-\cdot} \xrightarrow[2H^+]{e^-} HOOH \xrightarrow[H^+]{e^-} H_2O + OH^{\cdot} \xrightarrow[H^+]{e^-} H_2O$$

Figure 2.18 Four-stage oxidation involving production of reactive oxygen intermediates.

superoxide anion free radical ($O_2^{-}\cdot$) is formed by the transfer of a single electron to oxygen. It may be converted to hydrogen peroxide (HOOH) spontaneously or through catalysis by superoxide dismutase (SOD). Cleavage of HOOH to the hydroxyl ion (OH^-) and the superreactive hydroxyl radical $OH^{\cdot}$ is catalysed by a number of trace metals in their reduced form (e.g., Cu^+, Fe^{2+}, Ni^{2+}, and Mn^{2+}).

HOOH is also the direct or indirect by-product of several other enzyme reactions (e.g., monoamine oxidase). Most, but not all HOOH production occurs in peroxisomes (see Section 7.5.6), catalysed by oxidases that remove two electrons from substrates and transfer then to O_2. HOOH is also produced by the oxidation of fatty acids.

ROIs generated through normal metabolism or induced by environmental contaminants (e.g., metal ions, pesticides, air pollutants, radiation) can cause significant metabolic dysfunctions. For example, superoxide radicals can cause peroxidation of membrane lipids, resulting in loss of membrane integrity and the inactivation of membrane-bound enzymes. Although not highly reactive, HOOH can inhibit some metalloenzymes, including superoxide dismutase, and is capable of rapid penetration of cell membranes thereby creating toxic effects at several different subcellular locations. Its most important cytotoxic effect is its role in producing hydroxyl radicals, which can indiscriminately attack and damage every type of macromolecule in living cells including lipids, proteins, and DNA.

Carcinogens are categorised as initiators, promoters, progressors, or complete carcinogens.

Initiation is caused by damage to cellular DNA that results in a mutation. If the mutation is not repaired, it becomes permanent, although the cell is nonmalignant and may remain in the organism for several years. However, there is evidence to suggest that such premalignant cells may be selectively excised through a process of programmed cell death, or apoptosis. It is now believed that initiation is a common occurrence and as such may not be a limiting step in the development of neoplasia.

Promotion is the process of increased replication (hyperplasia) of initiated cells leading to the production of a precancerous condition. Several promoters have been identified, including several inducers of cytochrome P450, such as phenobarbitol, butylated hydroxytoluene, 2,3,7,8 TCDD, polychlorinated biphenyls (PCBs), and several chlorinated pesticides (DDT, aldrin, dieldrin, chlordane, and others). In contrast to the situation for initiators, for promoters there is no evidence that they act directly on DNA. Although the mode of action of promoters is poorly

understood, an important component seems to be their stimulation of cell division, which propagates the numbers of mutated cells. The end product is a preneoplastic lesion, which is regarded as a reversible condition insofar as removal of the promoter will halt the process or reverse the condition through apoptosis. Work with phorbol esters has indicated that promotion is influenced by the ageing process (Van Duuren et al., 1975) and by dietary (Cohen and Kendall, 1991) and hormonal factors (Sivak, 1979; Carter et al., 1988).

Although the promotion process is reversible, it often leads to a series of irreversible complex genetic changes known as *progression*. Progression can be a spontaneous process, but it is also enhanced by a variety of chemicals including 2,5,2′,5′-tetrachlorobiphenyl, hydroxyurea, arsenic salts, asbestos, benzene, and derivatives (Pitot and Dragan, 1996). These progressor agents differ from promoters in that they have genotoxic properties. A characteristic of progression is irreversible changes in gene expression, notably that of oncogenes. The continued presence of promoters following the onset of progression will result in an increased number of neoplastic lesions.

Examples of initiators, promoters, and progressors are given in Table 2.6. Chemicals that are capable of performing all these functions independently of other agents are called *complete carcinogens*. *Cocarcinogenicity* is a term that seems to be disappearing from modern usage, describing the potentiation of a carcinogen by a noncarcinogen. A common example of this is the potentiation of polycyclic aromatic hydrocarbons (PAHs), the main cancer-causing agents in tobacco, by catechols, another tobacco by-product. As more is learned about the process of carcinogenesis, the distinction between cocarcinogen and promoter becomes increasingly blurred, and the likelihood is that terms such as *promoter* and *progressor* will also evolve as more is learned about the mechanistics of carcinogenicity.

In Table 2.6, a subset of chemicals, identified with an asterisk, has no specific genotoxic action (i.e., they do not interact with DNA and have no apparent mutagenic activity). Little is known of the exact role(s) of these epigenetic carcinogens, although in view of their chemical diversity, it is likely that they may have several different modes of activity. Some have been shown to alter genetic transcription through a variety of receptor pathways (see Sections 2.3.6 and 7.5.2).

DNA repair. Mechanisms for DNA repair may be broadly categorised into excision-repair and direct repair. Excision-repair involves the removal of a sequence of several nucleotides including the damaged portion of the DNA strand and the insertion of the correct nucleotide sequence using the opposing, undamaged DNA stand as a template. Direct repair involves a reversal of the event which caused a DNA lesion. For example, the methylation of the O^6 position of guanine causes it to be misread as adenine, thereby resulting in a cytosine $\rightarrow$ thymine transition. The enzyme O^6-methylguanine-DNA methyltransferase reverses this process and restores the correct base-pairing (Shevell et al., 1990). There are

Table 2.6. Examples of different modes of action of chemical carcinogens[a]

Initiators	Promoters	Progressors
Dimethylnitrosamine	2,3,7,8 TCDD*	Benzene
2-Acetylaminofluorene*	Cholic acid	Arsenic salts
Benzo(a)pyrene	Polychlorinated biphenyls*	
Dimethylbenz(a)anthracene	Dichlorodiphenyl trichloroethane (DDT)	
	Peroxisome proliferators (see Section 7.5.6)	
	Dieldrin*	

[a] Chemicals with asterisks (*) show no direct genotoxic activity (i.e., they are epigenetic).

at least four genes (ada, AlkA, AlkB, and aidB) known to exercise control over this DNA repair system.

Cell death. Exposure to toxic chemicals results in two types of cell death: necrosis and apoptosis.

Necrosis is a form of mass cell death caused by high doses of toxic chemicals or severe hypoxia. It is an uncontrolled process characterised by the swelling of cells and organelles, random disintegration of DNA, acute inflammation of cell clusters, and secondary scarring. The time course of necrosis is several hours to several days.

Apoptosis is a highly regulated, energy-dependent homeostatic process that takes the form of a programmed excision of ageing, damaged, or preneoplastic cells. It is, therefore, responsible for maintaining the normal morphometric and functional integrity of tissues as well as acting as a means of preventing the onset of neoplasia. It is a controlled process involving individual cells rather than clumps of cells (as in necrosis) and acts on a faster time scale (<10 h) than necrosis. DNA is cleaved in an orderly manner, first in large, then smaller fragments. This process is mediated by members of the caspase family of protease enzymes (Enari et al., 1998). The cells shrink without the collapse of the tissue, and there is no associated inflammation or scarring. Apoptosis may be induced by low levels of chemicals that may cause necrosis in higher concentration [e.g., 1,1-dichloroethylene (Reynolds et al., 1984) and dimethylnitrosamine (Ledda-Columbano et al., 1991)]. Apoptosis may also be stimulated by small doses of ionising radiation, cancer therapeutic agents, changes in hormone balance, and hyperthermia.

Increasing evidence demonstrates that apoptosis may be induced in response to DNA damage, through the mediation of the p53 tumour-suppresser gene, although there is clearly a delicate balance between doses of chemicals that may induce apoptosis and those that may stimulate counteractive effects and necrosis. Tumour

promoters may reverse apoptosis (Bellomo et al., 1992), and oxidative stress and ROIs have been shown to stimulate the opposing effects of abnormal cell growth on one hand and apoptosis on the other.

2.3.2   Genotoxicity assays

Although there is no direct evidence that mutations result in carcinogenicity, the indirect evidence for this is very strong, and screening for mutagenicity is a valuable tool in assessing cancer-causing potential. The most widely used assay is the Ames test (Ames et al., 1975), which uses a mutant strain of *Salmonella typhimurium* incapable of synthesising histidine and unable to grow in a histidine-free medium. Reversion to a histidine-producing wild type provides a means of quantifying mutagenic activity. The Ames *Salmonella* assay is very versatile and has proven effective over a range of situations and media including water, gaseous emissions, tobacco smoke, marine sediments, fuels, and food. It possesses some limitations as a means of forecasting carcinogenicity. For example, epigenic carcinogens act in a nongenotoxic manner and would not be expected to give a positive result in an Ames test. Examples include arsenic, chlordane, and dioxins. Studies comparing *Salmonella* mutagenicity with tumour induction in rodents indicate a 70–80% coincidence between bacterial and rodent assays (Tennant and Ashby, 1991; Gold et al., 1993). This correlation improves to >90% with the exclusive selection of probable carcinogens identified through structure activity relationships (SAR) (see also Section 5.5), although it is recognised that forecasting carcinogenicity using the *Salmonella* assay is more reliable for some groups of compounds than for others. One inherent problem is that of time scale. The *Salmonella* test provides a rapid (ca. 2 days), convenient means of quantifying mutagenic potential. However, the development of a neoplastic condition is clearly a much longer process, often several years, and is subject to numerous checks and balances. Thus, the correlation is somewhat analogous to judging the outcome of a long and complex journey based on the first few steps. Nevertheless, the scientific community generally regards the Ames test as an effective screening assay for an important correlate of carcinogenicity. In accepting this model, the extrapolation is made from prokaryotes to eukaryotes. However, much smaller phylogenic jumps still cause interpretive problems for carcinogenicity data. For example, it is not always straightforward to predict human carcinogenicity from experiments on laboratory test animals such as rats and mice, even after normalising for body size. Tests on rodents result in an approximately 500-fold overestimate of human cancer risk from vinyl chloride, and there are many other mismatches of such data to a greater or lesser degree. Nevertheless, very often, such assays are all we have, and there is sufficient confidence in them to support a large body of regulatory legislation involving chemical registration and discharge.

Typically, full carcinogenicity bioassays involve 50 individual male and female rats and the same numbers of mice. Six-week-old animals are exposed for a 2-year period, with a further, postexposure observation period of up to 6 months. Each

test costs between U.S. \$0.5 million and \$1.0 million. To reduce reliance on such tests and to minimise, or at least better understand, phylogenetic extrapolations, most human mutagenicity testing is now performed using mammalian cell preparations. These include in vitro assays of mammalian cell lines, including human somatic cells, and in vivo tests on human and other mammalian lymphocytes. The latter tests may be followed by in vitro culturing of mutant cells or amplification of altered DNA using the polymerase chain reaction to provide sufficient material for molecular analysis.

2.3.3 Chromosome studies

Chromosomal aberrations have been detected by microscopic examination of a variety of cells, notably lymphocytes, from test animals and humans exposed to mutagens. Physical derangement of chromosomes (clastogenesis) takes a number of forms including breaks, omissions, and abnormal combinations of chromosomes or fragments. Sister chromatid exchange has been correlated with DNA strand breakage through chemical intercalation (Pommier et al., 1985) and is used as an assay for several different carcinogens. Many of these observations are carried out during mitosis when the chromosomes may be easily visualised with the use of differential stains. Discernibility of omissions, inversions, and exchanges has been greatly enhanced by the use of fluorescent probes, a technique known as fluorescence in situ hybridisation (acronym FISH).

Aneuploidy is a condition wherein chromosomes become unevenly distributed during cell division in either meiosis or mitosis. It is caused by damage to the meiotic or mitotic spindle, or to the point of attachment between the chromosome and the spindle. Known or suspected aneugens include X-rays, cadmium, chloral hydrate, and pyrimethamine (Natarajan, 1993). Aneuploidy may also arise from hereditary conditions such as Down's syndrome.

2.3.4 The concept of threshold toxicity

Characterisation of the dose or ambient chemical concentration at which toxic effects first appear (threshold) is a fundamental objective of toxicology, but one that has been approached in a variety of ways and is still the subject of controversy. The concept of threshold is embodied in a number of population-based empirical measures of toxicity (e.g., LOEC, NOEC). However, other models dealing with this concept usually adopt a more mechanistic approach. As more information is gathered on how toxic chemicals exert their effect at the molecular level, a variety of mechanistic models have been proposed. Most of these models move us away from the notion of the one-hit model, which was originally proposed to describe carcinogenicity, to multihit models that incorporate a value for the critical number of hits that must occur to elicit a toxic response. Such models may incorporate putative single or multiple receptors and form the basis of the linearised multistage (LMS) model which is used by the U.S. Environmental Protection Agency to determine life-time cancer risk. Embodied in this approach is the
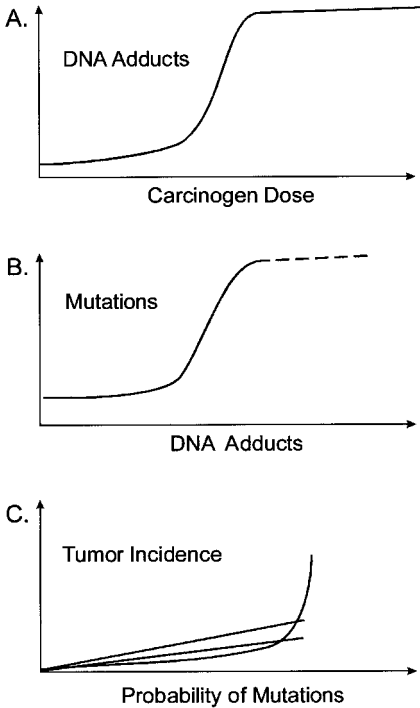
Figure 2.19 Nonlinear mechanistic model relating different factors associated with carcinogenicity. (A) The relationship between carcinogen dose and DNA adduct formation. (B) The relationship between mutation rate and adduct formation. (C) The linear line collectively represent the slopes of A plus B. The exponential increase in tumour incidence. Based on Lutz (1990).

notion that eukaryotic organisms have a whole arsenal of defence mechanisms to cope with an anticipated range of noxious environmental stimuli. The toxic threshold is, therefore, perceived as the point at which there is a preponderance of damaging overcompensatory (repair) effects. It may be seen as a gradual shift in balance or a more precipitous "spillover" effect.

Evidence from mammalian bioassays indicates a strongly sigmoid relationship between carcinogen dose (e.g., 2-acetylaminofluorene) and DNA adduct formation. Mutation rate, in turn, is nonlinearly related to adduct formation and shows a steep increase after adducts reach a certain concentration. Dose-response relationships associated with chemical carcinogenesis have been examined by Lutz (1990), who concluded that the sigmoid curves relating dose to adduct formation and adduct formation to mutagenesis would result in an exponential increase in tumour incidence as a function of probable mutations (Figure 2.19). It is, therefore, clear that evidence for a mechanistic basis for threshold toxicity is beginning to accumulate.

A more recent extension of this concept is the demonstration of an active response to low-level doses of toxic chemicals, which actually stimulate cell

growth and repair. This may happen to the extent that, at low levels of toxicant exposure, normal (i.e., nonneoplastic) cellular proliferation may even exceed control values. This is called hormesis.

2.3.5 Hormesis

Hormesis is a stimulatory effect of low levels of toxic agent on an organism. It may be expressed as an enhancement of some measure of physiological or reproductive fitness relative to control values and is superseded by inhibitory effects at increasing levels of toxicant exposure. Hormetic effects have been shown to result from exposure to trace metals and organic chemicals and have been reported from both plants and animals. Additionally, hormetic effects of radiation on animals, particularly humans, have been extensively studied.

Mechanistically, hormesis has been explained in two principal ways. A more specific, yet less common, occurrence stems from the fact that several inorganic contaminants such as trace metals, while toxic at high levels, are essential to the survival of both plants and animals at very low levels. Examples of essential metals include arsenic, cobalt, copper, iron, manganese, molybdenum, selenium, and zinc. In situations where an organism is deficient in one of these metals, a low dose may cause a beneficial effect. A less specific yet probably more broadly applicable explanation for hormesis is a transient overcorrection by detoxification mechanisms responding to inhibitory challenges well within the organism's zone of tolerance.

Chemicals known to stimulate nonneoplastic hepatocellular generation include allyl alcohol, bromotrichloromethane, carbon tetrachloride, chloroform, and ethylene dibromide. Hormesis has been interpreted as evidence for the functional separation of the "damage" component, and the "repair" component, and, although it is assumed that these in turn have subcomponents, this provides support for looking at cancer development as a stochastic two-stage process.

Radiation hormesis describes the stimulatory and arguably beneficial effects of low-level ionising radiation, generally doses less than $10\,\text{mSv day}^{-1}$. The concept that low levels of radiation might actually be beneficial is still radical and far from proven. However, various studies have shown that cells and organisms adapt to low-level ionising radiation (LLIR) (see Chapter 8) by the stimulation of repair mechanisms. Holzman (1995) described a radiation-induced adaptive response wherein rat lung epithelial cells exposed to low-level alpha radiation increased production of the p53 tumour suppressor protein. Several reports have been made of radiation-induced protein induction. Although some have speculated that this induction may serve to maintain optimal protein levels for cell repair and function (Boothman et al., 1989), further beneficial effects remain speculative at present.

2.3.6 Receptors

From a regulatory standpoint most legislation is based on toxicological information gathered at the whole organism or tissue level. However, from a mechanistic
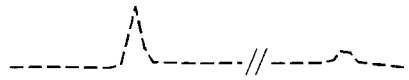
**Chromatographic Trace
for Protein Separation**

LMW = Low Molecular Weight
HMW = High Molecular Weight

LMW   HMW
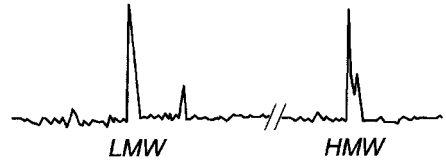
**Mercury Analysis of Fractions
Collected Concomitantly with
Protein Chromatography**

*Preliminary (sub-lethal)
Uptake / Distribution*

*Later (toxic) Metal
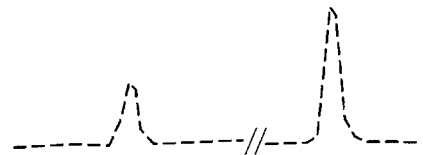Accumulation / Distribution*

Figure 2.20 The spillover concept in metal toxicity. The concept is illustrated by the saturation by a toxic metal of a low-molecular-weight, protective protein (e.g., metallothionein) and the subsequent association of the metal with a high-molecular-weight protein (e.g., enzyme).

point of view, we recognise that such information is often at best correlative and may tell us little about the mode of toxic action. We may document unusually high exposure to and bioaccumulation of potentially toxic chemicals and may be able to arrive at estimates of toxic dose [e.g., through the lethal body burden (LBB) approach described in Chapter 3]. Yet, in this approach, we recognise that such a dose represents only a crude indication that defence mechanisms are overloaded. In fact, much of the tissue or body burden of a toxic chemical or mixture of chemicals often results from its storage in a benign form (e.g., bioaccumulation of hydrophobic nonpolar organics in neutral storage lipids or storage of trace metals associated with metallothioneins). When chemicals move from benign storage to potentially damaging receptor sites, toxicity occurs. The actual toxic lesion may result from only a tiny proportion of the ingested toxicant acting on a narrowly circumscribed receptor. Sometimes, such a situation can arise as a spillover effect following saturation of available sites on protective proteins such as metallothioneins. Such an effect may be traced at the molecular level as a shift in metal associated with a low-molecular-weight protein to a high-molecular-weight protein such as an enzyme (Figure 2.20).

Receptors are defined as normal body constituents that are chemically altered by a toxicant resulting in injury and toxicity. Usually receptors are proteins, although they can also be nucleic acids or lipids. For example, alkylating agents such as *N*-nitroso compounds are activated by the mixed function oxidase (MFO)

Table 2.7. Inhibition of metabolic enzymes by xenobiotics

Toxicants	Effect
As	Inhibition of pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, blockage of mitochondrial electron transport, oxidative phosphorylation.
Sb^{3+}	Competitive inhibition of fructose-6-phosphate. The hydrated species $Sb^{3+} (H_2O)_3$ is a structural analog of fructose-6-phosphate and binds to substrate binding site on phosphofructokinase. Inhibition of glycolysis, partial blockage of energy production leading to loss of cell function and cell death.
Pentachlorophenol, 2-*sec*-butyl-4,6-dinitrophenol (Dinoseb)	Uncouples electron transport system (ETS) from control by oxidative phosphorylation. ETS reverses, resulting in ATP hydrolysis and heat generation.
Rotenone	Binds reversibly with NADH dehydrogenase resulting in lack of flow of reduced electrons and inhibition of energy production.
2,4-D	Inhibition of succinate dehydrogenase and cytochrome-c reductase. Damage to bioenergetic function of mitochondria.

system in the liver to carbonium ions which react spontaneously with N-, O-, and S-containing functional groups and with nonesterified O groups in phosphates. The N-7 of the purine DNA base guanine is particularly vulnerable to electrophilic attack (Figure 2.17), and methylation may result in the pyrazine ring being broken, thereby distorting the base or resulting in the excision of the purine with consequent mutagenic potential. Several chemicals may cause DNA lesions through covalent bonding with purine and pyrimidine bases. Examples are given in Chapter 7. For nucleic acid receptors, the end-point is mutation. In the case of protein receptors, toxic chemicals elicit a variety of responses depending on the function of the protein and the part it plays in the survival of the cell. Depending on the constituent amino acids, a number of different functional groups (e.g., amino, carboxyl, hydroxyl, sulphydryl) may be affected, leading to protein denaturation or blockage of an active site for the binding of endogenous substrates. In the latter case, the protein may be an enzyme and/or a transporter molecule. Enzymic function often confers a high degree of specificity on the receptor, and the bond formed with the toxicant is often reversible.

Where toxicants act competitively with endogenous substrates, the response may be dependent on the relative proportions of the substrate, antagonist, and available active sites or ligands. Enzyme-substrate kinetics may be diagnostic in determining the competitive or noncompetitive nature of the interaction (Section 3.5.5). Where the enzyme is catabolic in nature, occupation of the active site by a toxicant acting as an analog of the normal substrate may result in a build-up of that substrate or a failure in transmembrane movement of the substrate. For example,

Table 2.8. Receptors capable of handling organic compounds

Receptor	Characteristics
Aromatic (aryl) hydrocarbon receptor (AhR)	High-affinity aromatic (aryl) hydrocarbon-binding protein forms a complex with nuclear translator (ARNT) prior to DNA binding (Figure 7.20) and transcription of genes responsible for the production of various metabolic enzymes [e.g., glutathione-s-transferase, glucuronyl transferase, CYP1A1, CYP1A2 (see Section 7.8.3)].
Thyroid receptor (TR)	Thyroid hormone receptors are associated with the plasma membranes, mitochondrial membranes, and nuclear membranes in cells of hormone-responsive tissues. The principal activators are thyroxine and triiodothyronine. Activation of nuclear receptors results in the increased formation of RNA and subsequent elevated levels of protein synthesis and enzyme activity.
Retinoid receptors (RAR); Retinoic acid Receptor (RXR)	Retinoids are important for reproduction and development. They inhibit β-hydroxysteroid dehydrogenase, which converts estradiol to estrone. The peroxisome proliferator receptor, the RAR, and the thyroid receptor are capable of association with the RXR, resulting in the formation of a heterodimer.
Peroxisome proliferator–activated receptor (PPAR)	Peroxisomes are subcellular organelles in most plant and animal cells, performing various metabolic functions including cholesterol and steroid metabolism, β-oxidation of fatty acids, and hydrogen peroxide–derived respiration. Although endogenous activators of PPAR are fatty acids and prostaglandins, several industrial and pharmaceutical products have demonstrated affinity for the receptor and, therefore, peroxisome proliferating properties.
Estrogen receptor (ER)	This member of the thyroid/retinoic acid/steroid hormone receptor superfamily exhibits much structural homology with other receptors. Although its primary activator is the major female sex hormone 17β-estradiol, the ER is capable of interacting with a broad range of chemicals, including pesticides, PCBs, industrial phenols, and phthalates – the so-called xenoestrogens. Endocrine disruption through the mediation of ER is highly complex. Complications include cross-reactivity with other receptors and the fact that ER will interact with both estrogen agonists and antiestrogens.
Invertebrate receptors – ecdysteroid receptor, farnesoid receptor	Invertebrates possess unique enzymes such as the molting hormone (ecdysone) and the juvenile hormone (methyl farnesoate). Although these hormones bear little structural resemblance to the vertebrate hormone estrogen, they show a significant degree of homology with the retinoid receptor (RAR) and are capable of forming functional heterodimers with RXR.

an elevated level of aminolaevulinic acid (ALA) is regarded as diagnostic of lead intoxication through its inhibition of ALA-dehydratase. Lead may also displace calcium from binding sites on the intracellular protein calmodulin. Effects of various toxicants on enzymes involved with energy utilisation are shown in Table 2.7. In some cases, inhibition is achieved through occupation of the active site of the enzyme. In other cases, the effect is caused by the displacement of an integral component of the enzyme molecule. For example, trace metals such as copper and zinc are components of several enzymes, and their displacement by mercury may denature those enzymes. For some enzymes, the bond formed with the toxicant may be very strong and essentially irreversible. An example is the bond formed between an acetylcholine analogue such as paraoxon and the enzyme acetyl-cholinesterase. Here the loss of the nitrophenol group from the paraoxon increases the basicity of the serine hydroxyl group on the enzyme through an interaction with an adjacent imidazole nitrogen. This facilitates a phosphorylation, which is extremely difficult to reverse and functionally incapacitates the enzyme (Figure 7.13).

Intracellular receptors that regulate gene expression belong to a family of receptor proteins that apparently follow a similar phylogenetic lineage and that interact with a variety of endogenous substrates such as thyroid hormones, sex hormones (e.g., estrogen), and corticosteroids. The xenobiotic (Ah) receptor (see Section 7.5.2) may be a relative of these. These receptors are found intracellularly because their substrates are sufficiently lipid-soluble to cross the plasma membrane, and some, such as the estrogen receptor, are found in the nucleus itself. In the case of these receptors, the xenobiotic itself may be the substrate (e.g., Ah receptor) or may mimic the normal endogenous substrate. These circumstances would still fall within our initial (toxicological) definition of receptor if they led to an inappropriate function of the gene regulator, such as the induction of transsexual characteristics. Examples of different receptors capable of handling endogenous and exogenous organic compounds are shown in Table 2.8.

2.4   Questions

1. How is the term *dose* used in environmental toxicology? Describe the different ways dose has been related to toxicity in all branches of the subject.

2. What is an LC_{50}? Describe how toxicologists measure it, including some of the statistical models that have been used to arrive at this parameter. What are the advantages and disadvantages of this means of assessing toxicity?

3. Chronic or subacute toxicity bioassays have often been shown to be more sensitive than acute toxicity tests. Using examples from a broad range of assays, describe the types of end-points commonly employed in these tests. Give two examples of strategies employed to extrapolate from acute to chronic toxicity end-points.

4. Describe two different ways of arriving at a lowest observed effect concentration and a no (observed) effect concentration. What are the advantages of a generalised linear model approach compared with preceding population response distribution models?

5. Compare and contrast toxicity bioassays in plants and animals in (a) aquatic and (b) terrestrial environments.

6. Describe the major components of carcinogenesis, including factors that may advance or retard the process. Give an account of three assays that have been used as direct or correlative evidence of carcinogenesis.

7. How is the concept of threshold toxicity dealt with in population-based dose-response models compared with more mechanistic models? Give two explanations for hormesis. Discuss the pros and cons of current dose-response models in describing and quantifying this phenomenon.

8. The term *receptor* has acquired a broad array of definitions in environmental toxicology. At the molecular and submolecular level, give an account of the toxicological significance of different kinds of receptors, illustrating your answer with specific examples.

2.5   References

Abbott, W. S. (1925) A method of computing the effectiveness of an insecticide, *Journal of Economic Entomology*, **18**, 265–7.

American Society for Testing and Materials (ASTM). (1992) Standard guide for conducting three-brood renewal toxicity rests with *Ceriodaphnia dubia*, Designation E 1295–89. Annual Book of ASTM Standards, Vol. 11.04.669–684, ASTM, Philadelphia.

Ames, B., McCann, J., and Yamasaki, E. (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test, *Mutation Research*, **31**, 347–64.

Bellomo, G., Perotti, M., Taddei, F., Mirabelli, F., Finardi, G., Nicotera, P., and Orrenius, S. (1992) Tumor necrosis factor-inf induces apoptosis in mammary adenocarcinoma cells by an increase of intranuclear free Ca^{2+} concentration and DNA fragmentation, *Cancer Research*, **52**, 1342–6.

Bliss, C. I. (1934a) The method of probits, *Science*, **79**, 38–9.
   (1934b) The method of probits – A correction!, *Science*, **79**, 409–10.

Boothman, D., Bouvard, I., and Hughes, E. N. (1989) Identification and characterization of X-ray-induced proteins in human cells, *Cancer Research*, **49**, 2871–8.

Carter, J. H., Carter, H. W., and Meade, J. (1988) Adrenal regulation of mammary tumorigenesis in female Sprague-Dawley rats: Incidence, latency, and yield of mammary tumors, *Cancer Research*, **48**, 3801–7.

Caswell, H. (1996) Demography Meets Ecotoxicology: Untangling the Population Level Effects of Toxic Substances. In *Ecotoxicology. A Hierarchial Treatment*, eds. Newman, M. C., and Jagoe, C. H., pp. 255–92, Lewis, Boca Raton, FL.

Chapman, P. M. (1986) Sediment quality criteria from the sediment quality triad: An example, *Environmental Toxicology and Chemistry*, **5**, 957–64.
   (2000) Whole effluent toxicity testing – Usefulness of level of protection, and risk assessment, *Environmental Toxicology and Chemistry*, **19**, 3–13.

Christopher, S. V., and Bird, K. T. (1992) The effects of herbicides on development of *Myriophyllum spicatum* L. cultured in vitro, *Journal of Environmental Quality*, **21**, 203–7.

Cohen, S. M., and Kendall, M. E. (1991) Genetic errors, cell proliferation and carcinogenesis, *Cancer Research*, **51**, 6493–505.

Cox, D. R., and Oakes, D. (1984) *Analysis of Survival Data*, Chapman and Hall, London.

Dixon, P. M., and Newman, M. C. (1991) Analyzing Toxicity Data Using Statistical Models for Time-to-Death: An Introduction. In *Metal Ecotoxicology: Concepts and Applications*, eds. Newman, M. C., and McIntosh, A. W., pp. 207–42, Lewis, Boca Raton, FL.

Enari, M., Sakahira, H., Yokoyama, H., Okaura, K., Iwamatsce, A., and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis and its inhibitor ICAD, *Nature*, **391**, 43–50.

Finney, D. J. (1971) *Probit Analysis*, Cambridge University Press, Cambridge.

Fleming, W. J., Ailstock, M. S., and Momot, J. J. (1995) Net Photosynthesis and Respiration in Sago Pondweed (*Potamogeton pectinatus*) Exposed to Herbicides. In *Environmental Toxicology and Risk Assessment*, STP 1218, eds. Hughes, J., Biddinger, G., and Moses, E., American Society for Testing and Materials, Philadelphia.

Fletcher, J. S. (1990) Use of Algae Versus Vascular Plants to Test for Chemical Toxicity. In *Plants for Toxicity Assessment*, ASTM STP 1091, eds. Wang, W., Gorsuch, J. W., and Lower, W. R., pp. 33–9, American Society for Testing and Materials, Philadelphia.

Fletcher, J. (1991) Keynote Speech: A Brief Overview of Plant Toxicity Testing. In *Plants for Toxicity Assessment*, vol. 2, ASTM STP 1091, eds. Gorsuch, J. W., Lower, W. R., Wang, W., and Lewis, M. A., pp. 5–11, American Society for Testing and Materials, Philadelphia.

Gaddum, J. H. (1933) Reports on biological standards. III. Methods of biological assay depending on quantal response. Medical Research Council Special Report Series 183, HMSO, London.

Gannon, J. E., and Beeton, A. M. (1971) Procedures for determining the effects of dredged sediment on biota-benthos viability and sediment selectivity tests, *Journal Water Pollution Control Federation*, **43**, 393–8.

Garten, J., and Garten, C. T., Jr. (1990) Multispecies Methods of Testing for Toxicity: Use of the Rhizobium-Legume Symbiosis in Nitrogen Fixation and Correlations Between Responses by Algae and Terrestrial Plants. In *Plants for Toxicity Assessment*, ASTM STP 1091, eds. Wang, W., Gorsuch, J. R., and Lower, W. R., pp. 69–84, American Society for Testing and Materials, Philadelphia.

Gelber, R. D., Lavin, P. T., Mehta, C. R., and Schoenfeld, D. A. (1985) Statistical Analysis. In *Fundamentals of Aquatic Toxicology*, eds. Rand, G. M., and Petrocelli, S. R., pp. 110–23, Hemisphere, Washington, New York, and London.

Giesy, J. G., and Hoke, R. A. (1990) Freshwater Sediment Quality Criteria: Toxicity Bioassessment. In *Sediments: Chemistry and Toxicity of In-Place Pollutants*, eds. Baudo, R., Giesy, J. P., and Mantau, H., pp. 265–348, Lewis, Ann Arbor, MI.

Giesy, J. P., and Graney, R. L. (1989) Recent developments in the intercomparisons of acute and chronic bioassays and bioindicators, *Hydrobiologia*, **188/189**, 21–60.

Gobas, F. A. P. C., Lovett-Doust, L., and Haffner, G. D. (1991) A Comparative Study of the Bioconcentration and Toxicity of Chlorinated Hydrocarbons in Aquatic Macrophytes and Fish. In *Plants for Toxicity Assessment*, vol. 2, ASTM STP 1115, eds. Gorsuch, J. W., Lower, W. R., Wang, W., and Lewis, M. A., pp. 178–93, American Society for Testing and Materials, Philadelphia.

Gold, L. S., Stone, T. H., Stern, B. R., and Bernstein, L. (1993) Comparison of trafet organs of carcinogenicity for mutagenic and non-mutagenic chemicals, *Mutation Research*, **286**, 75–100.

Grant, W. F. (1994) The present status of higher plant bioassays for the detection of environmental mutagens, *Mutation Research*, **310**, 175–85.

Hamilton, M. A., Russo, R. C., and Thurston, R. V. (1977) Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays, *Environmental Science and Technology*, **7**, 714–19.

Hayes, W. J., Jr. (1975) *Toxicology of Pesticides*, Williams and Wilkins, Baltimore.

Holzman, D. (1995) Hormesis: Fact or fiction, *Journal of Nuclear Medicine*, **36**, 13–16.

Hurlbert, S. H. (1984) Pseudoreplication and the design of ecological field experiments, *Ecological Monographs*, **54**, 187–211.

Kenaga, E. E. (1982) Predictability of chronic toxicity from acute toxicity of chemicals in fish and aquatic invertebrates, *Environmental Toxicology and Chemistry*, **1**, 347–58.

Kerr, D. R., and Meador, J. P. (1996) Modelling dose response using generalized linear models, *Environmental Toxicology and Chemistry*, **15**, 395–401.

Ledda-Columbano, G. M., Coni, P., Curto, M., Giacomini, L., Faa, G., Oliverio, S., Piacentini, M., and Columbano, A. (1991) Induction of two different modes of cell death, apoptosis and necrosis, in rat liver after a single dose of thioacetamide, *American Journal of Pathology*, **139**, 1099–109.

Lee, G., Ellersieck, M. R., Mayer, F. L., and Krause, G. F. (1995) Predicting chronic lethality of chemicals to fishes from acute toxicity test data: multifactor probit analysis, *Environmental Toxicology and Chemistry*, **14**, 345–9.

Leicester, H. M. (1956) *Historical Background of Chemistry*, Dover, New York.

Litchfield, J. T., and Wilcoxon, F. (1949) A simplified method of evaluating dose-effect experiments, *Journal of Pharmacology and Experimental Therapeutics*, **96**, 99–113.

Lutz, W. K. (1990) Dose-Response Relationships in Chemical Carcinogenesis: From DNA Adducts to Tumor Incidence. In *Biological Reactive Intermediates IV*, ed. Witmer, C. M., Plenum Press, New York.

Mayer, F. L., Krause, G. F., Buckler, D. R., Ellersieck, M. R., and Lee, G. (1994) Predicting chronic lethality of chemicals to fishes from acute toxicity test data: Concepts and linear regression analysis, *Environmental Toxicology and Chemistry*, **13**, 671–8.

Mulvey, M., and Diamond, S. A. (1991) Genetic Factors and Tolerance Acquisition in Populations Exposed to Metals and Metalloids. In *Metal Ecotoxicology: Concepts and Applications*, eds. Newman, M. C., and McIntosh, A. W., pp. 301–21, Lewis, Chelsea, MI.

Natarajan, A. T. (1993) An overview of the results of testing of known or suspected aneugens using mammalian cells *in vitro*, *Mutation Research*, **287**, 47–56.

Newman, M. C. (1995) *Quantitative Methods in Aquatic Ecotoxicology*, Lewis, Boca Raton, FL.

Newman, M. C., and Dixon, P. M. (1996) Ecologically Meaningful Estimates of Lethal Effects in Individuals. In *Ecotoxicology: A Hierarchial Treatment*, eds. Newman, M. C., and Jagoe, C. H., pp. 225–53, Lewis, Boca Raton, FL.

Nyholm, N. (1985) Response variable in algal growth inhibition tests: biomass or growth rate?, *Water Research*, **19**, 273–9.

Nyholm, N., Sorenson, P. S., and Kusk, K. O. (1992) Statistical treatment of data from microbial toxicity tests, *Environmental Toxicology and Chemistry*, **11**, 157–67.

Peterson, H. G., Nyholm, N., Nelson, M., Powell, R., Huang, P. M., and Scroggins, R. (1996) Development of aquatic plant bioassays for rapid screening and interpretive risk assessments of metal mining liquid waste water, *Water Science and Technology*, **33**, 155–61.

Pitot, III, H. C., and Dragan, Y. P. (1996) Chemical Carcinogenesis, Chapter 8. In *Casarett & Doull's Toxicology: The Basic Science of Poisons*, ed. Klaassen, C. D., pp. 201–67, McGraw-Hill, New York.

Pommier, Y., Zwelling, L. A., Kao-Shan, C. S., Whang-Peng, J., and Bradley, M. O. (1985) Correlations between intercalator-induced DNA strand breaks and sister chromatid exchanges, mutations and cytotoxicity in Chinese hamster cells, *Cancer Research*, **45**, 3143.

Reporter, M., Robideaux, M., Wickster, P., Wagner, J., and Kapustka, L. (1991) Ecotoxicological assessment of toluene and cadmium using plant cell cultures. In *Plants for Toxicity*

Assessment, vol. 2, ASTM STP 1115, eds. Gorsuch, J. W., Lower, W. R., Wang, W., and Lewis, M. A., pp. 240–9, American Society for Testing and Materials, Philadelphia.

Reynolds, E. S., Kanz, M. F., Chieco, P., and Moslen, M. T. (1984) 1,1-Dichloroethylene: An apoptotic hepatotoxin?, *Environmental Health Perspectives*, **57**, 313–20.

Shevell, D. E., Friedman, B. M., and Walker, G. C. (1990) Resistance to alkylation damage in *Escherichia coli*: Role of the Ada protein in induction of the adaptive response, *Mutation Research*, **233**, 53–72.

Sivak, A. (1979) Carcinogenesis, *Biochemical and Biophysical Research Communications*, **560**, 67–89.

Sprague, J. B. (1973) The ABCs of pollutant bioassay with fish. In *Biological Methods for the Assessment of Water Quality*, STP 528, eds. Cairns, J. J., and Dickson, K. L., pp. 6–30, American Society for Testing and Materials, Philadelphia.

Swanson, S. M., Rickard, C. P., Freemark, K. E., and MacQuarrie, P. (1991) Testing for pesticide toxicity to aquatic plants: Recommendations for test species. In *Plants for Toxicity Assessment*, vol. 2, ASTM STP 1115, eds. Gorsuch, J. W., Lower, W. R., Wang, W., and Lewis, M. A., pp. 77–97, American Society for Testing and Materials, Philadelphia.

Tennant, R. W., and Ashby, J. (1991) Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 39 chemicals tested for carcinogenicity by the U.S. National Toxicology Program, *Mutation Research*, **257**, 209–27.

U.S. Environmental Protection Agency (U.S. EPA). (1991) *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, 4th ed., ed. C. I. Weber, EPA-600/4-90/027, U.S. EPA, Washington, DC.

Van Duuren, B. L., Sivak, A., and Katz, C. (1975) The effect of aging and interval between primary and secondary treatment in two-stage carcinogenesis on mouse skin, *Cancer Research*, **35**, 502–5.

Versteeg, D. J. (1990) Comparison of short- and long-term toxicity test results for the green alga, *Selenastrum capricornutum*. In *Plants for Toxicity Assessment*, ASTM STP 1091, eds. Wang, W., Gorsuch, J. W., and Lower, W. R., pp. 40–8, American Society for Testing and Materials, Philadelphia.

Wright, D. A. (1977) Toxicity of fluoride to brown trout fry *Salmo trutta*, *Environmental Pollution*, **12**, 57–62.

Wright, D. A., and Roosenburg, W. H. (1982) Trimelthyltin toxicity to larval *Uca pugilator*: Effects of temperature and salinity, *Archives of Environmental Contamination and Toxicology*, **11**, 491–5.