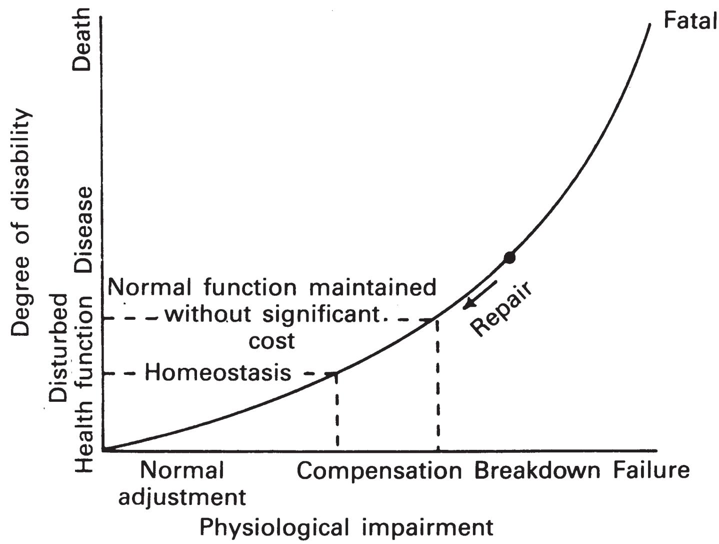

Figure 4.17 A possible relation between physiological impairment following increasing exposure to pollutants and the consequent disability of the fish (Lloyd, 1972)

wide range of modern diagnostic and analytical techniques, the state of health of the test animals can be relatively easily determined, but this is not necessarily the case. For example, in order to state that a particular value is abnormal it is necessary to know the normal range for that particular variable, and the way it is affected by the physiological status and environmental history of the animal. For aquatic animals, including fish, such detailed knowledge of their biochemistry and physiology is generally lacking. Thus although it is possible to say that a particular value is statistically different from that of the control animals, it cannot readily be inferred that the change has any ecological consequences. The 'abnormal' value may represent not damage to the fish, but a metabolic adjustment well within the animal's ability to compensate for varying environmental conditions, which are a normal feature of aquatic life and to which many aquatic animals have a wide range of tolerance. Mount and Stephan (1967a) succinctly stated the difficulty thus: 'An exposure causing death is obviously significant, but even the best fish physiologist would have difficulty establishing that a 10 per cent reduction in haematocrit would result in an undesirable effect on a population'.

A more detailed exposition of the problem was given by Lloyd (1972) with the aid of the diagram reproduced in Figure 4.17. The diagram shows the hypothetical relationship between physiological impairment following exposure to pollutants, and the consequent disability of the fish. Measured values of physiological or biochemical variables, or alterations in the behaviour of the animal or in the histological appearance of a tissue, may represent conditions within the areas of the graph marked 'homeostasis' or 'normal function maintained without significant cost', even though they may be statistically different from control values. The toxicologist's problem is to distinguish the point at which the value of a measured variable deviates so far from the control that it falls outside these zones. Unless this is clearly established, any change in the value of a measured variable is not necessarily an indicator of sublethal toxic effect. An example is provided by the report of Grant and Mehrle (1973) on the effect of exposure to sublethal levels of endrin on 19 physiological and biochemical variables in the rainbow trout. Although statistically significant differences occurred in 12 of these, the authors showed that nine out of 16 blood serum variables showed similar changes when the fish were subjected to moderate exercise, thus casting doubt on their usefulness as indicators of toxic effect.

Thus the validity of the approach to sublethal toxicity which is implicit in much of the work published in the last 30 years or so is questionable. Essentially this implicit approach has been to measure as many variables as possible, and to seek to determine the 'no observed effect concentration' (NOEC), that is, the highest concentration which has no observable effect on any of the variables measured. The 'maximum acceptable toxicant concentration' (MATC) is thus determined as lying between the NOEC and the next highest concentration tested. This rationale may be criticised on several grounds. First, as we have seen, a statistically significant difference in a measured variable between exposed and control fish does not imply that sublethal toxicity has occurred, unless it can be shown or at least reasonably expected that the change has actual or potential ecological significance. Second, there is a certain arbitrariness under this protocol in the decision as to whether or not a particular concentration exerts a sublethal toxic effect. If, for example, in an experiment 20 variables are measured, it follows that another 20, or 50, or 100, have not been measured. Any of these might, if they had been measured, have shown a difference from the control value. Thus the NOEC is determined partly by the choice of variables to be measured during the experiment. Further, there is no general agreement on what variables should be measured, so comparisons of results from different sources are difficult. Practising scientists will recognise that some variables are measured because they are easy to measure, some are selected in order to follow precedent, and some because of the availability of equipment or skilled personnel capable of making the measurement. Of course some measurements are made because there is a sound biological reason for making them, and as will be seen later it is becoming possible at least for some pollutants to identify on a rational basis specific and useful indicators of sublethal toxic effect. Finally, the use of the NOEC as the end-point of the experiment is a statistical absurdity since, as Skalski (1981) pointed out, it depends upon the non-falsification of the null hypothesis, a procedure which cannot be carried out with confidence (in the statistical sense). Such criticisms are not to deny the usefulness of the existing literature and practices in the study of sublethal toxicity. Rather, they are a reflection of the fact that the methodology is in a relatively early stage of development.

It is generally accepted that a pollutant effect on growth, reproduction or development of a species is an unequivocal criterion of sublethal toxic effect, since its ecological significance is reasonably clear. The first successful toxicity tests over a complete life cycle of a fish species appear to be those of a group of American workers (e.g. Mount and Stephan, 1967a, b) using the fathead minnow, *Pimephales promelas*. Since then, tests have been successfully carried out with about half a dozen fish species, mainly North American or small tropical species. It remains true that the number of species with which it is practicable to carry out such tests is, at the present time, a very small proportion indeed of the aquatic fauna as a whole. Apart from reproduction, it is clearly easier to carry out investigations on the effects of pollutants on growth rates, using a wider range of species. However, there are examples in the literature which show that growth rate is not necessarily a very sensitive indicator of sublethal toxic effect (Sprague, 1971), and there are even some examples of growth apparently being stimulated by sublethal concentrations of poison (e.g. McLeay and Brown, 1974).

Obviously experiments conducted over the whole, or a substantial proportion, of the life cycle of a species are both expensive and time-consuming, and it is not feasible to test all poisons, species and environmental conditions using such procedures. Consequently it remains important to develop and evaluate rapid methods for measuring sublethal toxicity. One approach is the so-called ‘critical life stage bioassay’. Analysis of the results of a large number of partial- and complete-life-cycle tests with various species and poisons shows that in the majority of cases, the early embryo and larval stages are the most sensitive part of the life cycle, and an estimate of the MATC based solely on the response of the embryo-larval stages generally lies very close to the value obtained when the whole life cycle is considered (Macek and Sleight, 1977; McKim, 1977). Thus the duration and scale of experiments can be considerably reduced, and the critical life stage bioassay (sometimes called the ‘embryo-larval test’) has been widely used. It also offers the possibility of increasing the range of species available for testing, since there are several species (e.g. many salmonids) whose eggs and early life stages can be maintained in the laboratory but which are difficult or expensive to maintain throughout an entire life cycle. Nevertheless there remain many species of interest which cannot be used, or which are only available during a relatively short period of the year. Thus interest remains strong in alternative criteria of sublethal toxicity.

For the reasons outlined above, such criteria should preferably be specific responses to the pollutant, that is related to the poison’s mechanism of toxic action, rather than non-specific responses which may merely represent physiological adjustment to new, but perfectly tolerable, environmental conditions. Ideally, sublethal toxicity tests should also be rapid, sensitive, relevant to actual environmental conditions, and based upon a measurable response which has, or may reasonably be expected to have, ecological significance; that is, likely to reduce significantly the fitness of the population. As we know relatively little about mechanisms of toxic action in fish and aquatic invertebrates, examples which meet all of these criteria are rare. Nevertheless there appears to have been a distinct change in emphasis in the study of sublethal toxicity over the last 15 years, away from the traditional approaches and towards a novel series of techniques based on improved knowledge of the physiology, biochemistry and cellular biology of aquatic species and of their interactions with toxic substances. Some examples are given below.

Heavy metals being among the most common of pollutants, the discovery of metallothioneins in the 1970s gave rise to interest in their use as 'biomarkers' of toxic effect. Metallothioneins are a group of proteins characterised by their low molecular weight (6000–20000), their high content of amino acids containing sulphydryl groups (especially cysteine), and their ability to bind to heavy metals. They are absent, or present at very low levels, in the tissues of vertebrates and invertebrates, but are produced at high levels when the animal is exposed to heavy metals (Kagi and Nordberg, 1979). They are relatively easy to isolate and identify using standard biochemical techniques. A tissue homogenate is separated into fractions of different molecular weight by gel chromatography, and the optical density of the fractions measured in the UV range at 250 and 280 nm. Fractions of the appropriate molecular weight which show a high absorbance at 250 nm compared to 280 nm (this is due to the sulphydryl groups), may be tentatively identified as containing metallothioneins if they also contain high levels of heavy metal; this last stage is destructive of the sample, as atomic absorption spectrophotometry is usually used to determine the metal content of the fractions. Figure 4.18 shows a typical result obtained from Plecopteran larvae isolated from a metal-contaminated river.

The potential value of metallothioneins is that since they appear only to be produced in quantity in animals under stress from heavy metals, they could be used directly to determine the level of heavy metal which a particular organism found unacceptable. Alternatively, it may be possible to determine whether the level of heavy metal present in a particular environment was above the limits of tolerance of the organisms living there. Some caution, however, is required at the present stage of knowledge. For example, many heavy metals which are toxic at a certain level are normal, even essential, metabolites at lower levels. Therefore organisms must have some means of metabolising them, and the induction of metallothioneins may represent a normal adjustment of the organism, or detoxification, rather than a manifestation of toxic effect. Although there are examples of the use of metallothioneins to assess the extent of metal pollution in field situations (Roch *et al.,* 1982, appear to have reported one of the first examples), there

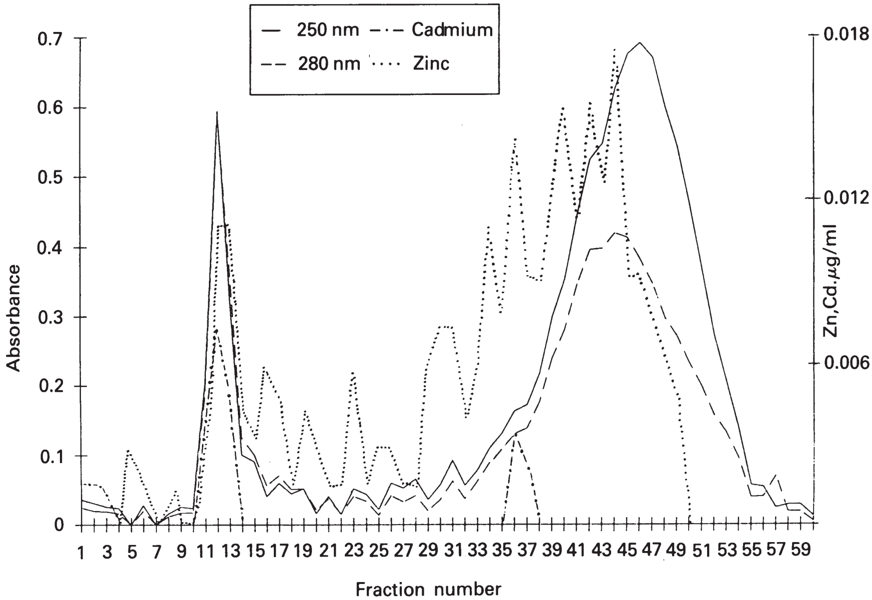

Figure 4.18 Elution profile of the cytosolic supernatant from homogenised tissue of a stonefly nymph, *Diura bicaudata*, taken from a metal-polluted river. The presence of metallothionein in fractions 11–13 is suggested by the corresponding peaks for zinc, cadmium and absorbance at 250 nm

is as yet no clear link between metallothionein levels and variables of direct ecological significance. Benson and Birge (1985) reported an association between metallothionein levels and metal resistance, in field and laboratory trials. Possibly further experience may allow the determination of levels of metallothionein which can be considered abnormal. Some other proteins of a generally similar nature, often called stress proteins, have been found to be induced by other forms of stress, such as temperature shock, and may be confused with metallothioneins (Sanders, 1990), if indeed they are different entities at all.

Mehrle and Mayer (1980) in a brief review of clinical tests in aquatic toxicology, drew attention to a promising series of investigations involving study of the effects of poisons on biochemical processes related specifically to growth in fish. They argued that growth in fish is the culmination of a series of biochemical processes which should show changes *before* any effect on growth rate is detectable by conventional measurements of weight and length. They showed that several organic toxicants affected the vertebral collagen content of fish, and the proline and hydroxyproline growth rate, and were more sensitive indicators of toxic effect than measurement of growth rate itself.