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No. 61

Assessing the toxicity of industrial wastes, with particular reference to variations in sensitivity of test animals

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reference to variations in sensitivity to test animals

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1. Introduction

Since the early 1960s the acute toxicity of various pollutants to marine organisms has been evaluated at the Ministry of Agriculture, Fisheries and Food (MAFF), Fisheries Laboratory, Burnham-on-Crouch using a static toxicity test with daily replacement of the test solution during a 96 h exposure period. The toxicities of a number of detergents, pesticides, polychlorinated biphenyls (PCBs) and pure chemicals to a number of species have been determined using this method (Portmann, 1972; Portmann and Wilson, 1971). These tests have been made because MAFF has exercised a responsibility for the control of marine pollution from the disposal of wastes from ships by dumping since 1965. Prior to 1974 this was done through a voluntary control scheme in which companies, or other organisations involved in the dumping of wastes, were invited to apply for MAFF's approval of their activities. Details of the volume and the physical and chemical nature of the waste were provided by the manufacturer and an assessment of the acute toxicity of a representative sample was made using a 96 h static test procedure. On the basis of this information a decision was made on the suitability of the waste for disposal in a specified area and, where no significant effects on the marine environment were expected, a consent for the disposal of the waste was granted (Wilson, 1972). The static test was also used for the evaluation of oil dispersants under the voluntary approval scheme which existed prior to 1974 (Portmann and Connor, 1968; Wilson, 1974). Dispersants with a satisfactory performance in efficiency tests carried out at the Warren Spring Laboratory of the Department of Trade and Industry and with a low toxicity in the static test were approved for use in the U.K.

In 1974, however, the Dumping at Sea Act (Great Britain-Parliament, 1974) came into force and MAFF became formally responsible, as the licensing authority for England*, for the control of waste disposal at sea by dumping. The Dumping at Sea Act makes it an offence to dump (or load for the purposes of dumping) any material in the sea from a vehicle, ship, aircraft, hovercraft or other marine structure without a licence from the relevant licensing authority, and except in accordance with the conditions of that licence. In determining whether to grant a licence the Act states that "the licensing authority shall have regard to the need to protect the marine environment and the living resources which it supports from the adverse consequences of dumping the substances or articles to which the licence, if granted, will relate: and the authority shall include such

*the other licensing authorities are the Welsh Office, the Department of Agriculture and Fisheries for Scotland and the Department of the Environment for Northern Ireland.
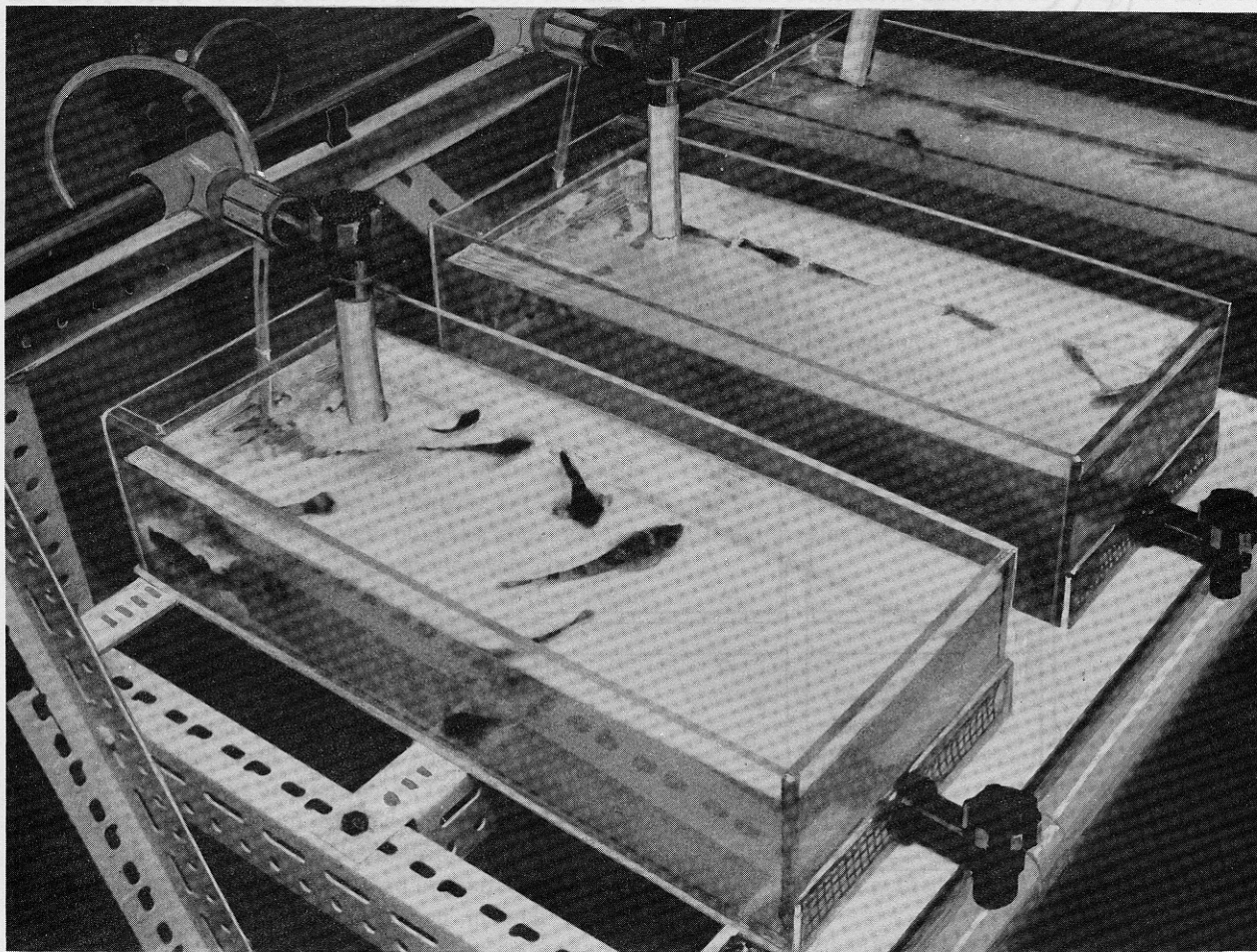

Figure 1   Test frame and tanks.

conditions in a licence as appear to the authority to be necessary or expedient for the protection of the environment and those resources from any such consequences". Substances which required licensing under the Act included not only wastes, such as sewage sludges, dredge spoils and industrial effluents, but also oil dispersants and related chemicals. This latter requirement led to the development of new toxicity tests for oil dispersants (Blackman *et al.*, 1977) and to standardisation of the static test method, which continued to be used for evaluating the toxicity of wastes.

Toxicity tests are generally required only for industrial wastes, since their complex composition rarely allows toxicity to be predicted, even if the toxicity of the individual components is known. It is essential either that the test method should be free from variations in sensitivity or that such variations should be recognised and their significance understood and taken into account when assessing the

results. Both water temperature and size of test animals are variables which can modify the results obtained (Portmann, 1968). These effects are eliminated in the standard test by carrying out all experiments at a constant temperature and using test animals within a specified size range. However, seasonal changes in sensitivity could be important, because licence applications for industrial wastes are received throughout the year. Such variations are impossible to eliminate when natural populations are used and any method used on a routine basis should be examined to find out whether such variations exist. To this end, tests to assess seasonal variations were carried out at three-monthly intervals between September, 1977 and September, 1979 using the standard method for industrial wastes with four reference compounds.

This report gives the results of these three-monthly tests, together with a detailed description of the standard static toxicity test procedure and its use in assessing the toxicity
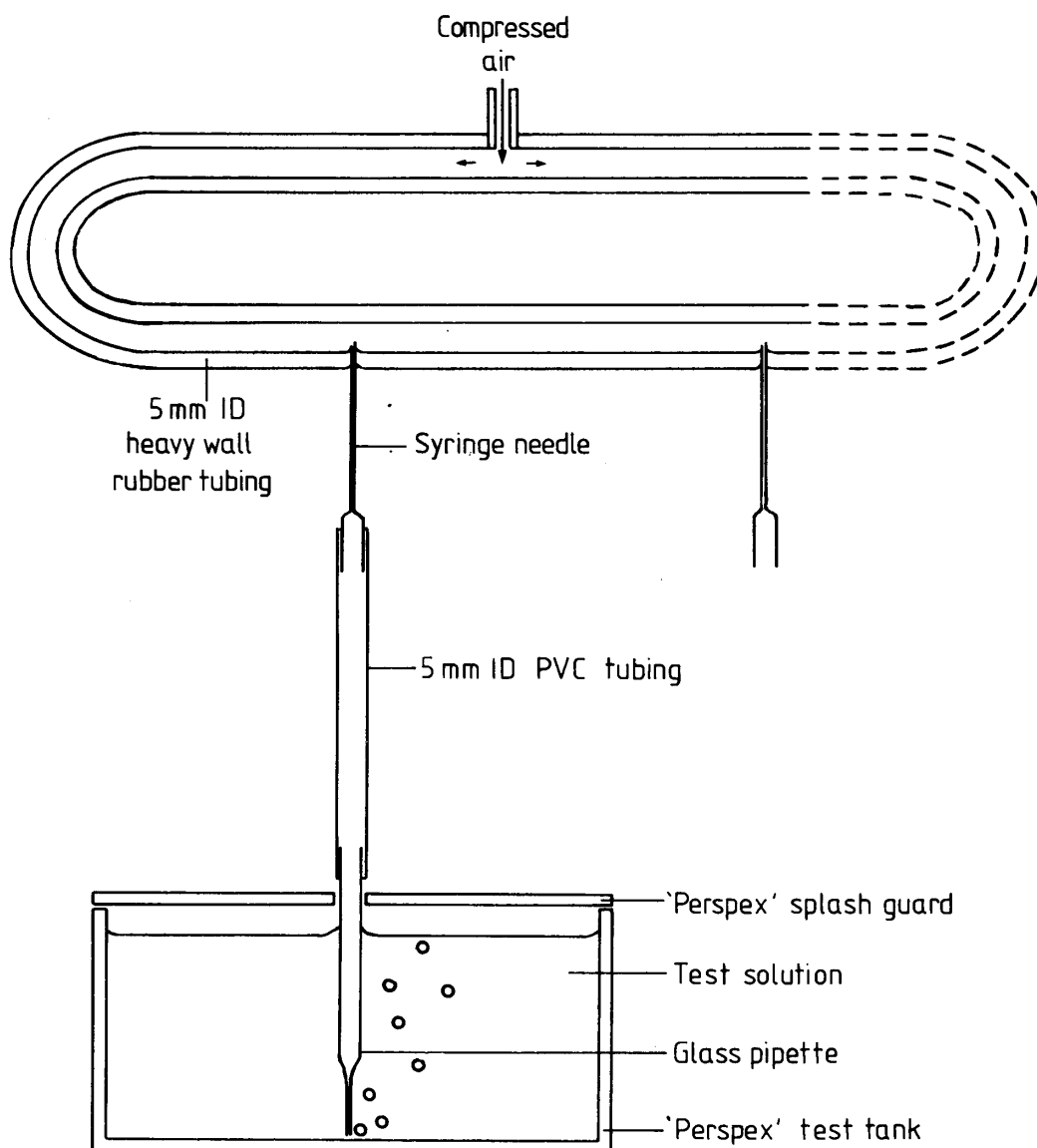

Figure 2   Aeration system for static test tanks.

of industrial wastes under the Dumping at Sea Act. The operation of the Dumping at Sea Act and the use of data generated by these tests in deciding whether or not to licence the dumping of wastes has been described elsewhere (Wilson, 1976; Norton, 1976).

2. Apparatus

Part of the test apparatus is shown in Figure 1. This consists of a number of 'Perspex' test tanks supported on an alloy frame over drainage channels. The tanks are fitted with nylon drain taps covered with a 5 mm plastic mesh screen to retain test animals. Each tank is calibrated to hold 10 l of test solution which is gently aerated through a pipette connected to the compressed air supply by means of the apparatus shown in Figure 2. The aerator pipette enters the tank through a 10 mm diameter hole in a 'Perspex' splash guard which prevents transfer of the test solution from one tank to another by the aerator spray. 'Perspex' lids are used to prevent escape of test animals during experiments. The room in which the test apparatus is housed is maintained at a constant temperature of 15°C (± 1°C) and is equipped with an automatic 12 h light: 12 h dark photoperiod.

The sea water which is used to make up the test solutions and for maintaining the test animals during acclimatisation is taken from the estuary of the River Crouch on a flood tide and stored in settling tanks for about 2 days to remove most of the silt particles. It is then pumped to the Laboratory's header tank and brought to the test temperature of 15°C (±1°C) before being used in the test animal stock tanks. A 10 μm in-line membrane filter situated close to the test tanks removes any remaining silt particles from the water which is then used to fill the tanks. The salinity of the water is measured daily; the mean value during the period of the tests described in this report was 31 parts per thousand (°/oo) with a standard deviation of 2.5°/oo.

3. Methods

3.1 Standard test for industrial wastes

The two species used for standard tests on industrial wastes are adult brown shrimp, Crangon crangon L (= Crangon vulgaris), and a fish, the armed bullhead (hooknose or pogge), Agonus cataphractus. These species were chosen for their small size, ease of handling and availability throughout the year. In addition, experience had shown that they have an average sensitivity to a wide range of pollutants. They are caught in the estuary of the River Crouch by the Laboratory's research vessel using a modified 2 m beam trawl with 10 mm mesh in the cod end. On arrival in the laboratory the animals are transferred to 40 l polyethylene stock tanks at a maximum density of 200 shrimps or 100 fish per tank. Water at the test temperature of 15°C is used to slowly fill the tanks and any dead or injured animals are removed. The animals are maintained in well aerated, gently flowing sea water for 2-4 days before the start of the test. They are not fed during their period in the laboratory, which never exceeds 9 days.

The pH of the undiluted industrial waste is measured; if this is less than 6 or greater than 8 an estimate is made of the dilution needed to bring it within the normal range for sea water. The pH values of the neat and diluted waste are recorded, together with a subjective description of its visual and olfactory characteristics; this information may be of use in interpreting the results of the toxicity test.

The test tanks are filled with 10 l of sea water and aerated for at least 1 h. Ten fish of 50-100 mm total length (about 2-8 g) or 20 shrimps of 40-70 mm (about 1-3 g) are then randomly added to each tank. Freshly moulted shrimps and diseased animals are excluded. A further acclimatisation period of 2 h is allowed before the test solutions are added. Test solutions are made up by gently stirring a measured amount of the well mixed test substance into the sea water in the test tanks. For each species, four concentrations of the waste are set up initially, with controls of clean sea water. Duplicates are used at each concentration. The concentrations chosen depend on what is known about the waste, but generally start at 10 000 μl l^{-1} (1%) and decrease at half-logarithmic intervals (i.e. 3 300, 1 000 and 330 μl l^{-1}). For wastes having a very high or low pH the maximum concentration is that which gives a pH within the range 3-11. Test solutions are renewed daily to discard metabolites and counteract losses of the test substance due to absorbtion by the test organisms, degradation or volatilisation. The dissolved oxygen concentration (DO) in each test tank is measured and recorded daily. If the DO in any of the test tanks drops below 70% air saturation value then the test is repeated with additional aeration. The pH of test solutions of very acid or alkaline wastes is measured and recorded 1 and 24 h after the start of the test. Each test continues for 96 h. A range of lower concentrations of the waste may be tested subsequently if necessary.

The tanks are inspected at frequent intervals, including 24, 48, 72 and 96 h after adding the test solution, and dead animals (defined as those not responding to gentle prodding) are recorded and removed. As cannibalism of freshly moulted shrimps may take place the number of animals remaining alive at the end of the experiment is also noted so that the total number of test animals in each tank may be calculated. At the time of each observation, and for each tank, the cumulative percentage mortality is calculated using the following formula:

$$\frac{2m - 1 \times 100}{2p}$$

where m is the cumulative mortality and p is the total number of animals in the tank. Thus where 10 animals are used in each tank the response of the first is regarded as 5%, the second as 15% and the tenth as 95%. This is because the median response time relates not to the response time of the fifth animal out of a total of 10, but to a response time between the fifth and sixth animal (Lloyd, 1979).

For each individual batch of fish the cumulative percentage mortalities are plotted against exposure time, using a probit scale for mortality and a logarithmic scale for time.
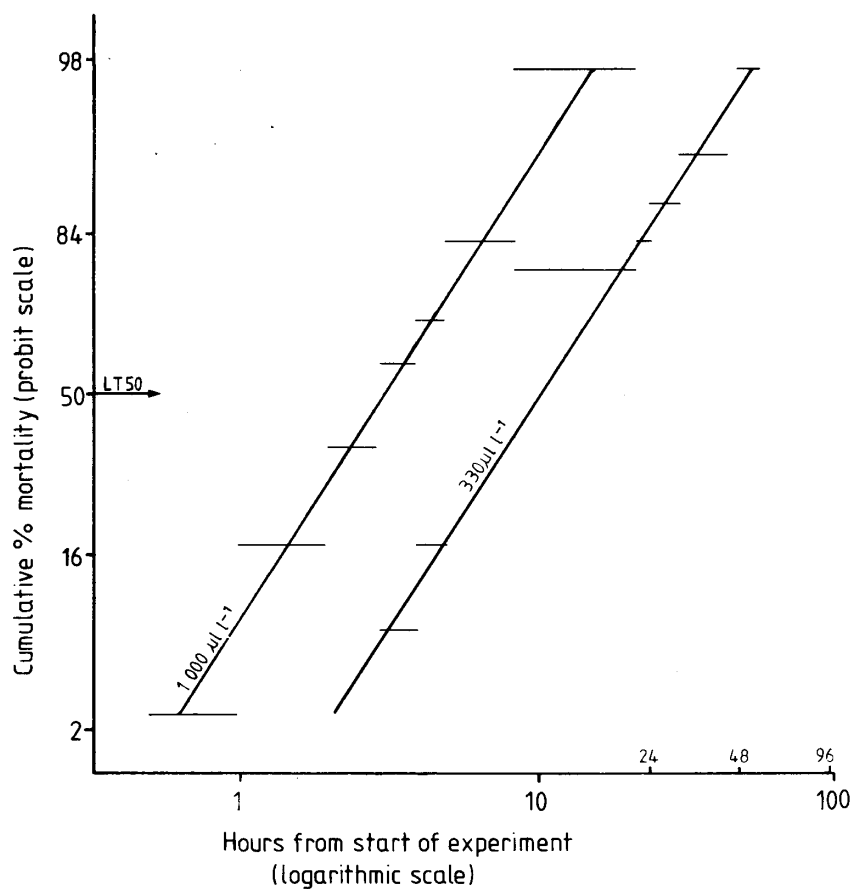
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Figure 3   Examples of time/mortality curves for *Crangon*.
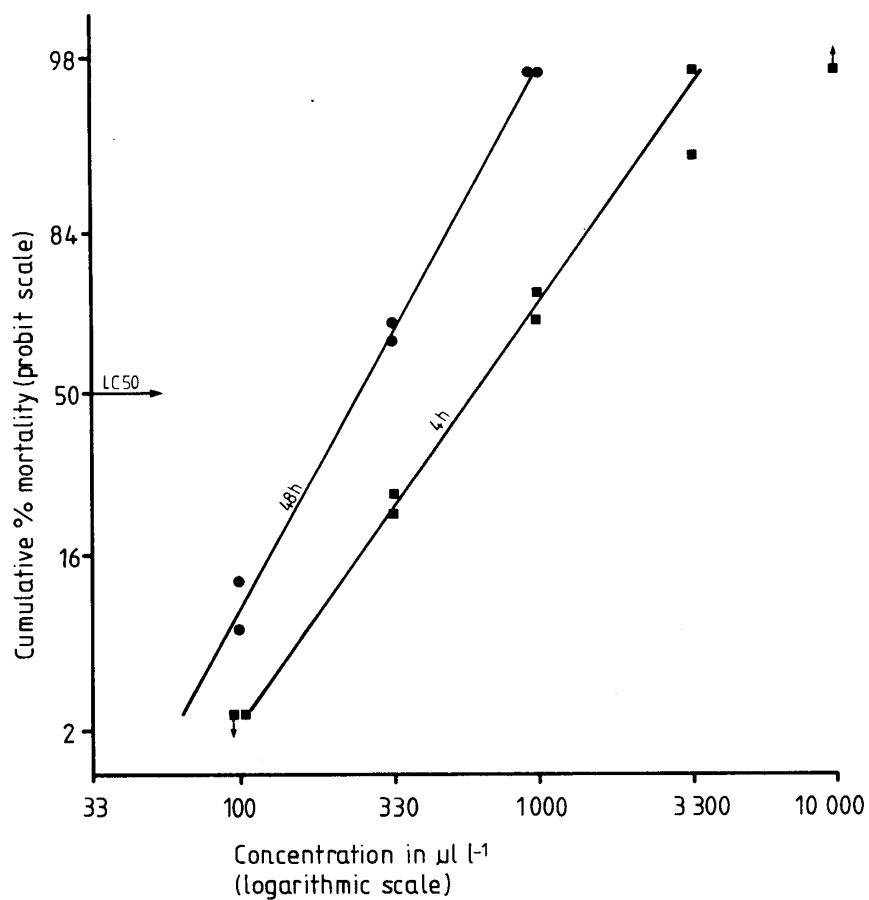

Figure 4   Examples of concentration/mortality curves
for *Crangon*.

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In instances when the exact time of death was not observed (for example, when overnight mortalities occurred) a line is drawn on the graph between the time when the mortality was recorded and the time of the previous observation (Figure 3). A line is then fitted by eye through the points (giving greater weight to those between 25% and 75% mortality) to give a time/mortality curve. The time for 50% mortality (LT50) for each tank may then be read off the graph and used to construct a concentration/median response curve.

Additionally, it is often useful to calculate the concentration lethal to 50% of the test organisms (LC50) at fixed observation times. In this case the cumulative percentage mortalities at, for example, 4 and 48 h, are plotted on a probit scale against concentration on a logarithmic scale (Figure 4). A line fitted by eye between the points allows approximate values for the LC50 at these time intervals to be estimated. A concentration/response relationship is then obtained for each species by plotting the values of the LT50 against concentration and the estimated values of the LC50 against the corresponding time, using log - log graph paper. Typical concentration/response curves showing LT50 against concentration for *Crangon* and *Agonus* are shown in Figure 5.

3.2 Three-monthly tests

The four reference compounds used for the three-monthly tests, chosen because of their different toxicological characteristics, were sulphuric acid (hydrogen ion, rapid action), phenol (non-ionised molecule, slow action), ammonium sulphate (non-ionised proportion toxic, rapid action) and copper sulphate (ionised metal, slow action). These were added to the test tanks as solutions of the analytical grade (AR) reagent in distilled water at the following concentrations: sulphuric acid, 10% v/v H_2SO_4; phenol, 1% w/v C_6H_5OH; ammonium sulphate, 10% w/v $(NH_4)_2SO_4$; copper, 5% w/v Cu from $Cu_2SO_4.5H_2O$. The tests were carried out in March, June, September and December of each year, using the standard industrial waste test method. Due to pressure of other work, it was not always possible to test all four compounds against both species, and some of the tests had to be carried out using a limited range of concentrations. When calculating the results of these tests, 95% confidence limits on the LT50 values were calculated according to the method of Litchfield (1949).
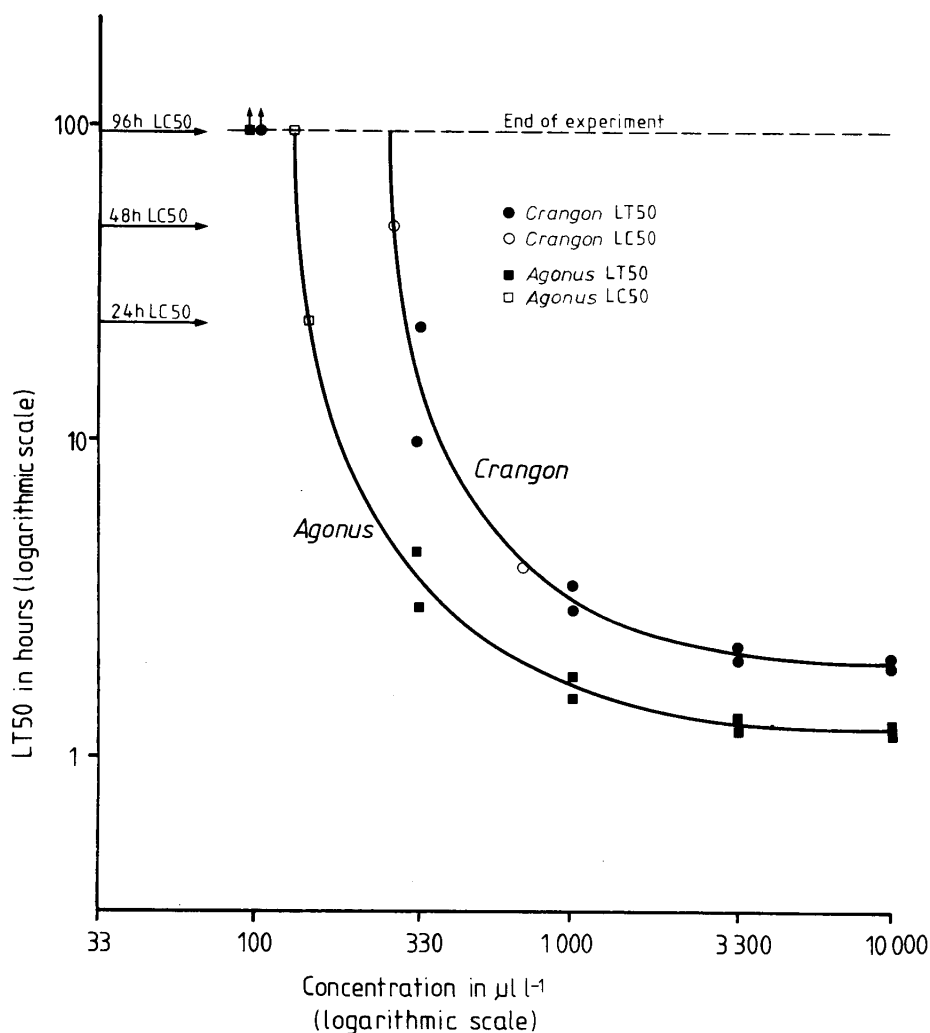

Figure 5   Examples of concentration/response curves for *Crangon* and *Agonus*.

3.3 Evaluation of the test data

In assessing the acceptability of a waste for sea disposal there are two items of information which are required from the standard toxicity test; first, a single concentration (LC50) for a standard exposure period which serves as an indication of the toxicity of the waste under the conditions of the test, and second, an indication of the rate of toxic action, as shown by the shape of the concentration/response curve.

The LC50 for any exposure period up to and including 96 h (e.g. 1, 24 and 48 h) may be taken from the response curve (see Figure 5). Since, with complex test substances like industrial wastes, it is impossible to measure the concentration of the test substance present in the tanks, the term LC(I)50, where C(I) is the initial concentration of the test solution, is more correctly used (Lloyd and Tooby, 1979). This indicates that, although the test solutions were renewed daily, the actual concentration of the test substance may not have remained constant during the period of the test.

The vast majority of wastes give rise to curves similar to those in Figure 5 in which a threshold, or asymptotic, LC50 can be measured within the exposure period 96 h, thus indicating that the toxic components of the waste can be excreted or detoxified within this time. At concentrations below the threshold LC50, animals of similar sensitivity to the test species are unlikely to be killed even after prolonged exposure to the test substance. This type of response is typical of wastes in which very high or low pH is the main cause of toxicity. Figure 6 gives examples of three other curve shapes which are sometimes obtained. Curve 1 is shallower than the example in Figure 5, and the threshold LC50 is not reached during the 96 h test period; concentrations lower than the 96 h LC50 may be lethal at longer exposure periods. This response indicates that the test organisms may detoxify only slowly or adapt to the waste.

A linear concentration/response relationship (Curve 2) indicates that no such detoxification is taking place, that no lethal threshold is evident under the test conditions and that prolonged exposure to concentrations well below the 96 h LC50 value would be expected to lead to some mortality. Wastes which give rise to this type of curve are usually bioaccumulative and therefore generally unacceptable for sea disposal. Curve 3 occurs infrequently; it indicates a more complex type of response, such as may result from a mixture of a fast-acting, low toxicity compound and a slower-acting substance of higher toxicity. Alternatively, this type of curve may result from the breakdown of an initially low toxic component into more toxic fractions.

4. Results

4.1 Industrial wastes

Nearly 140 industrial wastes have been tested under the Dumping at Sea Act since January, 1975. Over 90 of these were new applications for licences, the remainder being samples taken during routine inspection of wastes for dumping. The distribution of the 96 h LC(I)50 values for *Crangon* and *Agonus* are given in Figure 7. A significant proportion of the wastes (34% for *Crangon* and 26% for *Agonus*) had 96 h LC50 values greater than 10 000 μ l l^{-1} (1%), the maximum concentration used in the tests, whereas only 4% and 9% of the wastes had LC50 values less than 100 μg l^{-1} for the two species respectively. The relative susceptibilities of the two test species are shown in Figure 8. It is clear that *Crangon* and *Agonus* have, in general, similar sensitivities, with most of the 96 h LC50s falling within one order of magnitude of each other. A similar relationship has been observed for other species (Maki, 1979; Lee, C.M., personal communication).

4.2 Three-monthly tests

The results of the three-monthly tests to determine variation in sensitivity are given in Table 1. Neither *Crangon* nor *Agonus* showed any significant variation in sensitivity to sulphuric acid, phenol or ammonium sulphate but there was a considerable variation in the 96 h LC50 values of both species for copper (0.2 - >3.3 mg l^{-1} for *Crangon* and 0.06 - 0.5 mg l^{-1} for *Agonus*). This might be due to seasonal changes in the animals but it is possible that the variations in copper toxicity were caused by variations in the amount of copper available to the test animals, arising from fluctuations in the physical and chemical properties of the sea water. The availability of a metal such as copper to marine organisms depends on its solubility and whether it is in the ionic form or complexed with organic or inorganic compounds (Gnassia-Barelli *et al.*, 1978). Although copper as copper sulphate is highly soluble in distilled water, its solubility in natural water is limited by the formation of insoluble hydroxides, carbonates or phosphates (Andrew *et al.*, 1977).

In September, 1979 a simple experiment was carried out to determine:

    (i)   the concentration of copper in the various test solutions;

    (ii)   whether the apparent differences in toxicity reflected variations in the soluble copper concentrations;

    (iii)   whether the precipitated copper influenced the toxicity.

Two parallel toxicity tests were set up, with the test solutions being made up 2 days before the start of the test in order to allow equilibrium to be reached if any precipitation took place. The test solutions used to fill one set of tanks were then passed through a Whatman No. 1 filter paper to remove the precipitate. Animals were added to both sets of tanks and the toxicity experiments carried out using filtered and unfiltered water, but without daily replacement of the test solution. At the end of the experiment the concentrations of dissolved copper in the test solutions were measured by atomic absorption spectrophotometry (AAS).
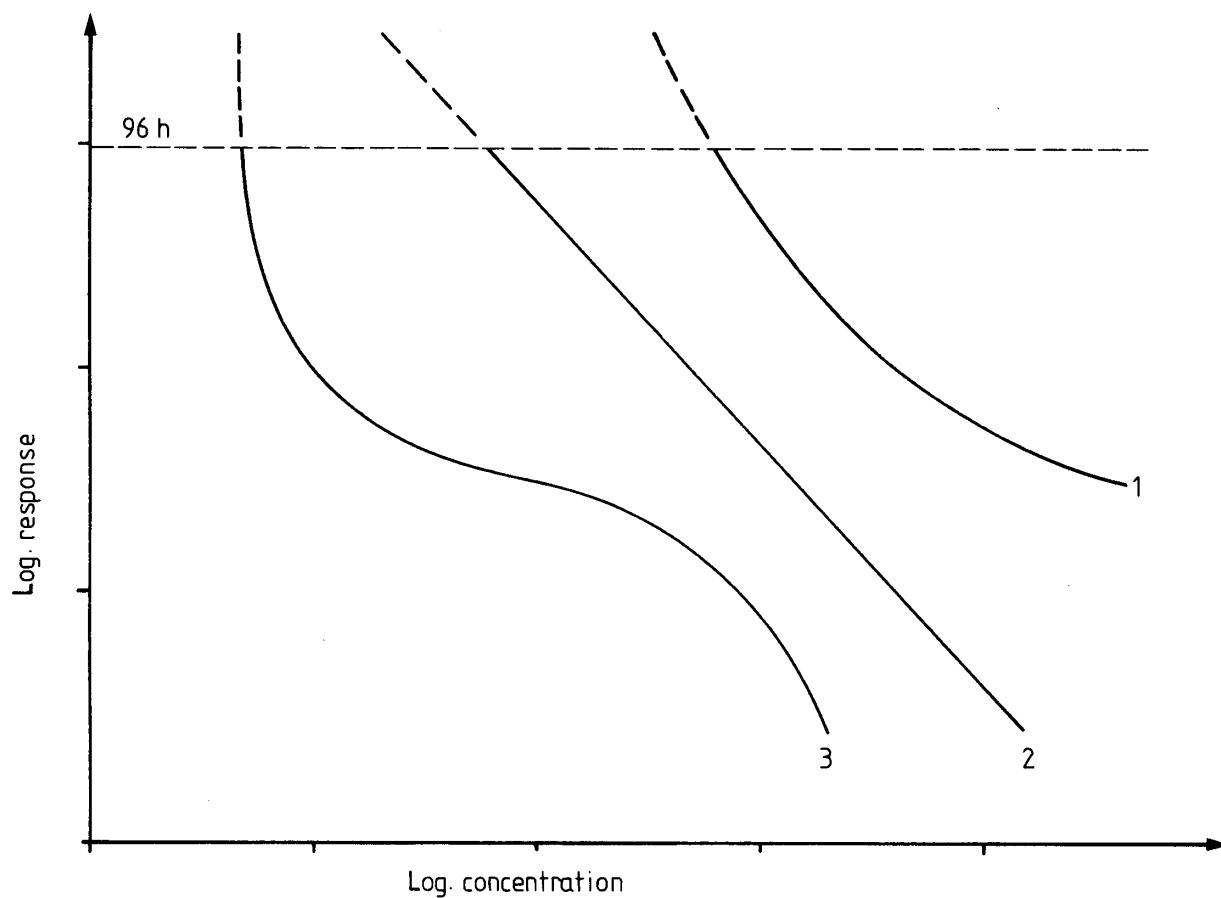
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Figure 6   Three types of concentration/response curves.

Table 1   Results of the three-monthly toxicity tests using four reference compounds

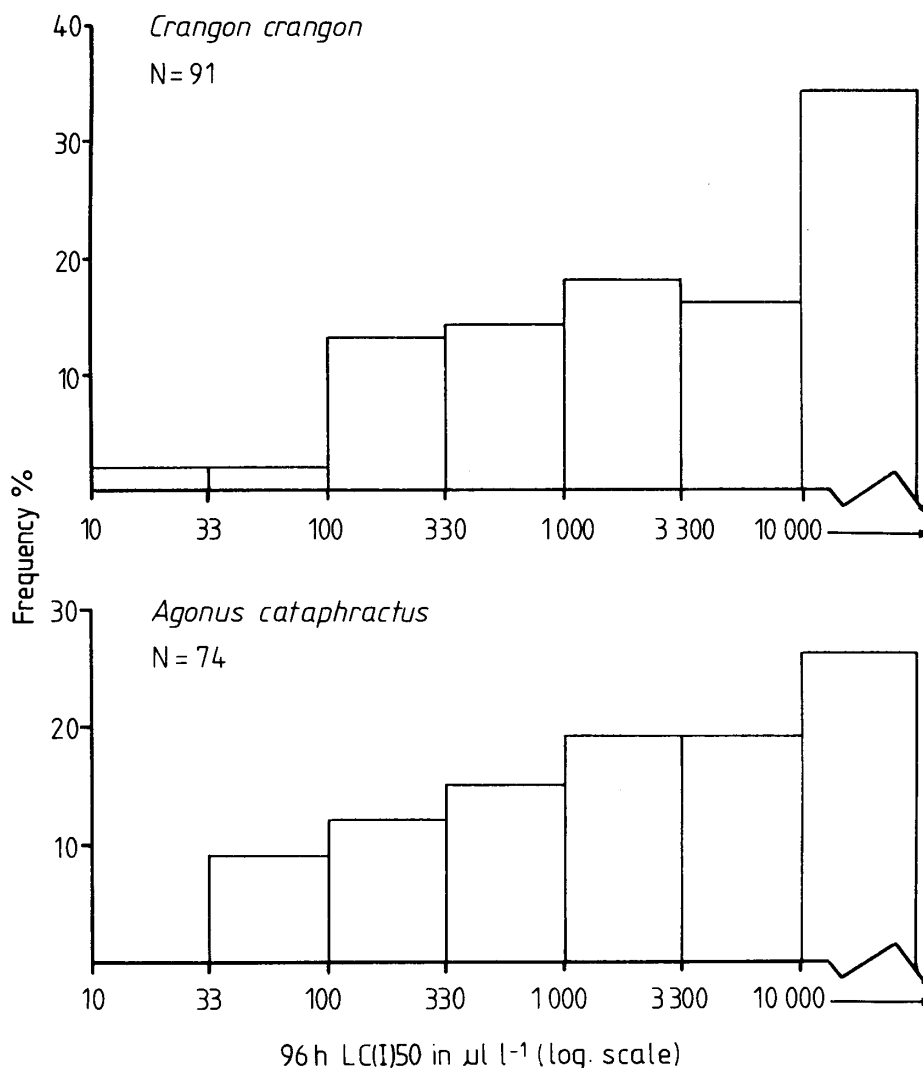| Date	96h LC(I)50 to *Crangon crangon*				96 LC(I) 50 to *Agonus cataphractus*			
	Sulphuric acid ($\mu l\,l^{-1}$)	Phenol ($mg\,l^{-1}$)	Ammonium sulphate ($mg\,l^{-1}$)	Copper ($mg\,l^{-1}$)	Sulphuric acid ($\mu l\,l^{-1}$)	Phenol ($mg\,l^{-1}$)	Ammonium sulphate ($mg\,l^{-1}$)	Copper ($mg\,l^{-1}$)
1977 September	33-100		380	1.6	33-100		210	0.5
December	33-100		600	0.2	33-100		200	
1978 March				0.3				
June	33-100		480	0.5	33-100		130	0.06
September	52	27	400	3.2	36	10		0.5
December	54	19						
1979 March	55	10-33	540	0.5				
June	33-100	20		>2.5	33-100	<33		0.5
September				>3.3				

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Figure 7   The distribution of 96h LC50s of industrial wastes to *Crangon* and *Agonus*.

The results of these experiments are given in Table 2. There was no significant difference in toxicity or copper concentration between the filtered and unfiltered solutions, suggesting that toxicity was due to dissolved rather than precipitated copper. The chemical analyses showed that between 0.5 and 90% of the copper in the tanks was present in solution, depending on the amount of copper added. A possible explanation of this is as follows. If the solubility of copper in sea water is limited by the formation of insoluble salts then the amount of copper in solution would be a function of both the concentration of the appropriate anions in the sea water and the solubility of the salts formed. Andrew et al. (1977) demonstrated a reduction in the toxicity of copper to Daphnia magna due to carbonate complexing and suggested that shifts in carbonate equilibria might be responsible for much of the variability of copper toxicity reported in the literature; it seems likely that the salt formed in these experiments was copper carbonate which has a very low solubility (thus: $CuSO_4.5H_2O + CO_3^{2-} \longrightarrow CuCO_3 + SO_4^{2-} + 5H_2O$). In the present experiment, when between 1 and 100 mg l^{-1} of copper were added to sea water, a maximum of about 1 mg l^{-1} went into solution and the remainder precipitated out. This suggests that carbonate was present in excess of the copper, forming salts soluble at about 1 mg l^{-1}. When copper was added at 1 000 mg l^{-1}, however, only about 200 mg l^{-1} precipitated out and the remainder (about 800 mg l^{-1}) was held in solution. This was probably due to the removal of all of the carbonate and to the excess copper remaining in solution.

The concentration of carbonate in sea water depends on a number of factors, including temperature, pH and dissolved carbon dioxide (CO_2) concentration. As all of these, particularly the CO_2 concentration, are likely to vary between tests, they could be possible causes of the variation in LC50 values for copper which were obtained over the two-year experimental period. An alternative, or more probably an additional, explanation for the variability in toxicity values could be differences in the forms of the copper in solution and the biological availability of these different copper species to the test organism. Variations in the organic components of sea water occur on a seasonal basis and the metal complexes associated with the organic fraction are likely to vary accordingly.
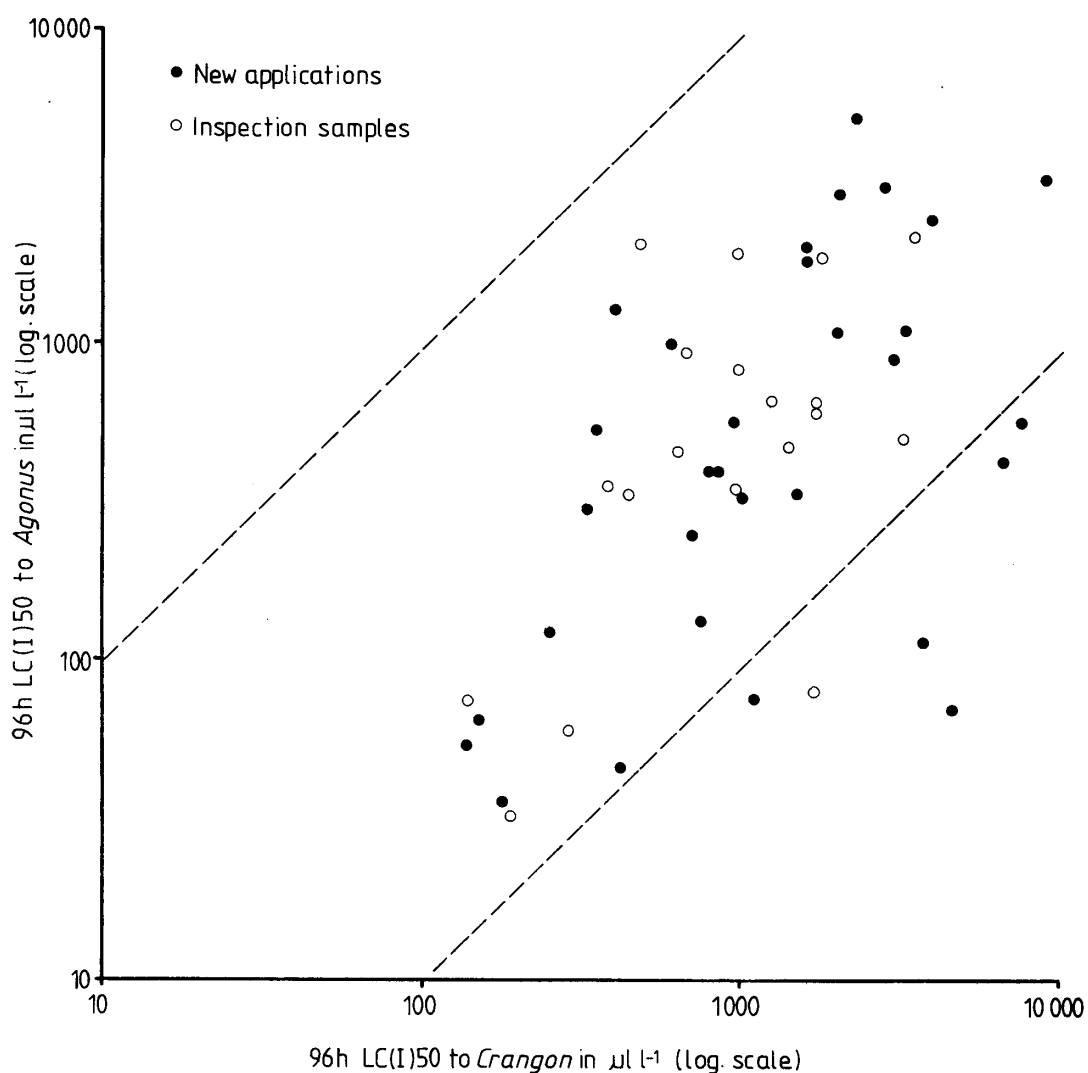
Figure 8   A comparison of the 96h LC50s for 51 industrial wastes to
            Crangon and Agonus.

Table 2 The toxicity and solubility of copper in sea water

Treatment	Copper added (mg l⁻¹)	Copper found in solution (mg l⁻¹)	pH of solution at 96h	% mortality at 96h	LT50 (h)
Filtered test	0		7.8	0	>96
solution	0.1		7.6	0	"
(i.e. precipitate	1	0.7	7.9	7.5	"
removed)	10	1.0	7.9	27.5	"
	100	0.5	7.2	7.5	"
	1 000	800	4.9	97.5	3.2
Unfiltered test	0		7.8	0	>96
solution	0.1		7.8	0	"
	1	0.9	7.9	12.5	"
	10 *	1.5	8.0	27.5	"
	100 *	1.6	7.2	32.5	"
	1 000 *	880	4.5	97.5	2.6

*blue precipitate present

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5. Discussion and conclusions

A major consideration in the selection of a method for determining the toxicity of wastes in relation to the Dumping at Sea Act was the need to process a large number of samples on a routine basis at a reasonable cost and to obtain reproducible data. This limits the toxicity tests to the use of one or two representative test species. Nevertheless, the LC(I)50 values derived from such tests provide a basis for assessment of potential hazard when compared with the predicted concentration of the waste at the dumping site.

If the LC(I)50 or the shape of the concentration/response curve indicates a potential risk to marine life, then the waste is not normally licensed. A licence for a waste found to be acceptable on toxicological grounds usually includes specification of the rate of discharge so as to provide a dilution rate which will give an acceptable margin of safety to species more sensitive than those used in the test. The rates of dilution required generally err on the safe side, because the toxicological data utilised are from a laboratory test in which the animals are maintained in relatively constant concentrations of the waste for 96h, whereas natural dispersion and degradation processes ensure a continually decreasing concentration of the waste at sea.

The results of the tests carried out at three-monthly intervals with four toxicologically different substances showed the method to be free of substantial seasonal variations in sensitivity of the test species. The toxicity of one heavy metal (copper) was not free of such variations, and it is possible that this may be in part due to the carbonate content of sea water. Variations in the latter, and other substances, alter the speciation of the metal, and are probably the result of natural fluctuations.

6. References

ANDREW, R.W., BIESINGER, K.E. and GLASS, G.E., 1977. Effects of inorganic complexing on the toxicity of copper to *Daphnia magna*. Wat. Res., *11*: 309-315.

BLACKMAN, R.A.A., FRANKLIN, F.L., NORTON, M.G. and WILSON, K.W., 1977. New procedures for the toxicity testing of oil slick dispersants. Fish. Res. Tech. Rep., MAFF Direct. Fish. Res., Lowestoft, (39) 7 pp.

GNASSIA-BARELLI, M., ROMEO, M., LAUMOND, F. and PESANDO, D., 1978. Experimental studies on the relationship between natural copper complexes and their toxicity to phytoplankton. Mar. Biol., *47*: 15-19.

GREAT BRITAIN - PARLIAMENT, 1974. The Dumping at Sea Act 1974. HMSO., London, 14 pp.

LITCHFIELD, J.T. Jr., 1949. A method for the rapid graphic solution of time - percent effect curves. J. Pharmac. exp. Ther., *97*: 399-408.

LLOYD, R., 1979. Toxicity tests with aquatic organisms. Paper presented at the Sixth FAO/SIDA Workshop on Aquatic Pollution in Relation to Protection of Living Resources. FAO, Rome, Report No. TF-RAF 112 (SWE) - Suppl. 1: 165-178.

LLOYD, R. and TOOBY, T.E., 1979. New terminology required for short-term static fish bioassays: LC(I)50. Bull. Environm. Contam. Toxicol., *22*: 1-3.

MAKI, A.W., 1979. Correlations between *Daphnia magna* and fathead minnow *(Pimphales promelas)* chronic toxicity values for several classes of test substance. J. Fish. Res. Bd Can., *36*: 411-421.

NORTON, M.G., 1976. The operation of the Dumping at Sea Act 1974. Chem. Ind., *2*: 829-834.

PORTMANN, J.E., 1968. Progress report on a programme of insecticide analysis and toxicity-testing in relation to the marine environment. Helgoländer wiss. Meersunters. *17*: 247-256.

PORTMANN, J.E., 1972. Results of acute toxicity tests with marine organisms, using a standard method. pp. 212-217. In: M. Ruivo (Editor) "Marine Pollution and Sea Life". Fishing News (Books) Ltd., London, 624 pp.

PORTMANN, J.E. and CONNOR, P.M., 1968. The toxicity of several oil-spill removers to some species of fish and shellfish. Mar. Biol, *1*: 322-329.

PORTMANN, J.E. and WILSON, K.W., 1971. The toxicity of 140 substances to the brown shrimp and other marine animals. Shellfish Inf. Leafl., MAFF Fish. Lab., Burnham, (22) 11 pp.

WILSON, K.W., 1972. Assessing the toxicity of industrial effluents. ICES CM 1972/E: 15, 9 pp. (mimeo.).

WILSON, K.W., 1974. Toxicity testing for ranking oils and oil dispersants. pp. 11-22. In: L.R. Beynon and E.B. Cowell, (Editors) "Ecological Aspects of Toxicity Testing of Oils and Dispersants". Applied Science Publishers, London, 1974, 149 pp.

WILSON, K.W., 1976. The role of toxicity tests for the prevention of marine pollution by dumping from ships. Paper presented at the fourth FAO/SIDA Training Course in Aquatic Pollution in Relation to Protection of Living Resources: Bioassays and Toxicity Testing. FAO, Rome, Report No. TF-INT 173(SWE) Suppl. 1: 199-210.