ASPECTS OF THE SENSITIVITY OF JUVENILE JASUS LALANDII AND DECAPOD EXCITABLE TISSUES TO THE WATER SOLUBLE FRACTION OF CRUDE OIL

by

S.R. LIPSCHITZ

Submitted in fulfilment of the requirements
for the degree of Master of Science in the
Department of Zoology at the University of Cape Town

August 1983

TABLE OF CONTENTS

SUMMARY

1. A flow-through aquarium facility, for testing the toxicity of the water-soluble fraction (WSF) of crude oil is described.

2. The 24 hour median lethal concentration (LC50) of WSF of crude oil for 2 to 3 year old (30 - 45 mm cephalothorax length) juvenile <u>Jasus lalandii</u> ranged from 3,7 to 5,9 mg ℓ^{-1}.

3. Behavioural experiments appear to provide a quick and convenient approach for estimating the relative toxicity of the WSF of crude oil.

4. The major effect of the WSF of crude oil on <u>Jasus</u> heart beat rate was bradycardia. Furthermore, the greater the initial exposure concentration the sooner the heart beat frequency decreased.

5. Exposure of <u>Jasus</u> muscle fibres to the WSF of crude oil resulted in a shift of the resting membrane potential of those fibres in a hyperpolarising direction. Preliminary investigations into the level of excitability of decapod neurons after exposure to the WSF, indicated a rapid increase, followed by a decrease in excitability, to a level below that of unexposed tissue.

6. Recommendations are made as to how the findings of this study could be applied to enhance the sensitivity of toxicity bioassays.

1. INTRODUCTION

The potential threat of oil pollution to the South African coastline is very significant as it lies on one of the world's busiest shipping routes. According to Chapman and Watling (1980) 8000 ships round the Cape annually, of which approximately 2500 are tankers, carrying an estimated 600 million tonnes of oil from the Middle East to Europe and America.

In December 1977, 21 000 tonnes of Iranian crude oil and 9000 tonnes of bunker oil were released when the Venpet and Venoil collided 60 km offshore from Plettenberg Bay. This incident, although by no means the first example of oiling of the coastline, served to highlight the almost total lack of information on the movement of oil slicks and the effects of oil and dispersants on the marine biota of South Africa (Chapman & Watling, 1980).

The National Oil Pollution Research Programme was established to collect the needed information for the protection of the coastline. This programme is directed by the Steering Committee for Oil Pollution Research and financed by the Department of Transport which is responsible for the prevention and combating of oil pollution in South African waters. Much of this research into the biotic effects of oil pollution is undertaken at the toxicity testing research aquarium at the Sea Fisheries Research Institute of the Department of Environment Affairs and Fisheries. The aquarium was especially constructed for this purpose and is situated at Sea Point, approximately 7 km from the centre of Cape Town.

Today, the concept of an environment as a sump with a limitless capacity to absorb and detoxify almost any material is no longer held. La Roche et al (1973) stated that two of the most pressing questions to be answered were what and how much of various substances could be released into the environment with predictable effects. They felt that these questions could be answered in the laboratory under simulated environmental conditions. Laboratory tests designed to measure the effects of toxicants on marine organisms are aimed at the drawing up of guidelines for the protection of the marine environment (La Roche et al., 1973; Sprague, 1969).

In general a distinction has been made between two broad categories of effect (Alderdice, 1967), i.e. acute toxicity which is usually lethal and chronic toxicity which may be lethal or sublethal. According to Warner (1967) there seemed to be a consensus of opinion that acute lethal toxicity occurred within the first hundred hours of exposure. Brown (1973) felt that there was an overwhelming need for chronic rather than acute tests, as acute tests merely provided estimates.

Chronic tests that measured the effects of pollutants on growth or reproduction, for example, although they may have been more sensitive, had a serious drawback, namely length of time that was required before useful information was obtained. This fact assumed increasing relevance when one considered the fact that management decisions for water quality were probably made even though adequate scientific information was not available (Bahr, 1973). The importance of short-term tests was that they served to supplement long-term bio-assay experiments by providing interim information as quickly as possible.

The concept of the median lethal concentration or LC50 has been well established and universally used in pharmacology and toxicology (Litchfield & Wilcoxon, 1949). It is a means by which exposure time and concentration can be linked (Gaddum, 1953). The LC50 can be regarded as a convenient and reproducable reference point, representing the concentration that would kill the average organism in a specified exposure time (Sprague, 1969). Under no circumstances should the LC50 be accepted as a "safe" concentration. Even though these short-term laboratory exposures are artificial, they have provided information about many types of pollutant. Their major limitation has been in using death as the end point of the experiment. Many authors have stressed that this type of experimentation is far from satisfactory for assaying pollution and that there should be more concern with sublethal effects (Alderdice, 1967).

The objective of research on lethal and sublethal effects has been aimed at the ability to predict levels that are "safe" under conditions of continual exposure (Tarzwell, 1962). To date estimations of "safe" levels have been based mainly on three considerations (Sprague, 1971);

predicted negligible mortality on long-term exposure, field observations of animals in polluted areas and the absence of sublethal or chronic effects in laboratory tests.

With some pollutants there may be a "no effect" level but, to date, there has been no way of determining whether the threshold is real or whether our ability to measure responses at low concentrations of exposure has been adequate (Waldichuck, 1979). Although it has been relatively easy to demonstrate small changes within an animal, it has been difficult to assess whether these changes are harmful to the animal or within the normal range of adaptation of the animal (Sprague, 1971). Mount and Stephan (1967) felt that research on sublethal effects was not complete until a statement could be made about the ecological significance of these effects.

More accurate predictions could be made about the effects of pollutants if their physiological modes of action were better understood (Alderdice, 1967; Sprague, 1971). Before this can be done, knowledge has to be obtained about the normal internal structure and functioning of the organism and existing techniques in other toxicological fields adapted to prevent incorrect generalisations being made. For example, lobster larvae have about the same resistance to pulp mill waste as salmon, but are apparently affected by different mechanisms, as waste treatment appears to remove the toxicity for salmon, but not for the lobster larvae (Sprague & McLeese, 1968).

Sublethal effects of pollution studied in the laboratory can be divided into four main categories (Waldichuck, 1979): Physiology, Biochemistry/Cell structure, Behaviour/Neurophysiology and Reproduction. Each of these approaches has advantages and disadvantages in terms of generating useful information.

Many physiological processes have been monitored, such as growth, swimming performance, respiration and circulation. Growth rate studies in terms of decrease in growth rate in response to pollutants have proved difficult to interpret, for in some cases pollution has been known to promote growth (McLeay & Brown, 1974). A better approach has been that of Davis and Warren (1968) who related food conversion efficiency, or scope for growth, to environmental stress. Similarly scope for activity, i.e. the difference between an organism's metabolism at rest and its active metabolic state has been shown to be a good means of evaluating performance (Brett, 1964; McLeod & Smith, 1966). A limitation of this method, however, has been the fact that only the performance of one animal at a time could be evaluated, which in turn raises the question of inter-animal variability.

Respiration or the ability to exchange CO_2 for O_2 across the gills is a valuable indicator of sublethal effects (Davis, 1973). There have also been a number of indirect approaches to the study of the respiratory system in which parameters such as ventilatory movements have been monitored (Rice et al., 1977b). Both respiration and activity responses are questionable unless the level of activity has been taken into account. Furthermore, resting rates are difficult to establish.

Much has been learned about the health of aquatic organisms and their responses to pollutants by monitoring heart beat and blood pressure, especially when the animal was forced to undergo rigorous activity (Davis, 1973). Careful evaluation of these experiments was necessary when relating the observed responses to exposure since it could not be assumed that a simple relationship existed between the two.

The responses of many physiological processes such as liver function, kidney performance, excretion, feeding, digestion and osmoregulation have been monitored (Waldichuck, 1979). The choice of a suitable physiological process to measure changes in response to pollutants should be based on that process which would give the most sensitive response at low concentrations.

Biochemistry together with physiology have been disciplines central to the understanding of mode of action of pollutants. Crisp et al. (1967) attempted to understand the mode of action at a chemical level and concluded that physical toxicity of organic compounds was dependent on their thermodynamic action. A number of biochemical changes have been documented in response to toxicants and range from an alteration in blood chemistry to enzyme changes (Waldichuck, 1979). Histology too has proved a good tool for the investigation of pollutant effects (Brown et al., 1968; Hawkes, 1977). The major difficulty with research carried out at this level has been in relating the responses to higher levels of organisation so that a statement could ultimately be made about survival of the species (Mount & Stephan, 1967).

As approaches to the study of sublethal effects, both behaviour and neurophysiology have been shown to be very sensitive to environmental influences (Anderson, 1971; Kleerekoper, 1976; Olla, 1974). Since the functioning of an animal's nervous system has been shown to be dependent on the normal functioning of the other physiological systems, it follows that neural monitors would be sensitive to a wide range of stressors (Bahr, 1973). Behaviour, on the other hand, as manifested in the activities of an animal, represents the integration of a diversity of physiological and biochemical processes. Monitoring behaviour has the advantage that measurements can be made without any harm to the test animal (Wilson, 1970). Both neurophysiological and behavioural investigaitons have suffered, however, with regard to quantification.

Reproduction has been considered to be the single most important function in the life cycle of an organism with regard to sublethal effects (Waldichuck, 1979). Davis (1972) reviewed the effects of pollutants on reproduction of marine organisms. Successful reproduction has been the real test of how severely a population has been impacted. Nonetheless, as stated previously such tests must run for a length of time before useful data are generated.

All the above approaches to sublethal toxicity testing have limited predictive value unless the implications of the data generated can be related to the field (Sprague, 1971). This has been done to some extent by taking into account the range of variables in a site specific situation and recreating them in the laboratory. Good bio-assays integrate all the variables, known and unknown, that affect water quality (Walden, 1976).

As a result of the volume of petroleum shipped by sea, and oil losses to the marine environment, there has been a growing concern over the impact of spilled oil on marine and estuarine environments (Anderson et al., 1974b; Neff & Anderson, 1981). Although all but the most volatile petroleum products can have a smothering effect, the main emphasis in research has been to investigate acute toxicity of the different crude and refined petroleum products on a chemical rather than a physical basis.

Unfortunately most of the published data on the acute effects of oil on marine organisms is such that it is difficult to compare the relative toxicities of these products and the different sensitivities of the marine species exposed to them. This has been largely due to a lack of standardisation of toxicity testing methodologies, in particular the way in which the oil was presented to the organism, and the fact that in many cases the actual exposure concentrations were not measured (Moore & Dwyer, 1974).

Petroleum being an extremely complex mixture of thousands of different hydrocarbons and related hetero-compounds, different crude and refined oils vary greatly in the relative concentrations of the different compounds and consequently show this variability in their solubility, dispersibility and persistence in seawater (Neff & Anderson, 1981). Furthermore, weathering of petroleum may substantially alter its physical and chemical properties, resulting in a change in the composition of the water accommodated fraction. Therefore, before meaningful comparisons can be made, it is essential to specify the actual concentration of the water-accommodated fractions used in bio-assays.

Trends that have emerged from this research indicate that crude oils are generally less toxic than refined products (Anderson et al., 1974a), and that this toxicity is related to the amount of water soluble aromatic hydrocarbons they contain (Neff & Anderson, 1981). However, it must be stated that the major impacts of oil pollution are probably those caused by long-term contamination from sewage plant effluents and sludges, land runoff, and deliberate discharges from vessels and atmospheric fallout, rather than acute spill events (Sinderman, 1979).

Brown and Greenwood (1978) proposed the need for a simple standard toxicity test that could be applied along the South African coastline, using unsophisticated equipment that would give statistically valid results in a short time. In concept this is ideal; however, before this stage can be reached it is necessary to develop complex technology to ensure that the simple standard test is indeed sensitive enough to low levels of pollutant. The studies carried out at the toxicity research aquarium were aimed at the development of methodology and technology to assay sublethal effects and to investigate some of the factors underlying the sensitivity of juvenile Jasus lalandii to the water-soluble fraction of crude oil.

Technological studies were centred around expanding the sensitivity range of existing methods where possible, and the development of new methods to detect and measure the effects of low levels of the water-soluble fraction on the rock lobster. Median lethal concentration experiments (LC50), although artificial, were performed to determine the experimental exposure range. Behavioural observations were made in an attempt to see if behavioural changes induced by the pollutant could be quantified, so that the toxicity of the water-soluble fraction could be estimated. Cardiovascular activity, as reflected by changes in heart beat rate, was monitored to try and relate it to the state of the health of the organism. The major difference between these experiments and those of previous workers was that in these experiments the heart beat rate of groups of animals were monitored. The mean group response for animals exposed to the same conditions served to diminish the effects of interanimal variability. Observations made during the above experiments, especially those on behaviour and heart rate, highlighted the need for neurophysiological investigations to integrate and explain the results obtained.

Brown and Greenwood (1978) also postulated that a species that was widely distributed, obtainable throughout the year and which was distinctive enough to allow casual recognition by relatively untrained workers be chosen for these experiments. On these grounds <u>Jasus lalandii</u> (H. Milne-Edwards) juveniles were found to be suitable experimental animals. This spiny lobster occurs mainly in the waters off the west coast of South Africa on rocky substrata at depths of 1 to 150 m and at temperatures varying from 8,9 to 15,9°C, in salinities ranging from 34,00‰ to 35,16‰ and oxygen content from 3,06 to

6,90 $m\ell\ \ell^{-1}$ (Heydorn, 1969). The ratio of males to females in the field approximates to 1:1 until the juveniles reach the 45 mm cephalothorax length size class after which the proportion of females to males declines (Pollock, 1973). Most of the experiments were performed on rock lobsters in the 2 to 3 year old age group (30 to 45 mm cephalothorax length).

1.1 THE TOXICITY TESTING AQUARIUM FACILITY

During the latter part of 1978 a tour of toxicity-testing laboratories in the USA, Canada and the UK was undertaken by P. Chapman with a view to setting up such a facility in South Africa. A research aquarium was subsequently built at Sea Point, Cape Town, based on the continuous flow system design of Vanderhorst et al. (1977) for Battelle Pacific Northwest Laboratories. This aquarium was situated at the site of an old public aquarium which was no longer in use. The pumping and water treatment plants were situated there as well as several of the old tanks which were renovated and used for water storage and provided header tanks for the new research aquarium.

Raw seawater was dosed with chlorine which prevented fouling of the intake pipeline (Fig. 1), and pumped via a prefilter, which served to remove kelp fronds, stray fish, etc. to two sedimentation tanks and then through two gravel filters. These filters removed organic detritus, sand and other debris associated with a fringing kelp bed which protected the intake pipeline from the full force of swells.

Any residual chlorine was removed by an activated charcoal filter. Storage reservoirs then fed the research aquarium. Effluent that resulted from toxicity bioassays was treated and toxic compounds removed before being returned to the sea.

The aquarium was designed to be as flexible as possible so that experiments could be performed with different kinds of pollutants on either a static or a flow-through basis. Three dosing rigs were constructed for this purpose with an operating range of 10°C to 30°C so that temperature regimes of the east and west coasts of South Africa could be simulated. Temperature control was effected by heat exchangers on each rig. The rigs (shown diagramatically in Fig. 2) were divided into two parts, namely a dosing side and a holding side (not shown) which was used for the acclimation of experimental animals prior to exposure to pollutants.

The dosing system (Fig. 2) was made up of the following components:

1. An effluent metering system for crude oil
2. Oil-seawater contacting area
3. A separation system to remove the floating oil
4. Dispersion metering
5. Dilutant metering
6. Exposure tanks

Although this system was almost an exact replica of the continuous flow apparatus used by Vanderhorst <u>et al</u>. (1977) for petroleum bioassays with fuel oil, the present writer had to modify this system to

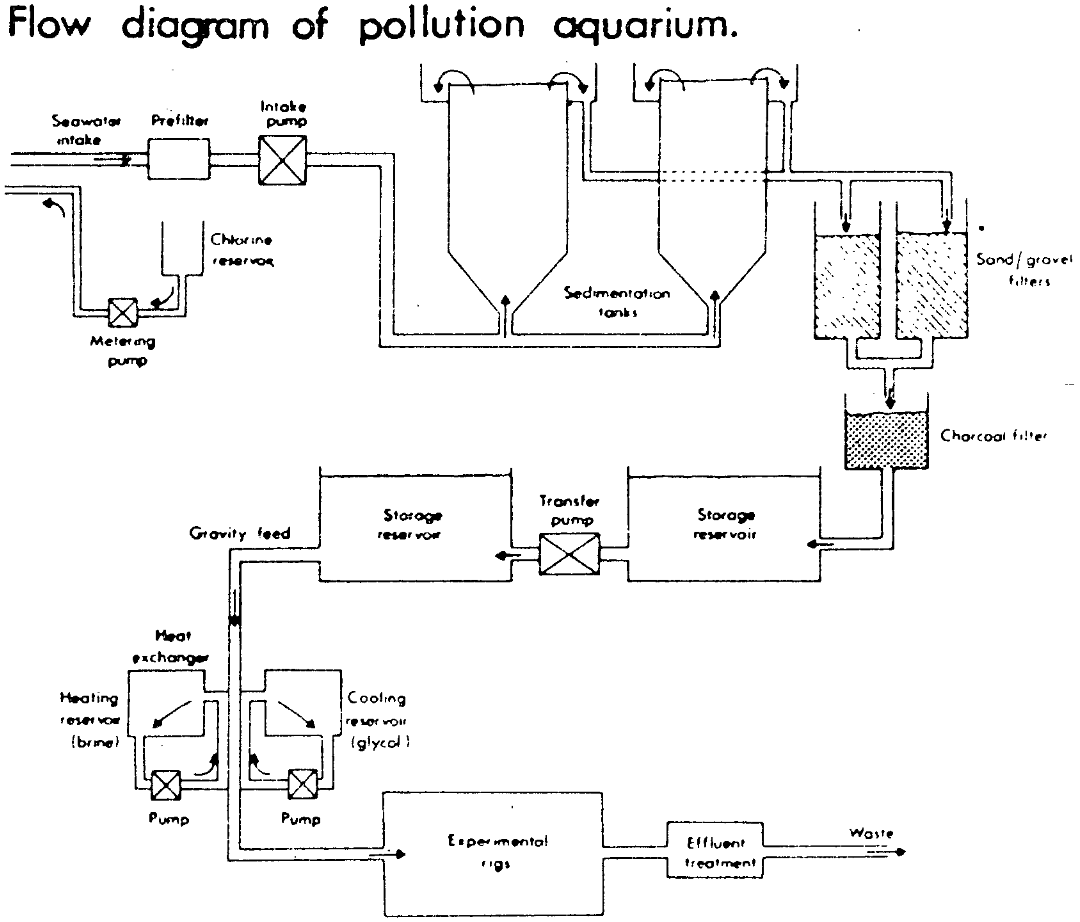
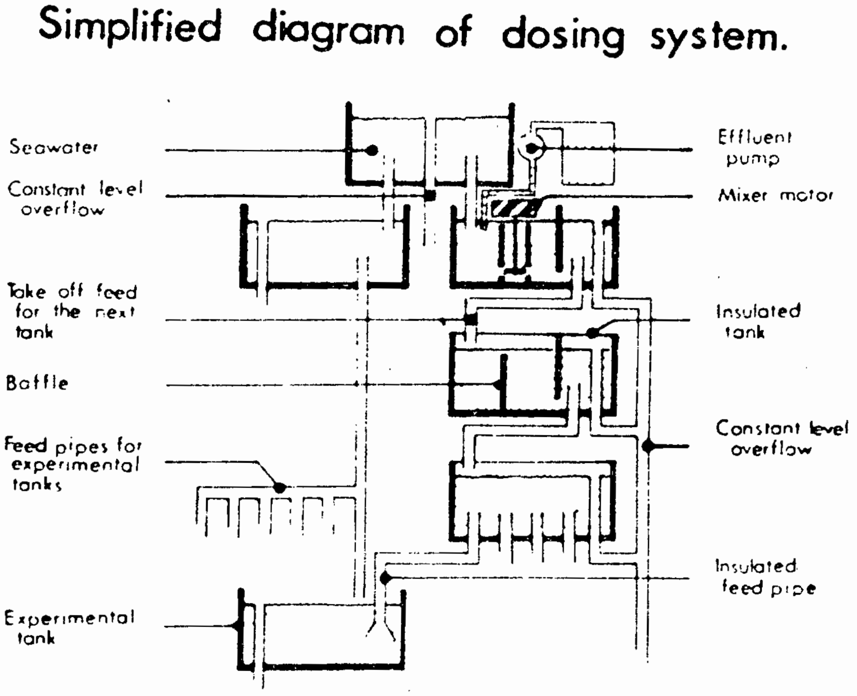


Fig. 1 Flow diagram of pollution aquarium.

cope with the crude oil used in the experiments described below.

The major problems that arose were firstly in getting the petroleum hydrocarbons into sollution at levels suitable for conducting experiments and secondly in keeping them in solution after this had been achieved. Oil discharged on a water surface was found to be in a non-equilibrium condition with respect to evaporation and solution. Hydrocarbons (alkanes, cycloalkanes and aromatics) were expected to dissolve from oil slicks in amounts related to their mole fractions in the oil, and inversely proportional to the molecular weights. This was not observed, indicating that dissolved hydrocarbons evaporated from the near surface waters (McAuliffe, 1977). Furthermore, it has been shown that loss rates for the lighter hydrocarbons (C_2 - C_{10}) were correlated with their vapour pressures. The rates of loss of benzene and cyclohexane from oils were shown to be the same, suggesting that loss by solution when compared with evaporation was minor in view of the fact that the compounds have very different solubilities but similar vapour pressures (McAuliffe, 1977).

The present writer carried out experiments in which crude oil was mixed in 1:9 ratio (Anderson et al., 1974a) with seawater for 10 hours. The mixture was allowed to stand for a further 2 hours and the water soiluble fraction or WSF, i.e. the dissolved, subparticulate and non-filtrable part of the mixture (Wells & Sprague, 1976), was drawn off. This was diluted with filtered seawater and kept at a temperature of 12°C in 34 ℓ glass containers. The dilutions ranged from 100% (14 mg ℓ^{-1} total hydrocarbons) to 40% and were sampled half hourly, and



Simplified diagram of dosing system.

Fig. 2 Simplified diagram of the dosing system.

analysed by infra-red spectrophotometry (Warner, 1978). Preliminary results indicated that the higher the initial concentration of WSF the faster the loss of hydrocarbons, which in some cases was as high as 50% of the initial concentration in the first 3 hours. These findings were similar to those of Anderson et al. (1974a). Aeration of the static tanks, which was necessary when animals were introduced, also contributed to a faster loss of the more volatile compounds (Rossi et al., 1976). Although there was an initial aromatic enrichment of the WSF (Bean et al., 1974; Boylan & Tripp, 1971; Whittle et al., 1980) the ratio of aromatic to aliphatic compounds declined with time during these static tests.

The unmodified flow-through dosing system was initially only able to deliver a maximum of 0,78 mg ℓ^{-1} total hydrocarbons when crude oil was used in the system. This was in part due to the short retention time of oil in the system (approximately 1 hour), but mainly due to the limited mixing of oil and water and the high rate of evaporation. The modifications listed below were developed by the present writer to bring the dosing system up to specification. They included the addition of a supplementary mixing system, alterations to the pollutant feedlines and general insulation of the system to cut down evaporation of volatile hydrocarbons.

The supplementary mixing system was installed in the oil-water contacting tank and consisted of an electric sewing machine motor with a variable speed control. This motor was connected to a small propellor on a shaft mounted inside a cylindrical perspex tube (ID 60 mm) with two horizontal rectangular holes (15 x 40 mm) at the water surface and a series of smaller round holes (3 mm) near the sealed base of the tube. The oil feed was led directly into this tube just below the surface of the water. This arrangement drew oil into the water and forced it out of the holes at the base of the tube in small droplets, effectively increasing the surface area of oil in contact with the water.The peristaltic metering pump was then set to deliver 120 mℓ of crude oil per hour and the mixer motor speed was adjusted so that the vortex created was one third the height of the water column within the tube. A system of baffles was used to separate the floatable oil from the WSF (Fig. 2). Removal of the floatable oil was improved by cutting a notch (20 x 30 mm) into each baffle at the water surface, this enabled the surface oil to drain via the overflow to the effluent treatment part of the system.

The separation and dispersion metering tanks were sealed from the atmostphere with aluminium foil and masking tape, as were all feed pipes from the dispersion to the dilution (exposure) tanks. These pipes all terminated in adjustable teflon taps which allowed the amount of WSF entering the exposure tanks to be controlled. Entry of effluent into the exposure tanks was through an inverted large-diameter funnel. This allowed the WSF to flow down the sides of the funnel and to lose velocity so that the final separation of any oil droplets that may have bypassed the baffles or that may have recoalesced in the feed lines, could occur, ensuring that only WSF was presented to the test animals. The WSF emerging from the funnel was checked by taking samples before and after filtration through glass wool (to remove oil droplets) and showed that only the WSF was indeed getting through.

The above alterations to the system enabled concentrations greater than 18 mg ℓ^{-1} of WSF to be produced and maintained in the system. Being an integrated system, a change in any part of the system had ramifications throughout the rest of the system. Therefore for the purposes of replicability the system was calibrated before each experiment and checked on completion of the run. The temperature for all experiments on Jasus lalandii was kept constant at 12°C (± 0,5°C) unless otherwise stated, and the total flow rate per experimental 50 litre tank, i.e. clean and contaminated seawater mixture was 1000 mℓ min^{-1}. Use was made of normal daylight and no other illumination was provided.

2. MEDIAN LETHAL CONCENTRATION TESTS (LC50)

2.1 AIM

Short-term median lethal toxicity studies were carried out to determine the range of tolerance of juvenile Jasus lalandii to the water-soluble fraction (WSF) of South African standard crude oil (see Appendix c), so that realistic exposure levels could be set for acute sublethal studies.

2.2 INTRODUCTION

The South African west coast lobster is an important commercially exploited species. Most of the published literature on this animal has consequently been orientated around population biology studies. The paucity of literature on the physiology and behaviour of Jasus lalandii made it necessary to investigate some of these aspects before responses to pollutants could be evaluated. This had to be done so that a distinction could be made between toxic effects and normal adaptational responses, which may be essential for the survival of the species through the various stressful conditions encountered in the animals life cycle (Waldichuck, 1979).

Despite its shortcomings, the acute bioassay has been shown to be a good means of categorising a mixture of toxic compounds as found in crude oil (Sprague, 1969; Walden, 1976). Median lethal toxicity tests do allow comparisons to be made between the toxicity of different oils, the sensitivity of different stages of the life cycle of a single species as well as the sensitivities of the different species (Rice et al., 1977a).

To date most studies have involved static exposures rather than flow-through tests because of the relative ease of use and inexpensiveness of such tests. Pilot studies were carried out by the present writer in which use was made of a static system to perform 96 hour LC50 experiments. Owing to problems encountered with this approach it was decided to use a flow-through system instead.

In the static tests a WSF was obtained by mixing oil and seawater in a 1:9 ratio for 10 hours. The WSF was drawn off after being allowed to stand for a further two hours before being diluted with clean seawater prior to presentation to the lobsters. The animals and WSF mixture were kept at a temperature of 12°C by suspending the test containers in water baths. This mixture was renewed every 12 hours for a period of 96 hours and analysed for total hydrocarbons by infra red spectrophotometry (Warner, 1978).

A number of problems relating to the concentration of the WSF were encountered. Firstly, the concentration of total hydrocarbons varied from mix to mix. This was probably related to the condition of the parent oil which had been decanted into small containers prior to addition to the mixing tank. Secondly, during the dilution phase further loss of volatile hydrocarbons occurred which was dependant on how vigorous the mixing process between the WSF and the clean seawater had been. Both these two problems contributed significantly to fluctuating starting concentrations in the test containers when the solutions were renewed at the end of each 12 hour period. Abram (1967) and Brown et al. (1969), however, concluded that the average of fluctuating concentrations could be used to adequately predict effects of exposure in such circumstances.

The next two problems encountered were related to the relative volume of the test containers and the size and number of Jasus juveniles per experimental tank. Aeration had to be introduced into the static tanks to prevent the effects of hypoxia, this also contributed to a rapid loss of the WSF to very low levels. Therefore the maintenance of effective concentrations of WSF would have required a greater renewal frequency of the test solutions. This would have definitely further stressed the test animals and possibly resulted in a significant effect on the experimental result. The final problem encountered related to the excretory products and other unidentified substances shed into the water by the animals in response to exposure to the WSF. These products built up in the static containers, to the point where it was no longer possible to analyse the water samples taken for hydrocarbon analysis.

Both Neff et al. (1976) and Vanderhorst et al. (1976) stated the need for flow-through systems as a result of difficulty in deriving an LC50 value from a static exposure when the concentration of the WSF is rapidly declining. A steady level of WSF would, however, approximate to a continuous input in the field situation.

2.3 METHODS AND MATERIALS

Jasus juveniles in the 2 - 3 year old age group were collected from the outer wall of the breakwater of Table Bay docks and from the kelp beds off Sunset Beach, Sea Point, by SCUBA divers. The animals were then transferred to flow-through holding tanks at the Sea Point aquarium and acclimated for a minimum period of 10 days at a temperature of 12°C. During this time they were fed twice weekly on live black mussel (Aulacomya magellanica) collected from the same locations as the lobsters. Soft-shelled (post-moult) animals and animals that were damaged during capture were not used in the experiments.

2.3.1 Apparatus

For the toxicity experiments the animals were transferred to the dosing side of the flow-through rigs (described in 1.1) to six fibreglass 50 ℓ tanks (Fig. 3). Four of the six tanks were supplied with predetermined dilutions of WSF and filtered seawater. The remaining two control tanks were only supplied with filtered seawater. Flow rate in each tank was set at 1000 mℓ min^{-1}, i.e. the total mixture of WSF and seawater together. This meant a complete turnover every

Fig. 3 Flow-through system used in toxicity bioassays.

fifty minutes. Temperature was set at 12°C, and monitored hourly. Dissolved oxygen (5,92 - 5,11 mg ℓ^{-1}), pH (8,1) and salinity (34,0‰) were monitored twice daily. Photoperiod was determined by the incident daylight entering through the windows of the aquarium (13 hours light:11 hours dark). The WSF was measured for total hydrocarbons by double extracting a 250 mℓ seawater sample into 25 mℓ of carbon tetrachloride (Warner, 1978). This was done twice daily by siphoning a sample from the middle of the water column. A "blank" of seawater was also run to exclude the effect of extraneous solvent extractable organics (Gruenfeld, 1973). Samples were run on a Beckman IR18 infrared spectrophotometer. Absorbances were measured between 2800 cm^{-1} and 3200 cm^{-1} using a 10 mm path length cell and values were read off a standard curve which was prepared from a reference sample of the original parent oil, from which the WSF was derived. Special attention was paid to the CH_2 group at 2930 cm^{-1} from which the value of total hydrocarbons was calculated. The CH_3 group at 2960 cm^{-1} and the CH aromatic group at 3030 cm^{-1} were also examined. The latter showed some aromatic enrichment of the WSF (Whittle et al., 1980).

2.3.2 Test Procedure

A flow-through exposure period of 24 hours was chosen, as under natural conditions the lighter fractions of crude oil evaporate quickly. This would result in animals not being exposed to a WSF that was representative of the parent oil for extended periods. A minimum of 10 sex and size matched animals per concentration were exposed to predetermined amounts of WSF ($<$ 10 mg ℓ^{-1}).

Mortalities were recorded every four hours and dead animals removed. Death was taken to be the cessation of all external movement. Particular attention was paid to the antennule flagella and the schaphognathites. It was interesting to note that electrocardiograms (ECG's) showed that the heart of Jasus continued to beat, albeit very slowly for some time after this stage was reached. However, once all external movement ceased the animals never recovered even when returned to clean water.

2.3.3 Data Processing

In toxicity testing special analytical procedures must be used. This is because of the double role played by time, i.e. both concentration and exposure time are part of the experimental stimulus, yet time is also part of the response (Gaddum, 1953). Since the concentration is fixed within each test tank, exposure time becomes the stimulus, controlled by the investigator according to the time chosen for inspection. The response therefore is percentage mortality. This has the effect of assessing each animal's response in an "all or none" or quantal way (Sprague, 1969).

If cumulated percentage mortality from such tests is plotted against exposure time a skewed sigmoid curve usually results. This skew is apparently due to the logarithmic nature of biological time (Gaddum, 1953). The sigmoid curve usually tails off more gradually towards longer survival times, reflecting variable responses of individuals (Finney, 1964). These sigmoid curves can usually be straightened by plotting the percentage response on a probability or "probit" scale instead of an arithmetic scale. Probits merely express mortality in terms of standard deviations above and below the mean response (Sprague, 1969). The mortalities in this study were analysed according to the methods set out in the MAFF Fisheries Research Technical Report No. 61 (Franklin, 1980). Percentage cumulative mortality, which is defined as $\frac{2\ m - 1}{2p}$ x 100 where m is cumulative mortality and p is the total number of animals in the tank was plotted against exposure time. A probit scale was used for mortality and a logarithmic scale for time. A line was then fitted by eye through the points (Litchfield & Wilcoxon, 1953), with a greater emphasis given to those points lying between the 25% and 75% mortality values to produce a mortality time curve (Fig. 4). The time for 50% mortality (LT50) for each concentration exposure was then calculated directly from the graph where the fitted lines cut the 50% mortality value (Litchfield, 1949).

The median lethal concentration or LC50 was calculated in a similar way by plotting percentage cumulative mortality (probit sclae) agaisnt concentration (logarithmic scale) (Fig. 4). Corrections could, where necessary, be made for control mortalities by using the method of Tattersfield and Morris (1924).

Concentraiton response curves were used to provide additional information about the rate of toxification (Franklin, 1980). These curves were constructed by plotting the LT50 values against concentration using log-log graph paper (Fig. 5). The important feature of these graphs is their shape (Franklin, 1980).

In the median lethal concentraiton test performed, confidence limits were not calculated for two main reasons. Firstly these tests were used to set the levels of petroleum hydrocarbons for sublethal studies, so absolute values were of limited importance. Secondly, confidence limits are of limited value as they merely indicate what might be expected if the same stock of animals were immediately retested under identical conditions (Anderson <u>et al</u>., 1974a; Finney, 1952).

2.4 RESULTS

Twenty-four hour median lethal concentraiton experiments (Flow-through system).

TABLE 1 Mortality/Time/Concentration

hr	0 mg ℓ^{-1}	3,0 mg ℓ^{-1}	4,3 mg ℓ^{-1}	5,7 mg ℓ^{-1}
0	-	-	-	-
4	-	-	-	-
8	-	-	1F (5)	2F (15)
12	-	2F (15)	1F, 3M (45)	1F, 2M (45)
16	-	-	1M (55)	2F, 2M (85)
20	-	-	1M (65)	1M (95)
24	-	-	2F (85)	-

GROUP A <u>Jasus lalandii</u>

Mean cephalothorax length: 32,7 mm (σ_{n-1} = 2,85)

Soruce: Sunset Beach, Sea Point

Number of animals per concentration: 10

Temperature: 12°C

F : Female

M : Male

( ) : Percentage cumulative mortality

See Fig. 4A

TABLE 2 Mortality/Time/Concentration

hr	0 mg ℓ^{-1}	2,9 mg ℓ^{-1}	4,0 mg ℓ^{-1}	4,8 mg ℓ^{-1}
0	-	-	-	-
4	-	-	-	-
8	-	-	1M (5)	1F (5)
12	-	-	1M (15)	1F, 1M (25)
16	-	-	-	1F (35)
20	-	-	-	2M (55)
24	-	-	-	1F, 1M (75)

GROUP B <u>Jasus lalandii</u>

Mean cephalothorax length : 37,0 mm (σ_{n-1} = 2,42)

Source : Outer breakwater Table Bay docks

Number of animals per concentraiton : 10

Temperature : 12°C

F : Female

M : Male

( ) : Percentage cumulative mortality

See Fig. 4B

TABLE 3 Mortality/Time/Concentration

hr	0 mg ℓ^{-1}	3,8 mg ℓ^{-1}	5,9mg ℓ^{-1}	8,5 mg ℓ^{-1}
0	-	-	-	-
4	-	-	-	-
8	-	-	-	1F, 2M (25)
12	-	1F (5)	2F, 1M (25)	2F (45)
16	-	-	-	-
20	-	-	2F (45)	1F, 2M (75)
24			1F (55)	1F, 1M (95)

GROUP C <u>Jasus lalandii</u>

Mean cephalothorax length : 37,9 mm (σ_{n-1} = 2,59)

Soruce : Sunset Beach, Sea Point

Number animals per concentration : 10

Temperature : 12°C

F : Female

M : Male

( ) : Percentage cumulative mortality

See Fig. 4C

TABLE 4 Mortality/Time/Concentration

hr	0 mg ℓ^{-1}	3,4 mg ℓ^{-1}	6,1 mg ℓ^{-1}	9,7 mg ℓ^{-1}
0	-	-	-	-
4	-	-	-	-
8	-	-	2F (7, 5)	3F, 3M (12, 5)
12	-	-	1M (12, 5)	2F, 2M (47, 5)
16	-	-	3M (27, 5)	1F (52, 5)
20	-	2F (7, 5)	1M (32, 5)	1F, 1M (62, 5)
24	-	2F (17, 5)	1F, 4M (57, 5)	2M (72, 5)

GROUP D *Jasus lalandii*

Mean cephalothorax length ; 40,6 mm (σ_{n-1} = 2,89)

Source : Sunset Beach, Sea Point

Number of animals per concentration : 20

Temperature : 12°C

F : Female

M : Male

( ) : Percentage cumulative mortality

See Fig. 4D

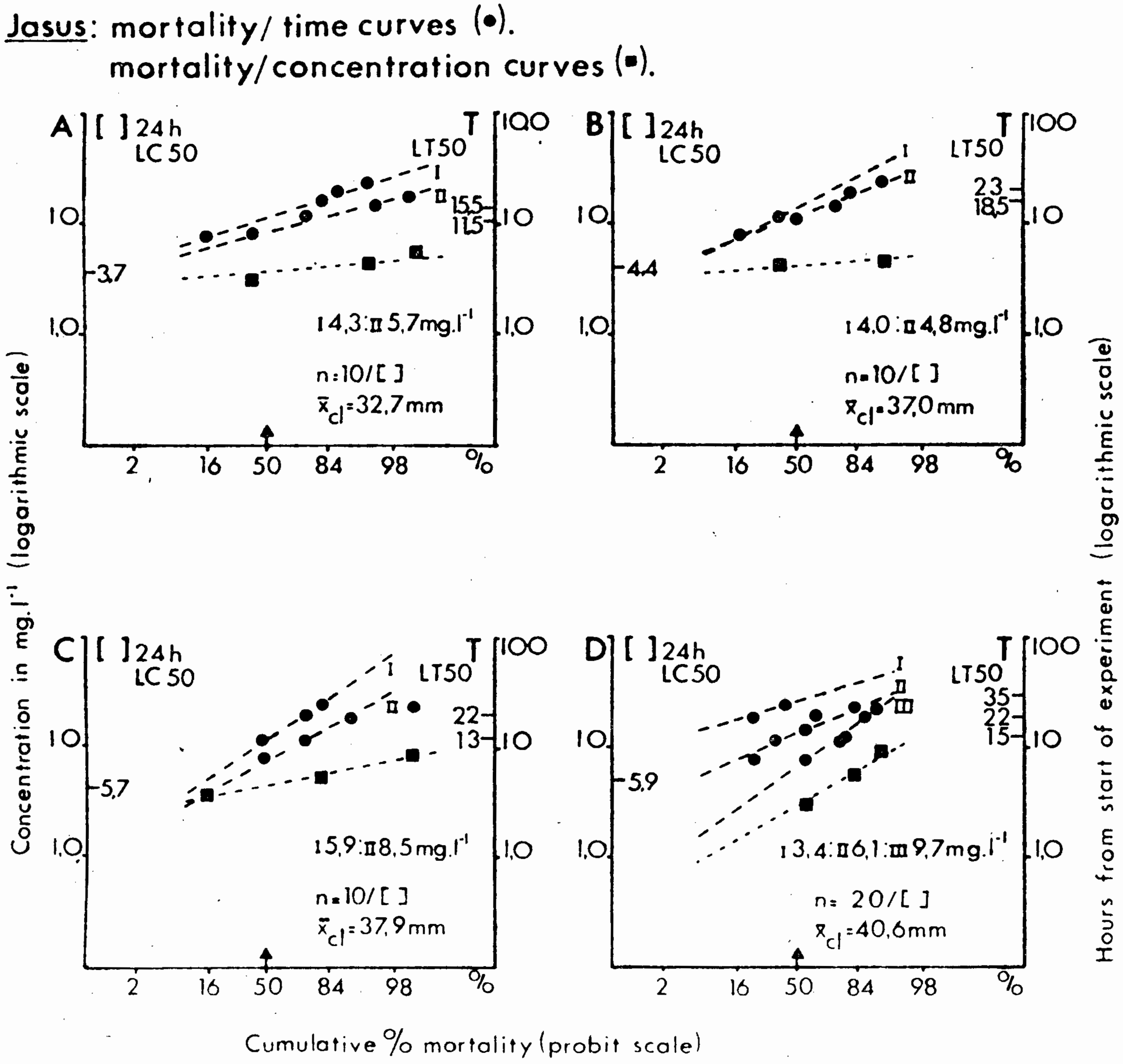


Fig. 4 Mortality/time curves, Mortality/concentration curves of Jasus exposed to WSF of crude oil

TABLE 5 Concentration/Response Data (LT50)

GROUP A

Concentration (mg ℓ^{-1})	LT50 (hr)
4,3	15,5
5,7	11,5

GROUP B

Concentration (mg ℓ^{-1})	LT50 (hr)
4,0	23,0
4,8	18,5

GROUP C

Concentration (mg ℓ^{-1})	LT50 (hr)
5,9	22,0
8,5	13,0

GROUP D

Concentration (mg ℓ^{-1})	LT50 (hr)
3,4	35,0
6,1	22,0
9,7	15,0

See Fig. 5

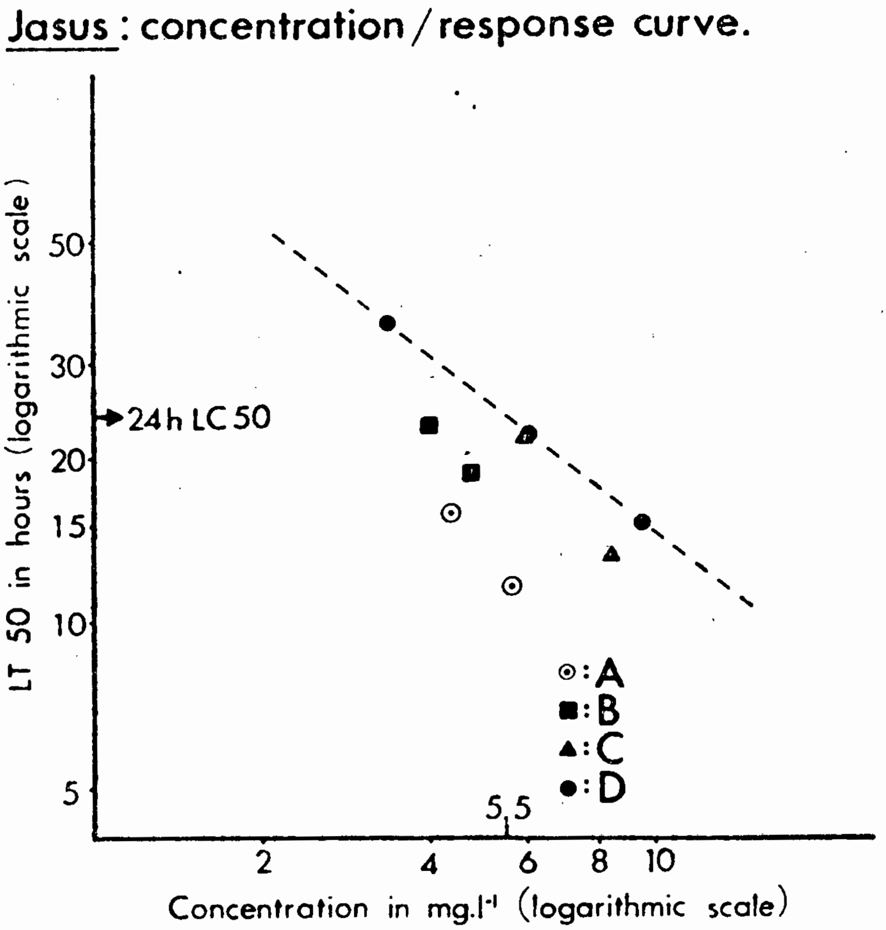


Fig. 5 Jasus: concentraiton/response curve for Group D (mean cephalothorax length - 40,6 mm).

2.5 DISCUSSION

Although limited data are available on quantitative comparisons between the sensitivities of adult and early life stages of animals, evidence indicates that the larval stages may be the most sensitive. Furthermore, comparisons between different kinds of animals indicate that the juvenile and larval crustaceans seem to be the most sensitive, especially during moulting (Anderson, 1979; Mecklenburg et al., 1977; Wells, 1972). Although Jasus moults throughout the year, there is a slight increase in moulting in the summer months (Heydorn, 1969). This must be borne in mind when evaluating experimental results. The above trends have emerged from median lethal toxicity studies, yet the mechanism of toxicant action is still not well understood. Johnson (1977) felt that the different permeabilities of arthropod skeletons between species and between different developmental stages in the same species may account for the wide range of sensitivities encountered.

Toxicity is the product of exposure time and dose, consequently relative sensitivity must be expressed in these terms. Besides the reasons given earlier for the acute exposure time of 24 hours, used in these experiments, Anderson et al. (1974b) found that most mortalities occurred in the first 12 to 24 hours. This was confirmed by the present writer using longer exposure periods (48 and 96 hours). However, bioassays with short time limits may be misleading as such tests may essentially be a measure of survival time at a given concentration and not toxicity as measured by the LC50 (Sprague, 1970).

The 24 hour median lethal concentraiton for the two to three year old group ranged between 3,7 and 5,9 mg ℓ^{-1}. This increase with age in the LC50 value was expected, as during its second year, juvenile Jasus increases its mean cephalothorax length by approximately 50% (30 - 45 mm, Pollock - personal communication).

The main criticism for using the LC50 as a research method is that data is used in a quantal form, i.e. animals are classified as either dead or alive at the end of an arbitrary period (Sprague, 1969). Consequently much information is lost about the graded time response and the precision of the experiment may be reduced accordingly (Bliss & Cattle, 1943; Finney, 1964).

Sex was not considered to be an important factor with this group (30 - 45 mm cephalothorax length), as the normal field ratio of males to females is approximately 1 to 1 (Polock, 1973). This was possibly an indication that animals belonging to this size class share the same ecological niche and consequently the same physiological tolerance range. However, a further disadvantage of LC50 tests was that they usually involved one toxicant under one set of conditions. This gave them limited predictive value as they did not usually take the effect of modifying conditions (temperature, pH, etc.) into account which could have greatly affected toxicity (Sprague, 1970).

The concentration response curve (Fig. 5) obtained for the Jasus juveniles (gorup D), by plotting LT50 against concentration (both on logarithmic scales) appeared to have a linear relationship. This suggested that under the acute experimental conditions *Jasus* was not able to adapt to the WSF and that no lethal threshold was evident (Franklin, 1980). This meant that some mortalities could have been expected at levels well below the LC50 value. Other workers in the field have shown that crustaceans are able to metabolise petroleum hydrocarbons (Stegeman, 1977), and so mitigate their effects to some degree. The results presented above may have meant that *Jasus* was unable to mount a significant adaptational response to the WSF in the short experimental period. According to Anderson (1977) there was no reason to believe that a pollutant had to be accumulated in tissues to produce an adverse effect but to his knowledge every pollutant studied has been found in tissues of exposed animals at levels above those of the surrounding waters. Tissue accumulation of petroleum hydrocarbons seemed to occur due to tissue lipid versus seawater solubilities of the WSF (Anderson, 1977).

During the course of the 24 hour LC50 experiments a number of observations were made which appeared to be directly related to the toxicity of the WSF. Firstly animals that were exposed to the WSF appeared in some cases to lose the ability to regulate their internal concentration of solutes. The disruption of osmoregulation manifested itself in that some animals became bloated shortly before death. Anderson (1977) made similar observations on the brown shrimp *Penaeus aztecus*. Secondly, in all experiments in which animals were exposed to the WSF of crude oil there was evidence of the induction of the autotomy reflex (Paterson, 1968). The nubmer of thoracic legs shed was related to the initial concentration of the WSF; the higher the concentration the greater the number of legs shed. Karenin and Rice (1974), who performed static bioassays with tanner crab juveniles Chionoecetes bairdi, obtained a similar response. This WSF induced autotomy would have been detrimental to the surval of the animal in the field.

All the above experiments were carried out at a temperature of 12°C. This too could have influenced the toxicity of the WSF due to the slower evaporation rate of the toxic low molecular weight compounds, resulting in LC50 values near the lower end of the scale (Anderson, 1979).

3. BEHAVIOURAL RESPONSES TO THE WSF OF CRUDE OIL

3.1 AIM

The purpose of these experiments was to quantify gross animal behaviour in response to known concentrations of water soluble fraction (WSF).

3.2 INTRODUCTION

A change in behaviour is the initial response of an animal to an environmental stressor (Slobodkin, 1968). Manning (1974) defined behaviour as all the processes by which an animal sensed the external world and the internal state of its body, and responded to changes that it perceived. Responses to a wide range of environmental stressors were found to be similar in that they were attempts to restore the normal metabolism that had been upset (Manning, 1974). For an animal to successfully mitigate the effects of a pollutant, the animal must be capable of sensing it, recognising it as harmful and responding appropriately (Olla *et al*., 1980).

Even though the detection of a pollutant has been shown to occur at very low concentrations (Blumer et al., 1973), the elicitation of more complex behaviour appeared to require much higher concentrations. Olla et al. (1980) demonstrated that Callinectes sapidus could detect naphthalene at concentrations of 10^{-7} mg ℓ^{-1} and the WSF of crude oil at 10^{-6} mg ℓ^{-1}. Yet this crab only showed locomotory activity at a concentration of 2 mg ℓ^{-1}, a value approximately seven orders of magnitude higher than the detection threshold. The measurement of an animal's sensory capabilities, however, only tells us what the system can do and not necessarily what it does. Nonetheless as marine animals respond to very low concentrations of natural chemical cues in the sea, oil pollution may interfere with chemotaxis in several maladaptive ways (Blumer et al., 1973).

Atema (1977) reported ambivalent behaviour of Homarus americanus to some fractions found in crude oil. Most of the fractions presented inhibited feeding; some, however, attracted lobsters at low concentrations and stimulated feeding (Atema & Stein, 1974; Atema, 1977), indicating the failure of the lobster to recognise them as harmful.

Fundamentally the success of a behavioural approach to the study of pollutant effects has been dependent upon the ability to quantitatively define normal patterns of behaviour (Eisler, 1979). It has often been difficult to distinguish pollutant induced behavioural modification from natural behavioural variation (Neff & Anderson, 1981). To date behavioural studies which have dealt with the effects of oil on locomotion, orientation, feeding and predation and alarm reaction have received particular attention (Eisler, 1979; Neff & Anderson, 1981).

Crisp et al., (1967) noted that organic contaminants produced varying degrees of narcosis. This was also given as the reason for detachment of the intertidal limpets Patella vulgata after exposure to Kuwait crude oil (Dicks, 1973, 1976). Percy and Mullin (1977) found that locomotory activity was impaired in the amphipod Onisimus affinis at concentraitons of 15 - 20 mg ℓ^{-1} of an oil in-water-dispersion. However, before the narcotic aciton of petroleum hydrocarbons took effect most authors reported an increase in activity (Swedmark et al., 1973). Hargrave and Newcome (1973) reported an increase in the crawling rate of the intertidal snail Littorina littorea, and Idoniboye-Obu (1977) also measured an increase in the motor activity of five species of crustacea when presented with low concentrations of n-hexane.

Pilot studies were carried out by the present writer in the aquarium in an attempt to find a suitable method of quantifying the gross behavioural changes observed when lobsters were exposed to the WSF. The initial increase in activity seemed to be directly related to the exposure concentration, i.e. the higher the concentration the greater the activity. The nature and rapidity of the movements of the animals made it impossible to quantify visually the amount of activity observed.

The short period of high initial activity gave way to a longer quiescent period in which activity decreased as the narcotic action of the WSF began to take effect. It was therefore decided to look in more detail at the period of decreasing activity with longer exposure to the pollutant. Plans were drawn up for the construction of special cages with intersecting light beams and photocells to detect animal movements, but these were found to be unnecessary, as a simple assay which did not require the use of any complicated technology was developed.

3.3 METHODS AND MATERIALS

These experiments were run simultaneously with the 24 hour LC50 experiments described in 2.3 using the same group of animals and the same experimental design.

Swedmark *et al*. (1973), when describing the effects of oil dispersants and oil emulsions on marine animals, noted that these effects manifested themselves in the following sequence. Firstly, increased activity which was attributed to avoidance reactions, followed by the successive impairment of activity. This in turn, was followed by an affected sense of equilibrium in mobile species and finally immobilisation and subsequent death of the animal at lethal concentrations.

In all cases this sequence appeared to be the same in *Jasus* when exposed to the WSF crude oil. The only variable was the length of the sequence which was inversely dependent on the concentraiton of the WSF.

In an attempt to quantify the stages of impairment, the behavioural sequence as described by Swedmark et al. (1973) was reworked into a behavioural scale with definite cut off points to lessen the subjective influence of the observer (Lipschitz, 1982). These stages were defined as follows:

0. Normal behaviour: compared with non-exposed control group (Fig. 6A).

I. Co-ordination impairment: not taking the severity into account.

II. Loss of equilibrium: animals on their backs and no longer able to right themselves, legs actively moving (Fig. 6B).

III. Moribund: animals on their backs barely moving.

IV. Death: the cessation of all movement including antenular flagellars and schapnognathites. Cardiograms performed by the present writer have shown that the heart beats, albeit very slowly, after the cessation of all external movements. Even so it was decided to use lack of movement as a criterion for death, because once this stage was reached, the aniamls did not recover even when transferred to clean environments.

Observations were made every four hours during the course of the median lethal toxicity experiments and the behavioural state of the animals recorded and assigned values according to the above behavioural scale.

Fig. 6A Control (no WSF) normal behaviour state.

Fig. 6B Exposed (WSF) animals showing loss of equilibrium.
Note the autotomised limbs.

These behavioural values were then related to exposure concentration by applying methods based on the MAFF Fisheries Technical Report No. 61 for assaying the toxicity of industrial wastes (Franklin, 1980). The major difference being that a behavioural value was substituted for the normal end point of death used in conventional assays. Brett (1967) found that log-probability techniques for analysing results of acute bioassays could also be applied to studies of swimming performance. The application of these techniques in the present experiments was done by plotting percentage cumulative loss of equilibrium against the exposure concentration (Lipschitz, 1982). For the purposes of these experiments, percentage cumulative loss of equilibrium was defined as

$$\frac{21e - 1}{2p} \times 100$$

where le was the cumulative number of animals displaying loss of equilibrium moribundity or death, and

p was the total number of animals per concentration.

Percentage cumulative loss of equilibrium was plotted on a probit scale and concentration on a logarithmic scale. A line was then fitted by eye through the points. By reading off the concentration value corresponding to the 50 percent cumulative loss of equilibrium, a value close to the 24 hour LC50 was obtained within a few hours of exposure. Comparisons can only be made between animals of equal sized groups that have been exposed to a concentration of a pollutant for the same length of time. Furthermore, neither the 0% value nor the 100% value can be used more than once, i.e. if 100% of animals at any sampling interval have passed beyond, or were at stage II, that group of animals may not be included in the following sampling interval.

3.4 RESULTS

TABLE 6 Behaviour/concentration

Hr	0 mg ℓ^{-1}	3,0 mg ℓ^{-1}	4,3 mg ℓ^{-1}	5,7 mg ℓ^{-1}	
0	10=0	10=0	10=0	10=0	
4	10=0	10=0	3=I, 7=II (65)	10=II (95)	Ø
8	10=0	10=0	1=I, 8=II, 1=IV	8=II, 2=IV	
12	10=0	7=I, 1=II, 2=IV (25)	4=II, 1=III, 5=IV (95)	5=II, 5=IV	Ø
16	10=0	5=I, 3=II, 2=IV	3=II, 1=III, 6=IV	I = II, 9=IV	
20	10=0	4=I, 4=II, 2=IV	2=II, 1=III, 7=IV	10=IV	
24	10=0	3=I, 5=II, 2=IV	1=III, 9=IV	10=IV	

GROUP A *Jasus lalandii*

Mean cephalothorax length : 32,7 mm (σ_{n-1} = 2,85 mm)

Source: Sunset Beach, Sea Point

Number of animals per concentration: 10

Temperature 12°C

0 : Normal, I : Coordination impairment, II : Loss of equilibrium III : Moribund, IV : Dead
( ) : Percent cumulative loss of equilibrium, Ø See Fig. 7A.

TABLE 7 Behaviour/concentration

Hr	0 mg ℓ^{-1}	2,9 mg ℓ^{-1}	4,0 mg ℓ^{-1}	4,8 mg ℓ^{-1}
0	10=0	10=0		10=0
4	10=0	10=0	6=0, 4=II (35)	10=II (95) Ø
8	10=0	10=0	9=II, 1=IV	7=II, 2=III, 1=IV
12	10=0	10=0	8=II, 2=IV	6=II, 1=III, 3=IV
16	10=0	10=0	8=II, 2=IV	4=II, 2=III, 4=IV
20	10=0	10=0	8=II, 2=IV	2=II, 2=III, 6=IV
24	10=0	10=0	8=II, 2=IV	2=II, 8=IV

GORUP B *Jasus lalandii*

Mean cephalothorax length : 37,0 mm (σ_{n-1} = 2,42 mm)

Source : Outer breakwater Table Bay docks

Number of animals per concentration : 10

Temperature : 12°C

0 =Normal; I = Coordination impairment; II = loss of equilibrium, III = Moribund; IV Dead; ( ) Percent cumulative loss of equilibrium

Ø See Fig. 7B

TABLE 8 Behaviour/concentration

Hr	0 mg ℓ^{-1}	3,8 mg ℓ^{-1}	5,9 mg ℓ^{-1}	8,5 mg ℓ^{-1}
0	10=0	10=0	10=0	10=0
4	10=0	10=0	3=0 , 7=II (65)	9=II, 1=III (95) Ø
8	10=0	7=0+I, 3=II (25)	9=III, 1=III (95)	7=II, 3=IV Ø
12	10=0	7=I, 2=II, 1=IV	7=II, 3=IV	4=II, 1=III, 5=IV
16	10=0	9=I+II, 1=IV	6=II, 1=III, 3=IV	5=III, 5=IV
20	10=0	9=I+II, 1=IV	5=II, 5=IV	2=III, 8=IV
24	10=0	3=I, 6=II, 1=IV	4=II, 6=IV	10=IV

GROUP C Jasus lalandii

Mean cephalothorax length : 37,9 mm (σ_{n-1} = 2,59 mm)

Source : Sunset Beach, Sea Point

Number of animals per concentration : 10

Temperature : 12°C

0 = Normal; I = Coordination impairment; II : Loss of equilibrium; III = Moribund; IV = Dead; ( ) Percent cumulative loss of equilibrium; Ø See Fig. 7C

TABLE 9 Behaviour/concentraiton

Hr	0 mg ℓ^{-1}	3,4 mg ℓ^{-1}	6,1 mg ℓ^{-1}	9,7 mg ℓ^{-1}
0	20=0	20=0	20=0	20=0
4	20=0	20=0	15=I, 5=II	7=I, 13=II
8	20=0	16=0, 4=I	8=I, 9=II, 7=II 2=IV (57,5)	14=II, 6=IV (97,5) Ø
12	20=0	19=0+I, 1=II	5=I, 11=II, 4=III	10=II, 10=IV
16	20=0	17=0+I, 3=II	4=I, 9=II, 1=II 6=IV	9=II, 11=IV
20	20=0	13=I, 5=II, 2=IV	12=II, 1=III, 7=IV	7=II, 13=IV
24	20=0	13=1, 3=II, 4=IV	8=II+III, 12=IV	5=II, 15=IV

GROUP D Jasus

Mean cephalothorax length : 40,6 mm (σ_{n-1} = 2,89 mm)

Source : Sunset Beach, Sea Point

Number of animals per concentration : 20

Temperature : 12°C

0 = Normal; I = Coordination impairment; II = Loss of equilibrium; III = Moribund; IV = Dead; ( ) Percent cumulative loss of equilibrium; Ø See Fig. 7D.

Jasus: behaviour/concentration curves.

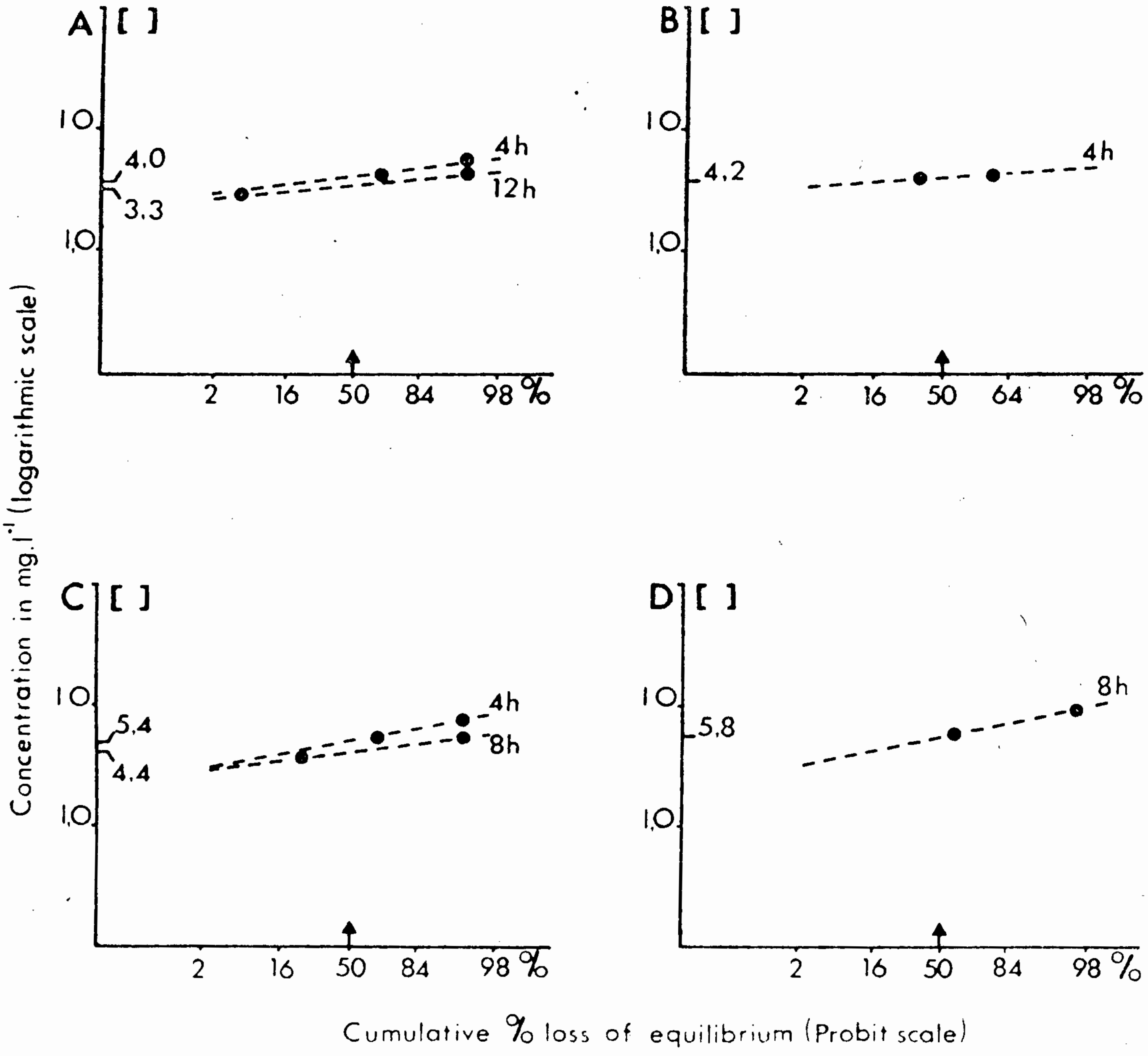


Fig. 7 *Jasus* behaviour/concentration curves for Groups A, B, C and D.

TABLE 10 A comparison of the $LC_{24}50$ values obtained by conventional means (Franklin, 1980) and the 'behavioural' $LC_{24}50$ values

GROUP	$LC_{24}50$ Calculated	$LC_{24}50$ Behavioural		
	24 hrs	4 hrs	8 hrs	12 hrs
A	3,7	4,0	-	3,3
B	4,4	4,2	-	-
C	5,7	5,4	4,4	-
D	5,9	-	5,8	-

Values in mg ℓ^{-1}

3.5 DISCUSSION

Although complex biochemical and physiological responses of an animal to a pollutant may be reflected in easily observable acts, behavioural measures generally suffer with respect to quantification because of the high degree of inherent variability. Nonetheless, behavioural studies can be directly interpreted as regards environmental quality (Olla, 1974). Before this can be achieved, however, adequate field studies are necessary to measure the real effects of stress on the behaviour of the animal. This has to be done so that the artificial influence of the laboratory situation on behaviour can be minimised by duplication of site specific features from the field situation. Nonetheless the laboratory situation will still influence the behaviour of the animal in immeasurable ways, for example, when it can not escape from a pollutant due to confinement in an aquarium, exposure will follow along with its accompanying effects (Olla *et al*., 1980).

Laboratory studies have, however, produced useful information by providing behavioural baselines. Significant departures from these baselines have been used to measure pollutant effects. According to Olla *et al*. (1980), "The applicability of a behavioural measure depends on the understanding of the animal's intrinsic behavioural capabilities and ecological requirements as well as the way in which the stressor acts on the animal's environment".

An interesting feature of the behaviour/concentration curves was that it did not matter when the observation of the animals was made after their exposure to the WSF. This was due to the fact that the concentration intercept on the log-probit curve corresponding to the 50 percent cumulative loss of equilibrium appeared to remain constant. What did change, however, was the slope of the line. The longer the period of time that had elapsed between the start of the exposure and the observation of behaviour, the shallower the slope of line appeared to be (Fig. 7). This approach for estimating the relative toxicity of crude oil is not only fast (a few hours), but simple to administer and interpret (Lipschitz, 1982). Furthermore, once a result is obtained the assay can be stopped and the animals returned to a clean water environment where most would recover from the toxic effects of the WSF.

In this study the concentrations of the petroleum hydrocarbons that caused significant behavioural changes were not significantly different from those which were acutely toxic or those that caused physiological impairment. Thus it would seem that the behavioural responses measured, although faster, were not significantly more sensitive than other biological responses as indices of sublethal hydrocarbon stress in *Jasus*.

A disturbing factor outside the scope of this investigation that requires further study is that of conflicting behaviour, namely the attraction and repulsion responses displayed by *Homarus americanus* towards some petroleum hydrocarbons in Atema's (1977) studies. Thompson *et al*. (1977) reported that scarlet prawns *Pleiopenaeus edwardsianus* were also attracted in large numbers to a benthic oil deposit. This maladaptive chemotactic response, although not observed in _Jasus_ in the limited laboratory tests that were performed, may nonetheless be a factor when a spill occurs in the field. If this is indeed the case, and _Jasus_ is attracted to the oil, the biological impact of oil on this resource could be far worse than anticipated.

4. THE EFFECT OF THE WSF ON RHYTHMIC BEHAVIOUR

4.1 AIM

The experiments described below were performed in an attempt to assess water-soluble fraction (WSF) induced stress by recording heart rate frequency changes from small groups of freely moving animals.

4.2 INTRODUCTION

Generally rhythmic behaviour can be evoked reliably without complex sensory stimuli. In many cases parameters such as frequency, amplitude and duration can be manipulated by imposing simple environmental changes. The repetitive nature of the activity generates large samples for categorising movements and the events that underlie them (MacMillan, 1977). Impedance techniques (Hoggarth & Truman, 1967) have been used to minotor repetitive activities such as heart and scaphognathite movements in various species of crustacea (Ansell, 1973; Uglow, 1973).

Although it was possible to monitor heart and scaphognathite activity from minimally restrained Jasus juveniles, the present writer decided against the use of this parameter to monitor stress. Firstly, being paired organs, both left and right scaphognathites had to be monitored. Such procedures, in particular the connection of the animal to the recording system, tended to impede the animal's behaviour in all but large animals (Dyer & Uglow, 1977). Although, in natantian crustaceans both scaphognathites have been shown to have similar synchronous responses (Dyer & Uglow, 1977), this was not always the case with brachyurans (Ansell, 1973; Arudpagasam & Naylor, 1964; Uglow, 1973). Secondly, even though scaphoghathites rate appeared to be more sensitive to changes in the animal's environment than did heart rate, it was far more difficult to quantify. Thirdly, McMahon et al. (1973) showed that water flow through the branchial chamber could be increased, by simply decreasing the resistance to water flow, without a change in beating rate. Studies on the effects of low oxygen water, performed by the present writer on Jasus, confirmed the above findings (Bailey & Beyers, in press).

Crustacean hearts are usually of the regulated neurogenic type, that is, they respond to a neural pacemaker but are also susceptible to modifications of heart beat from nerves from the ventral nerve cord (Bullock & Horridge, 1965; Warner, 1977). Heart rate has frequently been used as a sensitive test for environmental stress. Heart rate is normally dependent on body size (Maynard, 1960) but is also affected by external factors such as temperature, oxygen tension, carbon dioxide tension and activity (Warner, 1977).

4.3 METHODS AND MATERIALS

Heart rate was recorded from juvenile Jasus using a modified impedance pneumograph coupler (Z108) connected to the Washington Bioscience MD2 Oscillograph (Fig. 9). The impedance pneumograph produces a small oscillating current between the recording electrodes. Any impedance change between these electrodes produces a proportional voltage change which is then amplified before being fed into the galvanometer (Hoggarth & Truman, 1967).

Two holes (0,25 mm diameter) were drilled obliquely opposite each other on either side of the cardiac hump. The electrodes were made from 0,25 mm diameter insulated silver coated copper wire which had the ends exposed for 1,5 mm and crimped so that no copper was visible (Fig. 8). Techniques had to be developed to fix the electrodes into the holes in the cephalothorax. Dyer and Uglow (1977) also found that the exoskeleton of many crustaceans appeared to resist the adhesion of a variety of adhesives. The problem was further accentuated because periods of aerial exposure had to be kept to a minimum, so little time was available for the setting of the material used to anchor the electrodes.

The exoskeleton was dried in the region of the cardiac hump after the holes had been drilled with a watchmakers' drill. By adapting a method used by Savage (personal communication), it was found that a good bond could be obtained, with quick setting characteristics, by using a drop of a cyanoacrylate (Bostik® 7431) cement to which

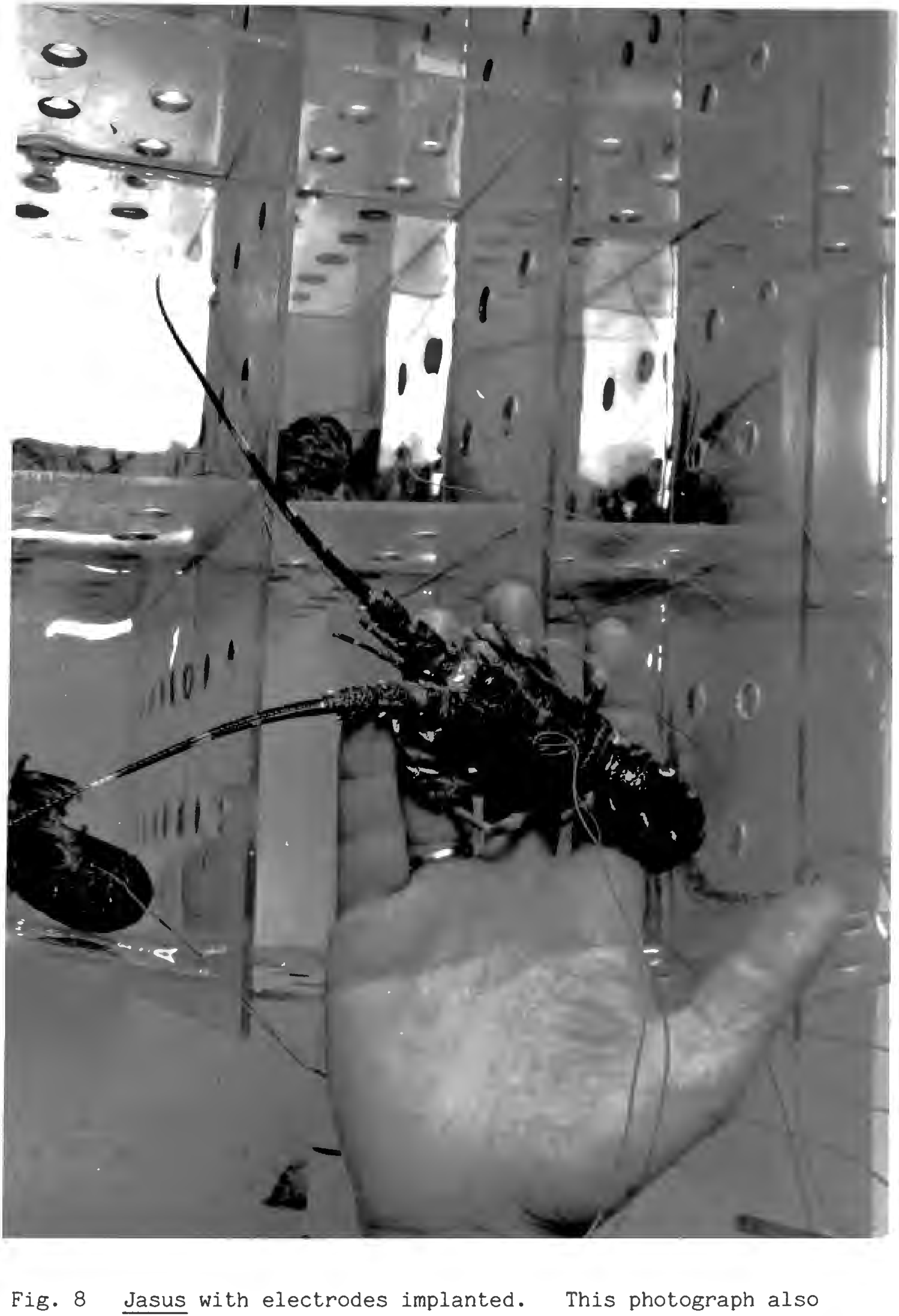


Fig. 8 *Jasus* with electrodes implanted. This photograph also shows the compartments used primarily to stop entanglement of the electrodes.

a drop of the fluid component of denture repair cement, a methylmethacrylate monomer (Kerr® Fastcure), was immediately added. This method has a remarkably fast drying time of a few seconds. As the setting or curing reaction is strongly exothermic, small drops of the two components must be added sequentially while making up the anchor plate, so as not to burn the animal. The electrodes were implanted very superficially so as not to damage the tissue beneath the exoskeleton. Any animals damaged during the electrode implantation were not used. The animals were placed in test solutions within an hour of implantation because it was found that when they were given time to acclimate they could detach the superficially implanted electrodes.

The major problem encountered by Dyer and Uglow (1977) during the heart rate recordings was the off-scale deflections of the recording pens during periods of sudden or violent activity, of the animal, which resulted in the loss of some information. The present writer found that it was possible to reduce this off-set problem considerably, by capacitance balancing the inverting and non-inverting electrodes with 3,3 µF capacators. This had the effect of returning the pen quickly to the baseline (balanced) position after such movements had occurred.

The rate of obtaining data from such a system is limited to the number of electrophysiological channels (pre-amplifiers) available. As many experimental parameters do not require continuous monitoring from all subjects, the investigator does not need to increase the

the number of pre-amplifiers, but can use a switching circuit, connecting different sets of experimental subjects to a single set of electrophysiological pre-amplifiers sequentially (Milligan, 1977). This was achieved by constructing a switch box capable of handling input from up to 32 subjects by means of relay switches (Fig. 9).

All animals were connected to a terminal box from where signals could be relayed to a single input pre-amplifier. Recordings were then made sequentially, taking into account the actual length of exposure to the WSF in each case so that in effect animals were actually being monitored simultaneously. This was achieved by sequentially staggering the starting times, of the animals' exposure to the WSF.

In experiments involving several animals partitions were constructed (Fig. 8) to prevent the electrodes from entangling. This restricted movement to a smaller area and allowed trace to be kept of individual animals. Although it could be argued that the restriction of movement imposed by the size of the compartment, may have further stressed the animal (Sprague, 1971), *Jasus* actually settled down sooner than when allowed to move freely about the test container with the partitions removed. Similar observations were made by Paterson (1969) who noted that these animals were highly territorial and actively sought out crevices in the aquarium. Furthermore, by decreasing the space in which the animal could move, so too was the level of activity that the animal could attain decreased. This last fact was important when one considered the relationship between level activity and heart rate. Inter-connecting holes 15 mm in diameter were

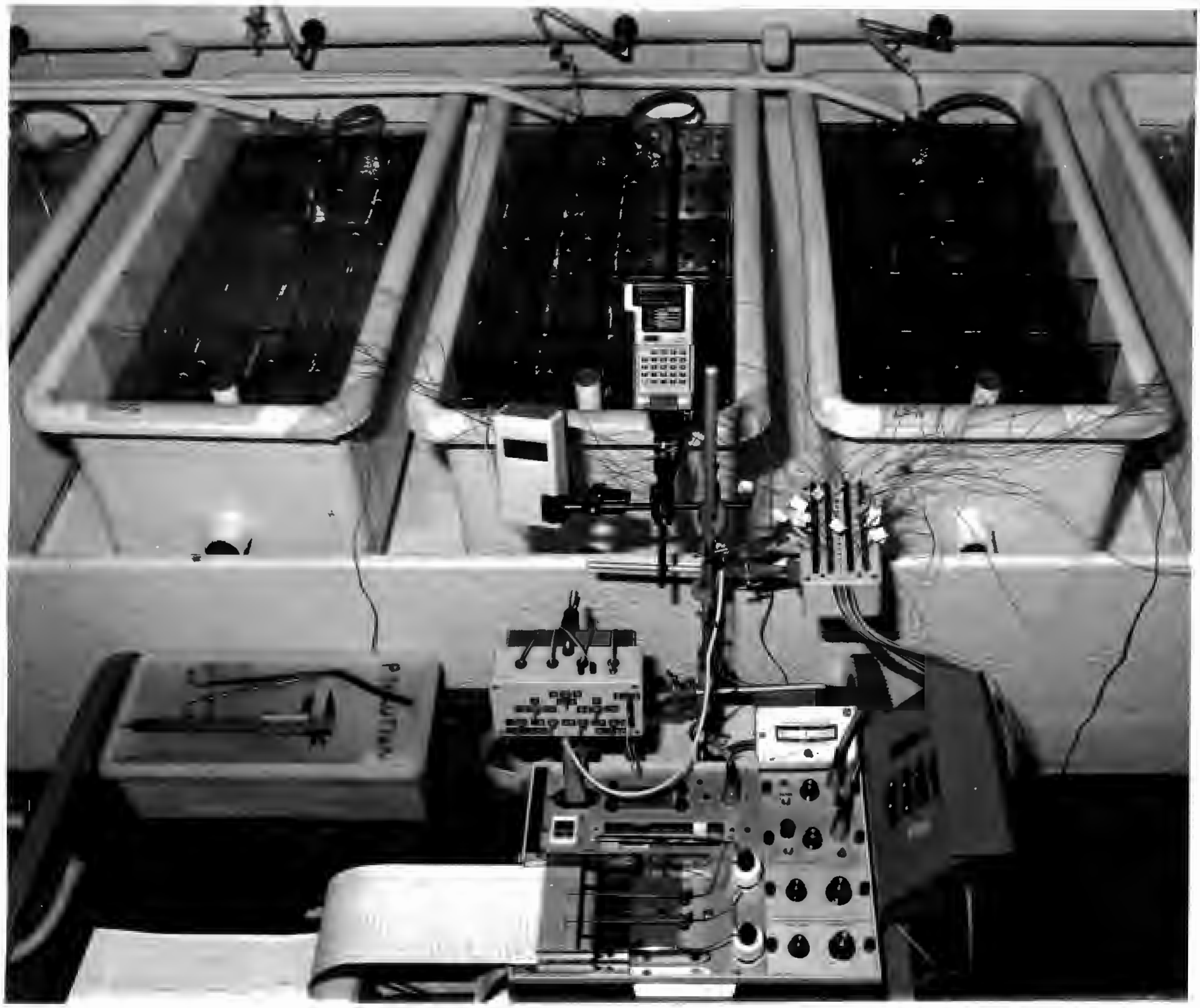


Fig. 9 Impedance pneumograph in operation. Note the electromechanical switchbox on the right and terminal box allowing sequential recording from 32 animals per pre-amplifier.

drilled at regular intervals in the walls of the compartments so as not to impede the flow of the water-soluble fraction.

The only cardiogram parameter that was measured during the exposure of the animals to the WSF was frequency. This was done for the following reasons. Firstly, frequency provided an easily accessible parameter for the assessment of stress in the cardiovascular system. Secondly, amplitude changes in the cardiogram, although they could have been due to an increase in the beat amplitude, could have reflected a change in the position of the heart, relative to the recording electrodes. Therefore amplitude changes could only have been used if recordings were made simultaneously from at least two sets of electrodes near the heart. Thirdly, wave form tended to be a function of the geometry of placement of the electrodes. Maynard (1960) stated that the decapod electrocardiogram varied from a simple monophasic wave to a complex pattern of oscillations. Furthermore its exact form depended in part on electrode placement, but even more on the condition of the heart. Savage (personal communication) stated that a change in the shape of the cardiogram could have been due to either a weakening of the heart or a movement of the electrodes relative to each other or, as stated previously, due to a movement of the heart itself.

4.3.1 Procedure

Sex and size matched animals were prepared an hour before exposure to the WSF by implanting two electrodes connected to leads that were

1 m long. Each pair of leads was connected to the impedance pneumograph coupler via the terminal box and the switch box. During the pre-test phase impedance pheumograph recordings were taken of the animals in clean water to ascertain whether the animals had been damaged during the preparation.

Animals were then transferred to the test tanks which contained WSF at different concentrations and monitored at 0,25, 0,5, 1, 2 and 4 hour intervals after the beginning of their exposure, for a period of 30 seconds per interval. No control group was used as preliminary experiments showed no pattern in their non-stressed heart rate frequency. The concept of this test was based on the physiological work that the heart had to do to restore the normal metabolic state which had been disturbed. Starting off the animal in a stressed condition served to limit the variability of cardiac behaviour. Brett (1964) used a similar concept to investigate the relationship between temperature, oxygen consumption and swimming performance by getting a fish to do work by swimming against a current. In the case of cardiac response experiments the heart rate was accelerated to an elevated level of activity by acute exposure to the WSF. If the animal was under no or minimal stress the heart rate would have been largely dependent on the level of the animal's activity.

A mean heart rate was then calculated for all the members of a group at each time interval and plotted against time for the four hour period. Using regression analysis a line was then fitted to these points. The slope of this line, which in all cases was negative, was taken to be the change in mean group heart rate per concentration. In an attempt to evaluate the effect of temperature on heart rate of exposed lobsters, the absolute value for the slope was plotted against concentration regime value for each group. A line drawn by regression analysis through these points joined animal groups tested at the same temperature. This technique enabled the synergistic effect of temperature and WSF to be evaluated.

4.4 RESULTS

Cardiac response to the WSF was the same in all cases monitored. Heart rate showed a rapid initial increase in heart rate frequency; this was followed by a progressive decrease in heart rate frequency (Figs 10A, B, C), which in turn gave way to a pattern of irregular beats interspersed with refractory periods of variable length (Figs 10D, E, F) and subsequent death of the animal (Lipschitz, 1982). The animals did, however, recover if transferred to clean seawater before the regular pattern collapsed. The collapse of the regular pattern was interpreted as a toxic effect. This was because in experiments carried out by the present writer (Bailey & Beyers, in press), it was shown that heart rate could be decreased to frequencies far lower than those encountered in the WSF experiments, by artifically decreasing the oxygen concentration in the water. Beat frequencies returned to normal without the animal apparently suffering any ill effects when the water was reoxygenated. In these exposures to WSF, the rate of decrease of heart rate frequency and subsequent disruption of the regular pattern was found to be related to the concentration

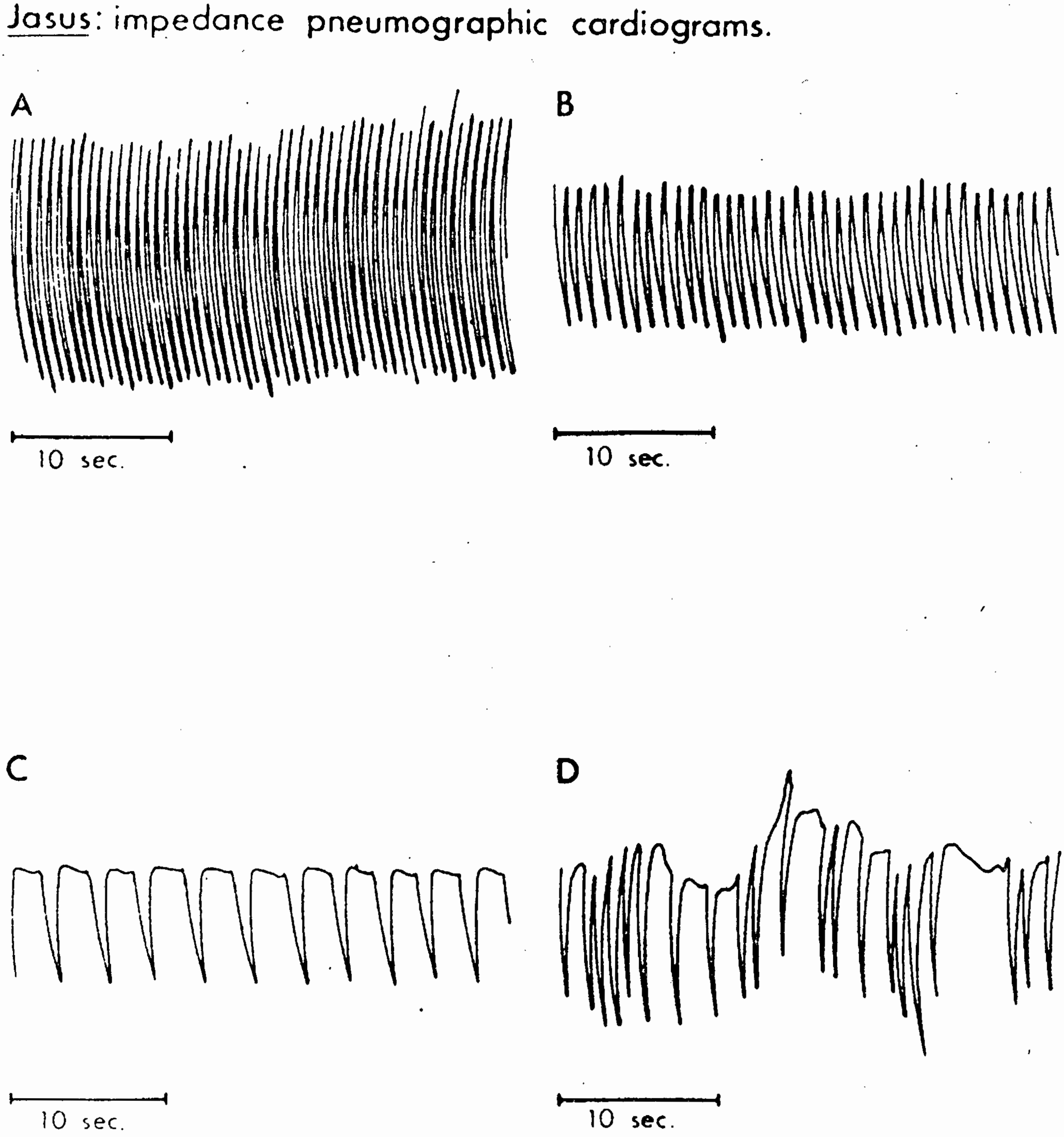


Fig. 10 *Jasus* impedance pneumographic cardiograms. Note the decrease in heart rate frequency (A-C) and subsequent collapse of rhythmic pattern (D).

Jasus: impedance pneumographic cardiograms.

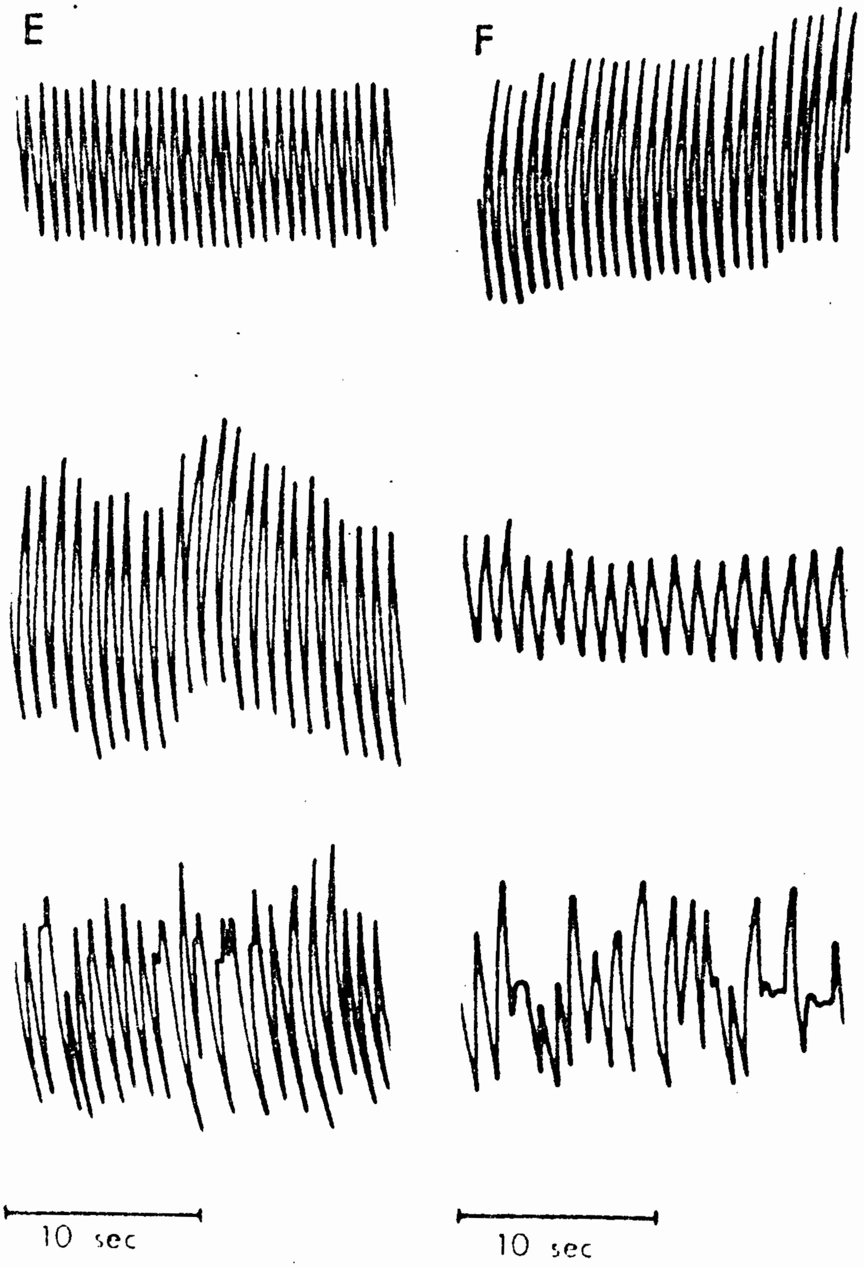


Fig. 10 E and F show the progressive decrease of heart rate frequencies in a further two Jasus juveniles followed by the collapse of the regular beat pattern.

of the WSF. The higher the concentration of WSF the sooner the heart beat frequency decreased.

TABLE 11A <u>Jasus</u>: Heart rate/time

t(hr)	0	0,25	0,5	1,0	2,0	4,0	
M	99	98	98	96	98	70	
M	117	106	106	100	100	θ	
F	73	110	106	106	98	θ	15,3 mg ℓ$^{-1}$
F	93	102	98	96	76	80	
Ave	95,5	104	99,5	99,5	93	77	
M	116	116	116	112	102	100	
M	110	110	106	106	106	98	
F	102	102	102	98	94	90	7,8 mg ℓ$^{-1}$
F	104	100	100	94	90	92	
Ave	108	107	106	102,5	98	95	
M	88	102	98	104	94	94	
M	68	104	106	104	102	98	
F	122	122	114	108	114	100	5,7 mg ℓ$^{-1}$
F	110	112	110	110	110	102	
Ave	97	110	107	106,5	105	98,5	

Mean cephalothorax length : 45,1 mm (σ_{n-1} = 2,42)
Number of animals per concentration : 4 (2 males, 2 females)
Temperature : 13°C
Heart rate : beats min^{-1}
M = male
F = female
θ = lost information (electronic noise)

TABLE 11B *Jasus*: Time vs rate (linear regression). 0 hr readings not taken into account

Concentration mg ℓ^{-1}	m (slope) average heart rate n animals	y (intercept)	r(correlation coef.)
15,3	-6,87	105,3	0,99
7,8	-3,18	106,5	0,95
5,7	-2,72	109,6	0,97

TABLE 11C *Jasus*: Absolute slope vs concentration (linear regression)

m(slope/concentration)	y(intercept)	r(correlation coef.)
0,45	0,04	0,99

See Fig. 109

TABLE 12A *Jasus*: Heart rate/time

t(hr)	0	0,5	1,0	2,0	4,0	
M	90	78	84	81	42	13,9 mg ℓ^{-1}
M	105	90	90	90	69	
M	105	93	90	102	78	
M	90	96	84	81	69	
F	80	87	90	42	24	
F	90	87	78	81	81	
F	90	87	87	72	81	
F	105	96	96	78	51	
Ave.	94	89	87	78	62	
M	90	99	96	96	81	7,9 mg ℓ^{-1}
M	90	93	93	99	96	
M	90	84	81	87	87	
M	80	90	87	99	87	
F	100	96	96	84	102	
F	100	96	102	102	92	
F	105	93	84	87	84	
F	80	93	90	90	87	
Ave.	92	93	91	93	90	
M	100	87	78	93	81	3,4 mg ℓ^{-1}
M	90	81	84	81	99	
M	90	96	102	99	99	
M	105	81	81	81	78	
F	105	102	93	96	96	
F	90	99	78	84	87	
F	105	96	105	99	108	
F	105	99	96	90	96	
Ave.	99	93	90	90	92	

Mean cephalothorax length : 44,5 mm (σ_{n-1} =2,53)
Number of animals per concentration : 8 (4 males, 4 females)
Temperature : 11,4°C
Heart rate : beats min^{-1}

M = male
F = female

TABLE 12B Jasus: Time vs rate (linear regression). 0 hr readings not taken into account.

Concentration mg ℓ^{-1}	m(slope) average heart rate n animals	y(intercept)	r(correlation coef.)
13,9	-3,59	82,3	0,90
7,9	-2,42	96,1	0,97
3,4	-0,78	93,1	0,73

TABLE 12C Jasus: Absolute slope vs concentration (linear regression).

m(slope/concentration)	y(intercept)	r(correlation coef.)
0,26	0,05	0,98

See Fig. 10G.

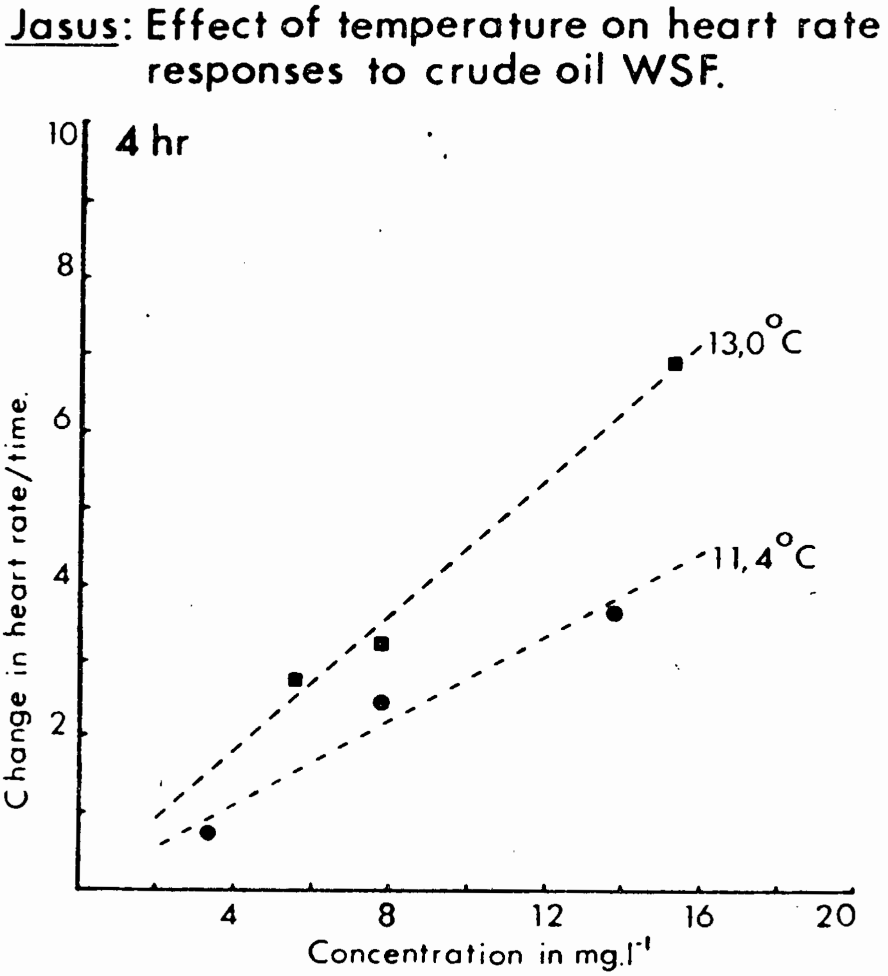


Fig. 10 *Jasus*: Effect of temperature on heart rate responses to WSF. These graphs were obtained by plotting the absolute value for change in heart rate with time (calculated by regression analysis; tables 11B and 12B) against concentration regime values. As the animals were sex and size matched, their responses to the WSF would be comparable. An increase in sensitivity to an additional stressor, e.g. temperature, would be indicated by a steeper slope.

4.5 DISCUSSION

In all cases studied, the common response to crude oil has been bradycardia or slowing of the heart (Anderson et al., 1977; Wang & Nicol, 1977; Meklenberg et al. unpublished). Anderson et al. (1977) looked at the responses of embryonic estuarine fish and found an immediate suppression of heart rate after exposure. They also noted that at lower concentrations no apparent relationships between heart rate and the WSF of crude oil could be established, whereas there appeared to be a relationship at near lethal levels. Caution must be exercised in interpreting laboratory studies associated with sublethal responses, as a simple relationship may not always exist between heart rate and metabolic rate (Maynard, 1960). Nonetheless the use of the cardiogram has been shown to be a useful technique for rapidly assessing the bioactive components in an array of chemical species (Bahr, 1973). It might well be argued though that this technique should be restricted to the identification of short-term effects that have no relevance in the long-term well-being of the animal (Bahr, 1973). Further work will have to be done before the relevance of such a technique can be evaluated in the long term.

Meklenberg et al. (unpublished) found that the heart rate of Alaskan King Crab exposed to the WSF of crude oil and its subfractions, declined during the first hours of exposure and then returned to near control levels when the WSF decreased. The severity of the heart rate depression correlated with the initial content of the aromatic hydrocarbons. Recovery was found to be dependent on the degradation of these toxicants (Meklenberg et al., unpublished).

Static tests carried out by the present writer at the Sea Point laboratory were in agreement with the above findings, however, instead of allowing the concentration of the WSF to drop, solutions were periodically replenished, which had the effect of a continued decrease in the heart rate. Here too the heart rate failed to recover until the concentration of the WSF was allowed to drop. It was decided therefore, in the interests of quantifying the cardiac responses, to present a constant level of pollutant by means of the flow-through system.

One of the major difficulties when dealing with small groups of animals is the establishment of a basal metabolic rate for that group. This was, as stated earlier, due in part to the expression of inter-animal variability and different levels of activity displayed by unstressed animals. It was therefore decided to dispense with a control group and also not to measure absolute change in response to a pollutant, but to use the rate of change instead. A control group could only have been used if the group had been exposed to a reference toxicant.

The initial acceleration of group heart rate on exposure, was probably directly correlated with the degree of stress evoked in response to a particular concentration of WSF. No attempt, however, was made to quantify this change. As the predominant response was bradycardia it seemed more important to understand the consequences of weak heart contractions and the slowing of the heart rate. It could be assumed that blood circulation was seriously reduced, and probably lacking in many tissues. These ischemic conditions would not only have resulted in an oxygen lack at the tissue level but would have also created a build-up of metabolic by-products such as potassium, lactic acid, carbon dioxide and others (Bahr, 1973). As most crustacean hearts are of the regulated neurogenic type, the above findings raise questions as to the mode of action of the WSF of crude oil, in particular as to where it acts. If the WSF acted directly on the heart then the ensuing effects of hypoxia and impairment of the functioning of the nervous system would follow. On the other hand, if the WSF acted on the nerves that modify heart beat then the bradycardia would be a consequence of a disturbed nervous system.

As stated earlier, most acute toxicity tests are unrealistic as they do not take other factors that may effect toxicity into account (Sprague, 1970). Consequently they have limited predictive value. The approach of plotting the rate of change of heart rate against concentration, appears to offer a convenient method for the evaluation of short-term synergistic effects. However, at this stage these results must be regarded as preliminary until more information has been collected.

5. SOME EFFECTS OF THE WSF OF CRUDE OIL ON EXCITABLE TISSUES

5.1 AIM

The main aim of this study was to investigate some of the electrophysiological changes that occurred in the membranes of excitable tissues when exposed to WSF.

5.2 INTRODUCTION

Acute median lethal toxicity studies indicated that juvenile Jasus was very sensitive to the water-soluble fraction (WSF) of crude oil This sensitivity was in part due to the prolonged exposure to monoaromatics at lower temperatures (12°C) and in part due to the finding that the most sensitive organisms to the WSF of crude oil appeared to be small crustaceans (Anderson, 1979). On closer inspection it appeared that Jasus mounted an inappropriate spontaneous autotomy reflex response to the WSF (Fig. 6B). The magnitude of this response appeared to be directly related to the concentration of the WSF. Karenin and Rice (1974) reported similar findings with newly moulted Tanner crabs. Autotomy or self-amputation of thoracic legs of decapod crustaceans has been well documented (Bliss, 1960). The fracture takes place at a breakage plane and the resulting stump is sealed off to prevent loss of haemolymph. Autotomy has also been reported in cases where crustaceans have been put into noxious preserving fluids (Wood & Wood, 1932). It was noted that not only did the animal have an inappropriate response, further prejudicing its own position, but that once the reflex had been triggered it did not always run to completion by sealing off the stump. This resulted in most cases in the animal bleeding to death.

The apparent interference with reflex activity, the successive neuromuscular impairment and bradycardia observed on exposure of *Jasus* to WSF all implicated modified functioning of excitable tissues, in particular the membranes associated with these tissues. The understanding of membrane function is central to all pollution studies as toxicants have to interact with membranes before they can interfere with metabolic processes.

5.3 METHODS AND MATERIALS

This study was carried out in the neurobiology laboratory of the Zoology Department of the University of the Witwatersrand.

5.3.1 Capture, Transport and Holding of Animals

Live juvenile *Jasus* (30 - 40 mm cephalothorax length) were collected by SCUBA divers from Table Bay docks, Cape Town. The animals were allowed to acclimate in a flow-through aquarium at Sea Point. Temperature was held at 12°C and the animals were fed during this period on black mussel.

The animals were cooled to 6°C over a period of 3 hours prior to packing in wet wood wool. These animals were then flown to Johannesburg in the cargo hold of a commercial airliner. At the University of the Witwatersrand the animals were introduced into artificial seawater (12°C), having been maintained out of water for approximately 5 hours. Care was taken to ensure that the animals were not exposed to thermal shock, by allowing their temperatures to rise to 12°C before introduction into the aquaria. In the period immediately after the flight there were a large number of mortalities. The remaining animals were then left for a further 10 days to adapt to their new surroundings before experiments began. During this time they were not fed.

5.3.2 The Dissecting Dish

In order to carry out experiments at a temperature of 12°C, a perspex dish (Fig. 11), 9 x 14 x 6 cm^3 was made with a drain and filling facility for pollutant presentation. Two cooling coils (ID 2 mm) of polythene tubing were moulded and anchored at opposite ends of the dish. These coils were then connected to each other and then to a reservoir of antifreeze inside a refrigerator at -15°C. Antifreeze was pumped through the circuit by means of a peristaltic pump (Fig. 12). The temperature in the dissecting dish, when filled with a saline

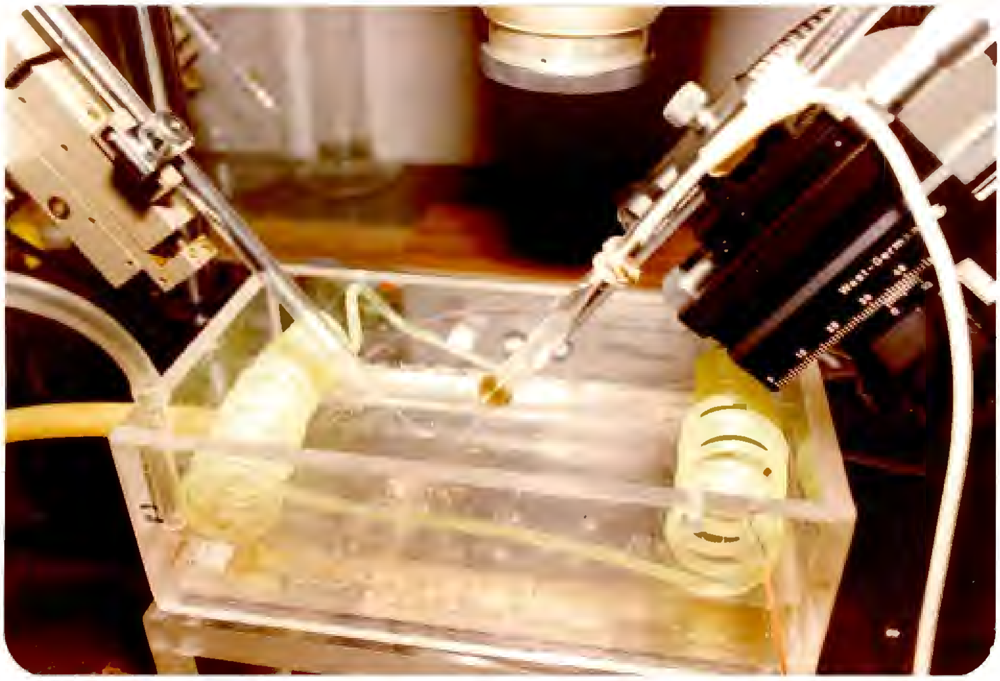


Fig. 11 Dissecting dish base filled with Sylgard® 184 encapsulating resin, cooling coils, filling and drainage tubes. Also visible are two micro manipulators; the RHS one with a 3M KCl filled micropipette

solution, was controlled by regulating the flow rate of the antifreeze by means of an adjustable clamp. The base of the dish was filled with Sylgard ® 184 encapsulating resin which was transparent and permitted the transmission of light through it, as well as allowing preparations to be pinned out on it.

5.3.3 Electrophysiological Recording and Stimulating Apparatus

Narishige horizontal electrode puller (Fig. 13).

High impedance probe (D C pre-amplifier).

Cathode follower (high input impedance amplifier) with built-in back-off, capacitance and current passing facilities through a bridge circuit (Fig. 14A). This system made it possible to stimulate and record through the same electrode.

Digitimer LTD. Isolated stimulator model DS2 (Fig. 14B).

Tektronix 5440 oscilloscope with 5A 48 dual trace amplifier and 5B40 time base (Fig. 14C).

Telequipment DM 64 two channel storage oscilloscope (Fig. 14D).

Nihon Koden 35 mm PC 2A continuous recording camera (Fig. 15).

Audio Monitor (Fig. 14E).

Faraday cage.

5.3.4 Preparation and Presentation of WSF

Crude oil was mixed with the appropriate crustacean ringer solution in a 1 to 9 ratio and then stirred for 20 hours on a magnetic stirrer. The water beneath the oil was siphoned off after being allowed to stand

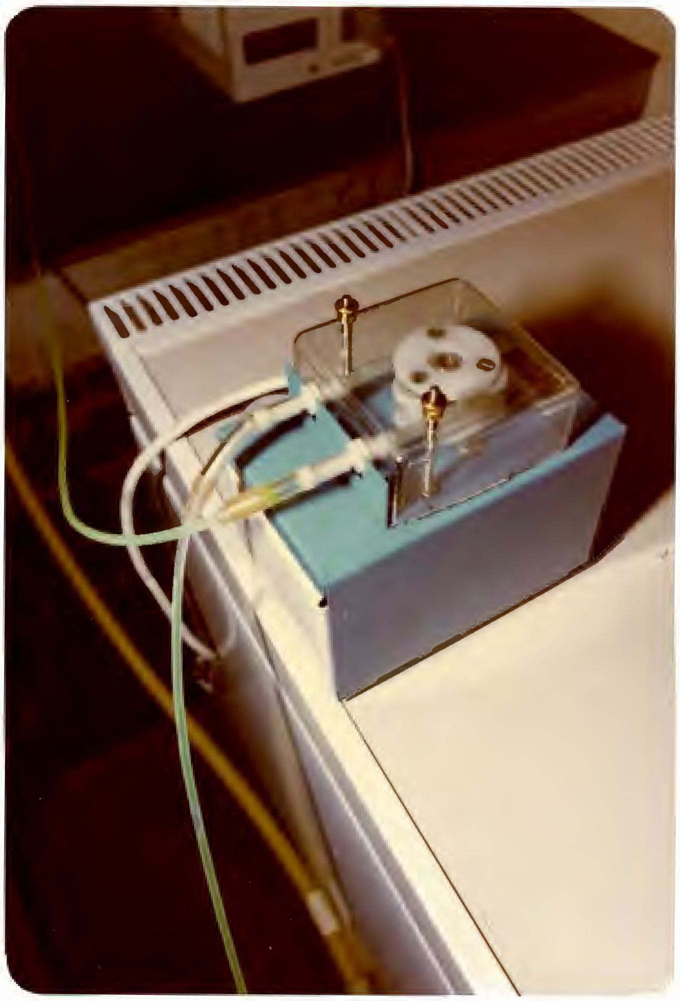


Fig. 12 Peristaltic pump for pumping antifreeze through the cooling system. The flow rate was controlled by means of a clamp.

for 4 hours, then filtered through glass wool to remove suspended oil droplets. A sample containing WSF was then extracted into CCl_4 and analysed on a Pye Unicam SP 300 spectrophotometer for total hydrocarbons (Warner, 1978). Dilutions were achieved by adding measured volumes of stock solution of WSF to known volumes of uncontaminated ringer solution in the dissecting dish, ensuring that both solutions were at the same temperature, namely 12°C.

5.3.5 Muscle Membrane Resting Potentials

The fifth pereiopod (walking leg) of Jasus was forced to autotomise by crushing the meropodite of that limb. The exoskeleton was then removed from the ventral surface of the propopodite until the underlying membrane was visible. This membrane was then dissected away exposing the adductor dactylopoditus muscle which moves the dactylopodite down and inwards. The preparation was then pinned out in the Sylgard® dissecting dish, with the ventral surface uppermost and the dactylopodite fully extended and immobilised. The preparation was covered with crayfish ringer solution (see Appendix a).

A 3M KCl filled micropipette with a tip resistance of between 10 and 20 $M\Omega$ was used to penetrate a muscle fibre in such a way that minimal damage was done to the muscle membrane. Penetration was achieved at right angles to the longitudinal axis of the fibre with the aid of a micromanipulator. Flexible electrodes, with long tapers were pulled on the horizontal electrode puller. These were found to be the most suitable for this purpose.

Fig. 13 Narashige horizontal electrode puller.

A chlorided silver wire was used to connect the glass micropipette to the positive terminal of a high impedance probe. The negative terminal was placed in the saline in the dissecting dish which was grounded. The probe was connected to the cathode follower and from there to the storage oscilloscope and audiometer. The back-off facility was used to eliminate any DC standing potentials prior to penetration.

The preparation and probe were placed inside the Faraday cage (a screened wire box) to improve the signal to noise ratio by screening them from outside sources of electrical noise such as 'hum'. Penetration was performed as cleanly as possible. A good penetration was represented on the oscilloscope screen by a sudden change in potential from ground to a level between minus 60 and minus 90 mV (Fig. 16).

Resting muscle fibre membrane potential was monitored for 10 minutes to ensure that it was stable before a known amount of WSF was added. After the addition, the resting potential was monitored at minute intervals for a period of 5 minutes. All readings were referred to ground potential. Changes in ground level that occurred during the experiment were compensated for at the end of the experiment by comparing the position of the ground before and after withdrawal of the electrode. It was decided to limit the observation period to 5 minutes after the addition of WSF to minimise the cumulative effects of changing ground potentials, evaporation of the WSF (static exposure), and possible deterioration of the preparation. In practice, however, the

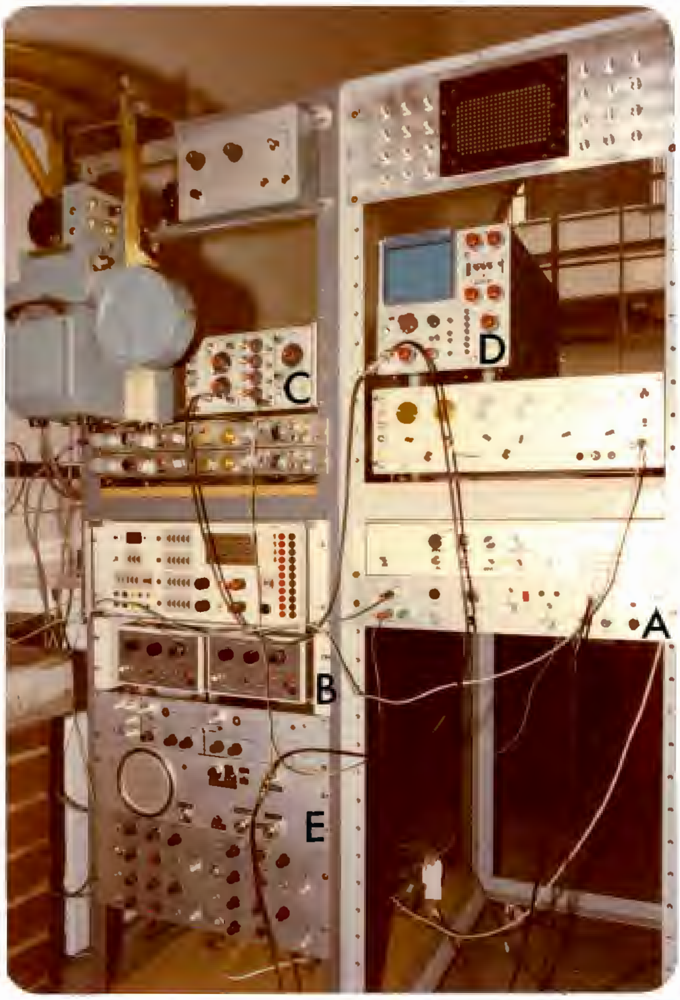


Fig. 14 Electrophysiological recording and stimulating system.

A : Cathode follower
B : Digitimer Ltd. Model DS2 stimulator
C : Tektronix 5440 oscilloscope
D : Telequipment Storage oscilloscope
E : Audiometer.

experiment was monitored for as long as 1 hour when possible, to see if recovery would take place as the concentration of the WSF decreased.

5.3.6 Motor Neuron Action Potentials

It was decided to record motor neuron action potentials from the local river crab Potamon sidneyi (Rathbun) for a number of reasons. Firstly, in view of the limited time available for this study it was not possible to become completely familiar with the fine ultrastructural neuro-anatomy of Jasus. Secondly, this relevant information was available for Potamon (Cannone, 1974; Lipschitz, 1977). Thirdly, the tough connective tissue sheath around the nerve bundles made penetration difficult in the case of Jasus, and fourthly Potamon muscle membranes behaved in the same way as those of Jasus in response to the WSF of crude oil. At the outset it must be stated that the following experiment, although it holds much promise as a technique for investigating sublethal effects, the results, at this stage, must be regarded as preliminary.

The preparation consisted of an isolated section of ventral thorax of the crab with the base of the posterior right pereiopod dissected in such a way as to leave the promotor muscle in situ with its motor supply intact. Pereiopods were made to autotomise by crushing the M-C joint. The abdomen, carapace, mouthparts, viscera and gills were then removed and the thorax washed out with cold Potamon ringer solution (see Appendix b, Schiff & Ewer, 1952).

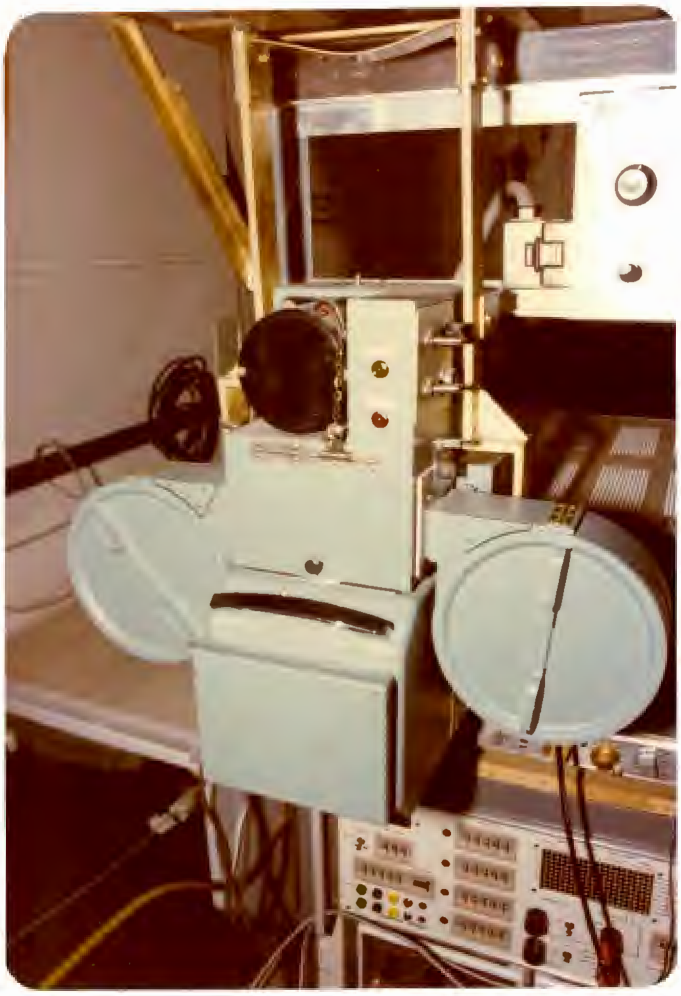


Fig. 15 Nihon Koden 35 mm (PC-2A) continuous recording camera.

Care was taken to avoid damaging the ventral thoracic ganglion and the nerves running from it that innervated the walking legs. The ventral thorax was then pinned out in the Sylgard ® dish and covered with ringer solution. The dissection was illuminated from below and the thoracic ganglion freed from the connective tissue holding it in place. All the nerves except those supplying the fifth walking leg, on the right side, were severed. The base of the fifth walking leg, together with its nerve and the thoracic ganglion, were isolated from the ventral thorax. The fifth pereiopod was then dissected from the dorsal surface. All the muscles and their innervations were progressively removed until only the promotor muscle and its innervation were left. Ringer solution in the dissecting dish was changed frequently during the dissection, as many substances were released into the bathing solution, which could have altered the extracellular ionic composition, and so have caused changes in the resting potential of excitable tissue membranes.

The dissection was performed as quickly as possible to counter the effects of the preparation deteriorating with time. Dissection was carried out using a Wild M5 microscope (Fig. 17). The early stages of the dissection were carried out at magnifications of 12 times, the final stages at 25, while in some cases 50 times was required. Electrodes with a short taper and resistances ranging from 10 to 20 MΩ were pulled for the purpose of penetrating the tough connective tissue sheath around the nerve bundle. Penetration was achieved with the aid of a micromanipulator positioned in the same axis as the nerve, while the nerve was supported with a micromanipulated glass

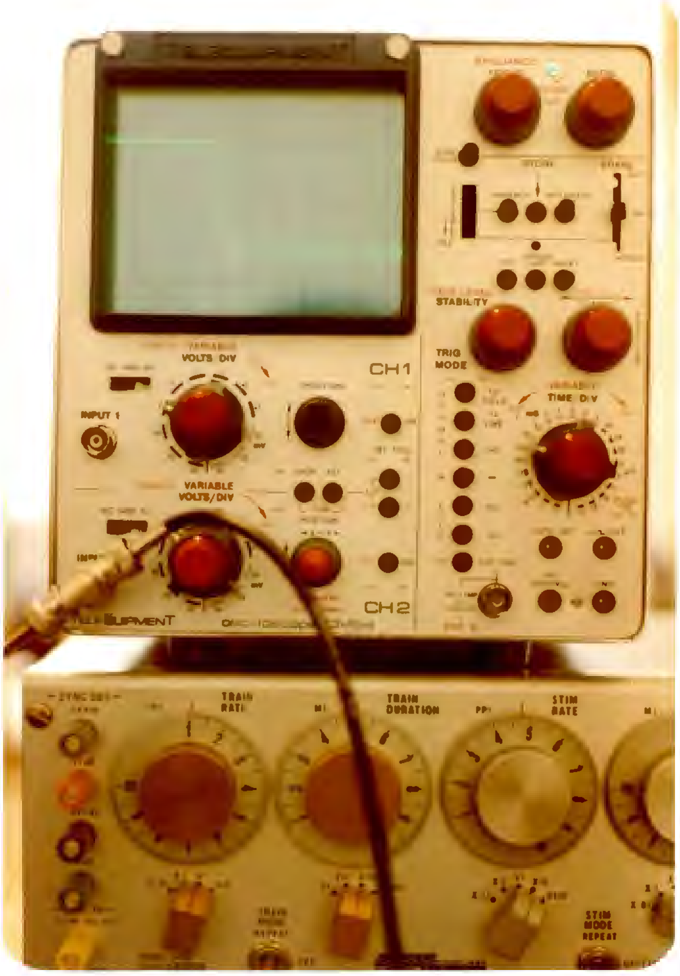


Fig. 16 DM 64 storage oscilloscope showing a good penetration of the muscle membrane. Resting membrane potential = ± 80 mV (20 mV/Division, Y axis).

hook close to the point of penetration.

A chlorided silver wire was used to connect the 3M KCl filled micropipette to the positive terminal of the high impedance probe. This probe was connected to the cathode follower with current passing facilities, through a bridge circuit. During the experiments the bridge was not necessarily balanced. The negative terminal of the probe was shorted to ground via the saline in the bath. This system was used to record action potentials that were evoked by passing an electrical current stimulus through the same electrode. The stimulus was generated at the Digitimer Limited isolated stimulator model DS2. For the purposes of this experiment, once a good penetration had been achieved the resting membrane potential was backed off to ground potential. The frequency of action potentials was now monitored instead of absolute membrane potentials. The cathode follower was in turn connected to the Tektronix 5440 oscilloscope, the DM 64 storage scope and the audiomonitor. The use of the audiometer enabled the penetration to be monitored aurally while guiding the elctrode onto the membrane under the microscope.

A permanent record of the responses to electrical stimulation was made by means of a Nihon Koden 35 mm PC 2A continuous recording camera attached to the Tektronix 5440 oscilloscope. Single frame photography was used in all cases and the camera was loaded with light sensitive paper film which was developed by conventional means.

After a stable penetration had been achieved, the resting potential

Fig. 17 Dissecting arrangement showing the Wild M5 dissecting Microscope, Dissecting dish, Micromanipulators, filling and drainage facilities. All apparatus was placed inside the Faraday cage to screen out 'hum'.

was backed off to ground. The motor neuron was then stimulated by injecting a rectangular current pulse of suitable duration and amplitude, to elicit a train of action potentials in which the interspike interval could be clearly seen (Fig. 18). In these experiments adaptation was not taken into account. The method of stimulating and recording through the same electrode gave rise to the response being superimposed on the stimulus.

The preparation was then stimulated at 30 second intervals for 5 minutes to ensure that the response to the stimulus was constant. A volume of known concentration of WSF, made up with Potamon saline was added to the known volume of clean saline in the dish.

The shutter of the camera was opened and the sweep triggered. Using a suitable time base and sweep speed the preparation was stimulated in such a way that the beginning and end of the stimulus could be captured on film. Readings were taken every 30 second for 20 minutes.

The film was then developed and the total number of action potentials per fixed stimulus were plotted against exposure time. The change in the level of excitability was then measured (Fig. 19).

Fig. 18 Train of action potentials recorded from Potamon promotor nerve superimposed on a rectangular current pulse stimulus. Recording and stimulation was through the same electrode.

Fig. 19 Train of action potentials recorded from the same Potamon promotor nerve as shown in Fig. 18, 30 seconds after exposure to the WSF of crude oil. The stimulus was of the same magnitude as that in Fig. 18. Note the immediate increase in the frequency of action potentials indicating a change in excitability of the neuron membrane.

5.4 RESULTS

5.4.1 Muscle Membrane Resting Potentials

TABLE 13 Jasus muscle membrane resting potentials

WSF mg ℓ^{-1}	Unexposed Initial Potential mV	Exposed Potential after 5 min mV	Absolute % change
3,0	-67	-71	5,97
3,0	-71	-74	4,23
4,0	-64	-71	10,94
4,0	-64	-72	12,50
4,0	-67	-72	7,46
7,0	-60	-70	16,67
7,0	-70	-78	11,43
7,0	-70	-80	14,29
7,0	-62	-69	11,29

Leg muscle preparation

Temperature 12°C

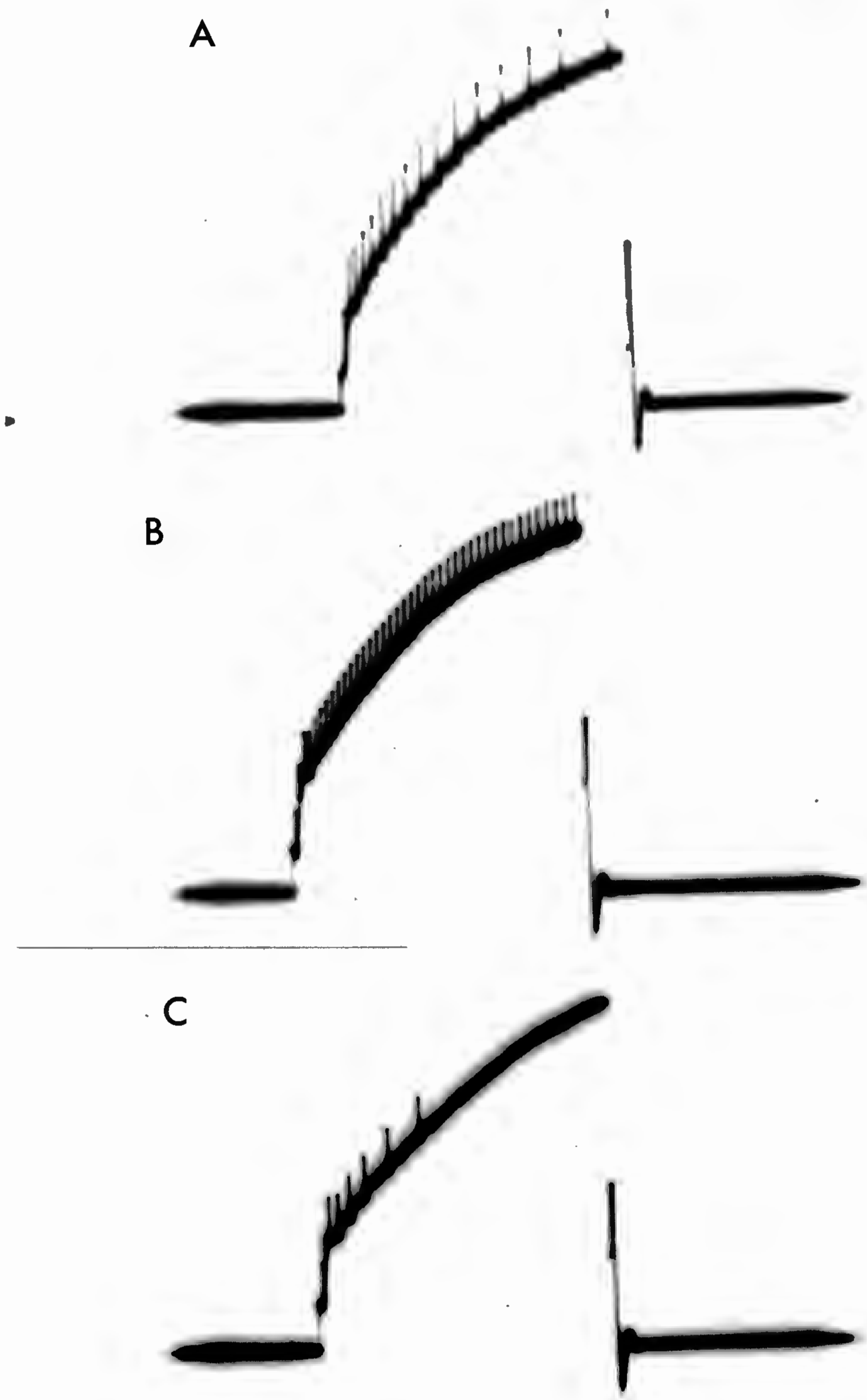


Fig. 20 *Potamon* motor neuron action potentials in response to a stimulus of 32nAx0,43s. The action potentials appear to climb as the bridge circuit moves out of balance. This was ignored as it was constant. A. Normal response prior to exposure. B. 2 minutes after exposure to WSF (5,6 mg ℓ$^{-1}$). C. 17,5 minutes after exposure.

5.4.2 Motor Neuron Action Potentials

TABLE 14 Potamon motor neuron action potentials

t(sec)	No. AP's	t(sec)	No. AP's
0,0	15 Fig. 20A	10,0	17
0,5	25	10,5	14
1,0	33	11,0	14
1,5	40	11,5	14
2,0	41 Fig. 20B	12,0	15
2,5	38	12,5	16
3,0	37	13,0	17
3,5	35	13,5	15
4,0	33	14,0	13
4,5	31	14,5	12
5,0	29	15,0	10
5,5	28	15,5	10
6,0	27	16,0	9
6,5	26	16,5	8
7,0	24	17,0	7
7,5	23	17,5	8 Fig. 20C.
8,0	22	18,0	8
8,5	21	18,5	8
9,0	20	19,0	8
9,5	19	19,5	8
		20,0	8

Promotor nerve preparation
WSF : 5,6 mg ℓ^{-1}

Stimulus: 32nA x 0,43s
Temperature 12°C

See Fig. 21 (A, B and C)

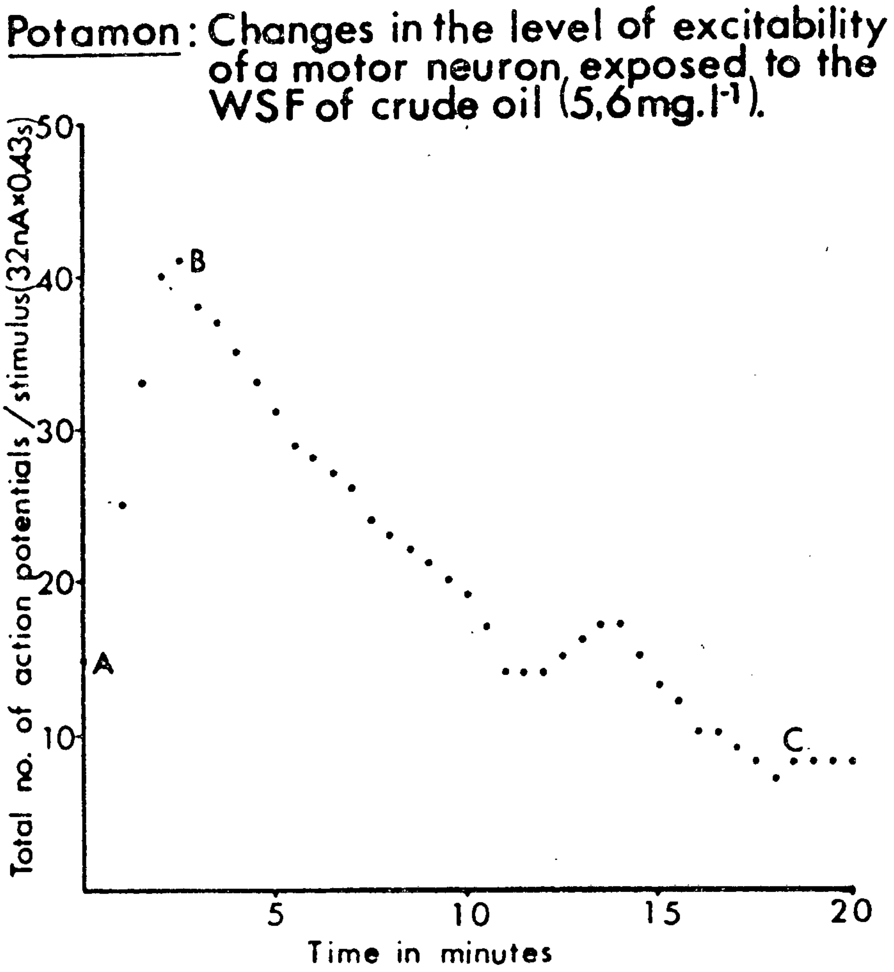


Fig. 21 Changes in the level of excitability of a motor neuron. A, Band C correspond to Fig. 20 A, Band C respectively. Each point represents the total number of action potentials per stimulus (32nAx0,43s) recorded at 30 second intervals after exposure.

5.5 DISCUSSION

The functioning of the nervous system is sensitive to the integrity of other physiological systems in the animal, therefore toxicants need not be specific neurotoxins for their impacts to be identified in a neurophysiological assay (Bahr, 1973). Furthermore, as lobsters have been traditional subjects of neurobiological research there is a wealth of information which can be adapted to the investigation of toxicant effects. Lobster preparations have provided a valuable system for the study of basic neurobiological phenomena because of their behavioural complexity coupled with the experimental accessibility of their nervous systems (Ache & MacMillan, 1980).

Preliminary neurophysiological experiments on the effects of WSF on the excitable tissues of Jasus and Potamon appear to have provided an explanation for the behavioural, cardiac and autotomy responses observed. Both neuron and muscle fibre showed a decrease in the level of excitability after exposure. In the case of the neuron there was an initial short-lived increase in the level of excitability. Muscle fibres showed an increase in the resting membrane potential (hyperpolarisation), i.e. a decreased level of excitability. The degree of hyperpolarisation was directly related to the concentration of the WSF; the higher the concentration, the greater the hyperpolarisation. About 45% of muscle fibres investigated did show a brief depolarisaiton which in all cases lasted less than sixty seconds. It was not possible to assess, however, whether this was an artifact caused by the physical disturbance of the WSF being added to the ringer solution bathing the preparation. Although the direct causes of the observed autotomy were outside the scope of this study it was possible to visualise a hypothetical series of events that could have triggered this inappropriate reflex. An initial increase in the level of excitability of the thoracic leg neurons could have been misinterpreted by the central nervous system (sternal ganglion) as injury potentials. This would have triggered the autotomy reflex (Bliss, 1960). If at the same time the autotomiser or remotor muscle fibres (Paterson, 1968) had already reached a stage of hyperpolarisation so that a proper contraction could no longer be effected, the stump would not have sealed off. The phenomenon of an increase followed by a more pronounced decrease in the level of tissue excitability (narcosis) was mirrored in the observed depression of heart beat rate and gross animal behaviour (Goldacre, 1968; Idoniboye-Obu, 1977; Swedmark et al., 1973; Meklenberg et al., unpublished). Idoniboye-Obu (1977) made similar observations, using remote electrodes. He recorded action potentials of marine decapods exposed to n-hexane. He found that activity increased to a point where the animals exhibited non-stop movements, generating distress signals together with a spectacular increase, and largely maintained action potentials whose sudden or gradual drop signified the collapse of the animal in a given medium. Atema (1977) also detected abnormal bursting patterns of action potentials in exposed Homarus americanus antennules. All the above findings appear to point to a common mode of action which reflect changes at a membrane level.

Not much literature has been published on the effects of petroleum hydrocarbons on cells. Sub-cellular changes have been monitored and shown to be a useful approach for determining the existence or severity of damage to the organism (Hawkes, 1977). Sabo and Stegeman (1977) and Moore and Dwyer (1974) both concluded that petroleum hydrocarbons produced subtle changes in both chronically exposed and briefly exposed fish, which strongly suggested an altered membrane structure, either intracellularly or at the cell surface. Although cell and membrane tissue damage could have resulted from pollutant contact alone, according to Anderson (1977), it seemed to occur as a result of the accumulation of these hydrocarbons because of their tissue versus seawater solubilities. Roubal (1974), using spin labelling techniques, showed a significant association of the hydrocarbons with the spinal cord, lateral line nerves and the brain of fingerling salmon. The association of hydrocarbons with fatty tissues has also been demonstrated in crustacea where the main site of hydrocarbon accumulation was found to be the hepatopancreas (Lee, 1977).

Roubal (1974) concluded that the aromatic hydrocarbons, to which the toxicity of crude oil is largely attributed (Rice et al., 1977a), altered membrane surfaces, while paraffins appeared to intercalate with membrane interiors and therefore exhibited longer residence times.

According to the fluid mosaic model of membrane structure (Singer & Nicolson, 1972), membrane phospholipids are aligned in a bilayer with their polar heads at the surface and their fatty tails extending into the fluid centre, with globular proteins imbedded in this phospholipid matrix. To preserve membrane integrity polar regions face the hydrophobic interior and interact with the fluid phase of the membrane. Because of the hydrophobic interactions between proteins and lipids, changes in the fluidity of the lipid phase, caused, for example, by drugs or petroleum hydrocarbons, may alter protein conformations and enzyme activities (Miller & Kinter, 1977). Changes as suggested above could have conceivably affected the proper functioning of excitable tissues by interfering with membrane permeability. This in turn would have brought about changes in the electrochemical gradients maintained by membranes that provided the energy for the propagation of action potentials (Aidley, 1971). Goldacre (1968) looked at the effects of crude oil subcomponents on the plasma membrane of Amoeba proteus and concluded that the lower members of the paraffin hydrocarbons belonged to the fat soluble anaesthetics and had a narcotic effect on a large number of animals. In man such anaesthetics, for example, benzene, toluene and phenol, have an action resembling to some extent that of alcohol, and produce, first restlessness, then excitement and inebriation, which is followed by drowsiness, depression and sleep. These conditions are reversible, but if the concentration is allowed to rise death from respiratory failure could follow (Goldacre, 1968).

The methods set out above for the investigation of the neurophysiological effects of the WSF represented a significant refinement in technique, in that they generated quantifiable data about sublethal responses. Previous authors (Atema, 1977; Idoniboye-Obu, 1977) only showed a qualitative, albeit dramatic, neurophysiological response. This was possibly due to the limitation imposed by a poor signal to noise ratio encountered at high gain; a property of the recording system, and the coarse techniques employed. Intracellular recording to a large degree minimised electrical noise in the system. By monitoring a single cell the direct neurophysiological effects of the toxicant could be measured, as the modifying influence of other physiological systems were absent. These factors contributed to the high sensitivity of this recording system. A major advantage of this approach was that the significance of changes observed at a cellular level could be extrapolated to the population level (Mount & Stephan, 1968). Atema (1977) showed that the association of petroleum hydrocarbons with Homarus antennular chemoreceptors affected chemotaxis. The significance of this fact could easily be extrapolated to the population level by considering, for example, the role of chemotaxis in interanimal communication or food detection.

Nonetheless the above system had certain disadvantages. The preparation was a lengthy and delicate procedure requiring a working knowledge of electrophysiology and a familiarity with the anatomy of the animal. Furthermore the dissection had to be performed as quickly as possible to prevent the preparation becoming hypoxic. There were, however, simpler techniques which would have yielded useful quantitative neurophysiological data, some of which have been mentioned below. It has also been argued that the results obtained from such acute exposures, approximated to peak concentrations encountered in the field and would only have had significance to the immediate survival of the animal. Another problem that arose was that at this level of sensitivity it was not possible to distinguish between normal adaptational changes and those caused by the toxic action of the WSF. This last problem could have been solved by statistically defining the 'no effect' level (Sprague, 1971). As an approach, however, the neurophysiological assay holds much promise as a sensitive way of investigating the effects of those pollutants that interfere with membrane integrity.

6. CONCLUSIONS AND RECOMMENDATIONS

1. Petroleum hydrocarbons are not easily introduced or maintained in the water column.

2. Acute median lethal toxicity studies (LC50) provide a useful starting point for physiological and behavioural assays. Even though they have limited predictive value they do enable comparisons to be made about animal sensitivity.

3. The 24 hour median lethal concentration for Jasus lalandii juveniles ranges from 3,7 - 5,9 mg ℓ^{-1}.

4. The high mortality encountered at low levels of exposure can be attributed to the combined effects of narcosis, the longer retention time of WSF at lower temperatures and the inappropriate autotomy response displayed by some animals.

5. Behavioural assays provide a quick and convenient approach for estimating the relative toxicity of the WSF.

6. Methods used for the analysis of log-probit curves can successfully be adapted to behavioural responses.

7. The major effect of WSF on heart rate is bradycardia; the higher

the exposure concentration the sooner the heart beat rate slows down.

8. Heart rate is not more sensitive than other techniques for evaluating toxic effects; a relationship between concentration and heart rate is only evident at lethal levels of exposure.

9. Preliminary investigations suggest that the rate of change of heart beat in response to different concentrations of the same crude WSF can be used to evaluate the contributary effects of other environmental stressors, e.g. temperature.

10. Intracellular neurophysiological techniques can provide sensitive and quantifiable assays for monitoring membrane mediated pollutant effects.

11. Exposure of *Jasus* muscle fibres results in a shift of the resting membrane potential in a hyperpolarising direction. The magnitude of the shift is dependent on the concentration of the WSF.

12. Preliminary results of the effects of WSF on decapod neurons indicate that there is an initial increase in excitability followed by a pronounced depression to levels below that of unexposed neurons.

13. It is relatively easy to extrapolate the significant changes at a neuron or muscle fibre level, to higher levels of organisation.

14. The 'no effect' level of a pollutant should be statistically defined, as it is easy to induce changes in physiology and behaviour, but not to say at what point those changes fall outside the animal's normal adaptational capabilities.

15. In view of the sensitivity of electrophysiological techniques, more of these should be adapted to toxicological studies in an attempt to elucidate the mode of action of pollutants.

16. An attempt should be made to develop a 'condition index' for pollution impacted animals that can be used in the field. This could possibly be done using changes in resting cell membrane potentials or nerve conduction velocities.

ACKNOWLEDGEMENTS

I wish to thank Professor A.C. Brown for his encouragement and many constructive ideas. My thanks to Dr A.J. Cannone for devoting much of his valuable time to this study and for making his facilities available to me. My sincere thanks to the Director of the Sea Fisheries Research Institute, Mr G.H. Stander and the Assistant Director, Dr L.V. Shannon, without whom this study would not have been possible. The equipment used was supplied by the Pollution Section of the Sea Fisheries Research Institute and the Zoology Department of the University of the Witwatersrand. The black and white photographs were taken and printed by Mr M.J. Swart.

APPENDIX

a) *JASUS* RINGER SOLUTION (Personal communication A.J. Cannone)

NaCl	500 mM
KCl	12 mM
$CaCl_2$	20 mM
$MgCl_2$	10 mM

pH adjusted to 7,4 with 0,1 M $NaHCO_3$

b) *POTAMON* RINGER SOLLUTION (Schiff & Ewer, 1952)

NaCl	320 mM
KCl	8 mM
$CaCl_2$	20 mM
$MgCl_2$	4 mM

pH adjusted to 7,5 with 0,1 M $NaHCO_3$

c) SOUTH AFRICAN STANDARD CRUDE OIL

Qatar light crude oil from 1966 cargo set aside to be utilised for scientific research. Density ,8233.

(See Fig. 22)

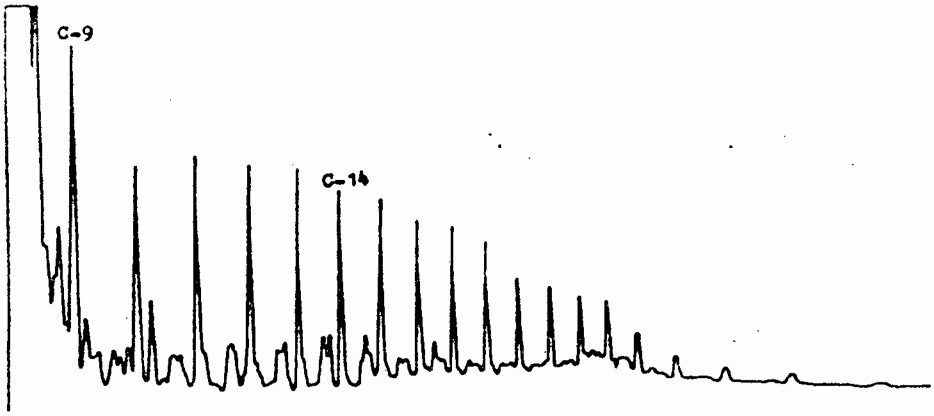


Fig. 22 Gas Chromatographic recording of Qatar light crude oil run through a 3% Dexil packed column on a Packard 7400 Chromatograph with flame ionization detector by T.F. Bidleman of the University of South Carolina, U.S.A.

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