THE UNIVERSITY OF HULL

AN INVESTIGATION OF HEAVY METAL TOLERANCE AND REPRODUCTION IN *NEREIS DIVERSICOLOR* WITH REFERENCE TO THEIR USE FOR BIOMONITORING

being a Thesis for the Degree of

Doctor of Philosophy

in the University of Hull

by

JONATHAN GLYN HATELEY, B.Sc

November, 1989

TABLE OF CONTENTS

All relevant Tables and Figures are at the end of each chapter.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Industrially-derived heavy metals are increasingly responsible for contamination of coastal and estuarine waters. All stages of metal production are sources of contamination, the main contributors being acidic mine drainage waters and smelting works. Other major sources are industrial water discharges, sewage sludge, the atmosphere, shipyard paints and electricity power stations (Bryan, 1984).

The most contaminated sites are the rivers and estuaries that directly receive the industrial outfalls. It is a common misconception that metal wastes are simply washed out to sea and dispersed. Estuaries are in fact efficient traps of heavy metals. The scrubbing processes of precipitation, chelation and adsorption onto particulate materials ensure that only small amounts of metals escape to the open sea (Turekian, 1977). The accumulation of heavy metals in estuaries raises the question; at what levels do metals have a detrimental effect on the biota?

Heavy metals have been classified according to the risks they pose to aquatic organisms. The classification is not just based on the intrinsic toxicity of the metals but also on their availability. Metals considered to represent a high risk to the biota as they are both very toxic and relatively accessible are; Be, Co, Ni, Cu, Zn, As, Se, Pd, Ag, Cd, Sn, Te, Pt, Au, Hg, Tl, Pb and Bi (Forstner and Wittmann, 1981).

Despite extensive research on heavy metal contamination of estuarine and marine waters, there is remarkably little direct evidence for ecological impact by metals (Bryan, 1984; Luoma, 1977). The problem lies in the inability of current pollution monitoring schemes to provide anything more than circumstantial evidence that high metal levels are adversely affecting natural populations (i.e. distinguishing between 'contamination' and 'pollution'; G.E.S.A.M.P., 1980).

Recently there have been two broad approaches to assessing the impact of contaminants at a site; biological effects monitoring, and

the study of population/species distributions. Both approaches have their drawbacks.

Biological effects monitoring aims to detect and measure a response of an organism to a contaminant through the study of physiological, biochemical and cytological indices of its condition. A range of variables have been considered suitable for a variety of organisms, including metallothionein levels, feeding rate, scope for growth, lysosomal stability and the presence of ulcers (G.E.S.A.M.P., 1980; Moore et al, 1986).

The main disadvantage of biological effects monitoring is that the naturally large variability of responses in living organisms obscures all but the most obvious effects (McIntyre, 1984). Also, it is almost impossible to directly attribute an effect to any one substance if the test organism is subjected to a mixture of contaminants (e.g. in mine drainage waters, where a variety of heavy metals are present) (Bryan, 1984). An additional problem peculiar to estuaries is the requirement that the test organism does not have a limited distribution because of salinity intolerance (Bryan, 1984; Phillips, 1977). There are comparatively few species available that penetrate estuaries sufficiently to enable pollution effect surveys to be made over the whole length of the estuary.

Other workers have attempted to develop monitoring schemes based on the distribution of populations and species. Deviations from 'expected' distributions are suggested as being indicative of ecological impacts. A variety of proposals have been voiced but all have either practical or theoretical weaknesses (I.C.E.S., 1978; Lewis, 1978).

It has been suggested that the presence or absence of key indicator species can demonstrate a polluted environment. For example, Capitella capitata is found in large numbers at sites where an environmental disturbance has reduced the resident population (Reish,

1979). Raffaelli and Mason (1981) considered that unexpectedly high nematode to copepod ratios in meiofaunal samples from polluted beaches could be used as a biomonitoring tool. Gray and Mirza (1979) and Warwick (1986) respectively proposed that deviations in the log-normal model of numbers of individuals among species and the relative distributions of biomass to numbers among species demonstrated a response to disturbance.

Quite apart from their own inherent weaknesses (see Discussion), all the population/species distribution monitoring proposals share the same failing with biological effects monitoring; they are unable to distinguish the relative contribution of each toxicant in a mixture of contaminants.

To provide direct evidence for ecological impact by metals a different approach is required. Luoma (1977), drawing parallels with pesticide resistance in insects and metal tolerance in angiosperms, advocated the study of tolerance to toxicants as a means of helping determine if trace contaminants are affecting organisms in a given situation. Essentially, if a population is tolerant of a toxicant, then the toxicant or a close chemical relative must be having a significant impact on that population. The tolerance approach has the added benefit of specificity in determining which contaminants are having the more significant effects.

The main body of this work is concerned with determining whether, "viewing toxicant resistance as an indicator of selective pressure in an ecosystem may provide a new tool for assessing the causes of simplification in aquatic communities." (Luoma, 1977). This was done through the practical investigation of one example of aquatic tolerance; namely the tolerance exhibited by populations of the estuarine polychaete Nereis diversicolor to copper and zinc (Bryan and Hummerstone, 1971, 1973). The toxicity of these metals to tolerant populations from a

heavily contaminated estuary (Restronguet Creek) were compared with populations from a moderately contaminated estuary (Humber) and a clean estuary (Kingsbridge Avon).

Resistance to toxicants has been demonstrated in a variety of organisms. The best documented studies have been on the tolerance of insects to a range of insecticides (O'Brien, 1967; Brown and Pal, 1971) and the tolerance of grasses growing on mine wastes to heavy metals (McNeilly, 1968; Rocovich and West, 1975). Luoma (1977) identified four similarities in these tolerances;

(i) Resistance results from selection for resistant genotypes; it cannot be induced over the lifetime of individuals.

(ii) Resistance usually involves physiological mechanisms specific for a single toxicant or group of toxicants, rather than selection for vigorous strains.

(iii) Resistance reduces the overall fitness of the population in clean environments.

(iv) The degree of tolerance is related to the level of exposure to the toxicant.

The first aim of this thesis was to determine how closely heavy metal tolerance in N. diversicolor conforms to these four characteristics. An assessment can then be made as to the effectiveness of toxicant resistance as a variable for determining ecological impact, as any significant deviation from these four characteristics makes its validity questionable. For the approach to be viable the resistance must have a heritable component, as an ecological impact must select against individuals within a population. If all individuals were capable of developing tolerance over their life-time (through induction) there would be no selection and hence no impact. The tolerance must have a physiological basis and not be due to selection for vigorous strains as these strains may be found in unpolluted environments. Ideally, the

physiological mechanism will be specific for each toxicant to allow determination of which toxicants are having the more significant effects. Tolerance must reduce the overall fitness of the population in uncontaminated environments otherwise tolerant populations could develop there by chance. Finally, the magnitude of tolerance should reflect the level of contamination to enable estimates to be made as to the degree of environmental contamination that requires tolerance.

Chapter 1 is concerned with determining if the level of tolerance reflects the degree of contamination (Luoma point (iv)) and if tolerance is due to selection for vigorous strains or through specific physiological mechanisms (Luoma point (ii)). The degree of tolerance to copper and zinc in N. diversicolor was determined at a number of sites down the heavily contaminated Restronguet Creek and related to the environmental levels of the metals. It was reasoned that if the distributions of copper and zinc tolerance were different then different physiological mechanisms must be involved.

Chapter 2 considers whether tolerance to copper and zinc results from selection for resistant genotypes or if it is due to adaptation of individuals over their life-times (Luoma point (i)). This was studied through the use of specific and non-specific crosses between tolerant and non-tolerant animals and induction experiments.

Chapter 3 attempts to determine if tolerance reduces the overall fitness of N. diversicolor populations (Luoma point (iii)). Competition experiments and studies of population differentiation were used to assess the relative contributions of restricted gene-flow and competitive interaction in determining the distribution of tolerance.

Population structure and gametogenic cycle have also been considered potentially useful study areas for biomonitoring purposes (G.E.S.A.M.P., 1980). The reproductive biology of N. diversicolor from

contaminated and uncontaminated estuaries were therefore compared to determine their potential use as more sensitive biological effects monitoring schemes. Heavy metals are known to impair reproduction in aquatic organisms even in very small amounts (Oshida et al, 1976; Kumar and Pant, 1984; Gibbs et al, 1987) and therefore perturbations in reproduction are potentially sensitive, although non-specific, indicators of pollution. Reproduction in N. diversicolor has been well studied (e.g. Olive and Garwood, 1981; Kristensen, 1984; Moller, 1985) but there have been few attempts to link it with heavy metal contamination (Ozoh, 1986). The aim of Chapter 4 and the second aim of this thesis, therefore, is to discuss the impact of heavy metals on reproduction in N. diversicolor and consider its use as a biomonitoring tool.

SITE DESCRIPTIONS AND GENERAL METHODS

SITE DESCRIPTIONS AND GENERAL METHODS

i) DESCRIPTION OF STUDY AREAS

Restronguet Creek is a branch of the Fal estuary in South West Cornwall (Figures 1 and 2). This area was in the heart of a metalliferous mining region that first became extensively worked in the early 18th century. In its heyday nearly 50% of the world supply of copper, arsenic and tin was produced in this area but output declined rapidly by the end of the 19th century. Currently, only one mine is still in operation, at Wheal Jane (for tin). Drainage of old mine adits and erosion of spoils have ensured that high concentrations of metals are still found in the freshwater streams that drain the area. Two rivers converge at the head of Restronguet Creek; the River Kennall contains negligible amounts of metal but the Carnon River is heavily contaminated with a variety of metals. The estimated daily inputs into Restronguet Creek from this source are:- cadmium, 2 $kg.day^{-1}$; copper, 6 $kg.day^{-1}$; lead, 4 $kg.day^{-1}$; zinc, 700 $kg.day^{-1}$ and arsenic, 15 $kg.day^{-1}$ (from Bryan and Gibbs, 1983).

The Avon estuary is situated near Kingsbridge, approximately 20 kilometers east of Plymouth. It receives drainage water from Dartmoor, but as there are no mines in the area the metal content is very low (Bryan and Gibbs, 1983).

The Humber has the largest catchment area of any estuary in the United Kingdom (Figures 1 and 3). It includes the cities of Birmingham, Bradford, Derby, Leeds, Leicester, Nottingham, Sheffield and Stoke-on-Trent and contains 60% of the country's coal production and 40% of crude steel production. The main freshwater inputs therefore contain high levels of industrially-derived metals. The tidal waters of the estuary also directly receive trade effluent high in metal content. On the north bank at East Clough there is a metal smelter and there are a

variety of chemical complexes nearer the estuary mouth, including two plants producing titanium dioxide pigments. The estimated inputs of metals into the Humber estuary from both freshwater sources and direct trade/sewage effluents are:- cadmium, 20 $kg.day^{-1}$; copper, 430 $kg.day^{-1}$; lead, 400 $kg.day^{-1}$; zinc, 2500 $kg.day^{-1}$ and arsenic 1100 $kg.day^{-1}$ (from Edwards, Freestone and Urquhart, 1987).

Metal input into the Humber is therefore a factor of 10-100 greater than that into Restronguet Creek. When making comparisons between the two, however, the enormous differences in geographical scale and degree of historical contamination must be considered as these affect the estuaries ability to absorb metal contamination. The Humber is 62 kilometers long and receives freshwater at a flow-rate of approximately 200 $m^3.s^{-1}$. In contast, Restronguet Creek is 3 kilometers long with a freshwater flow-rate of approximately 2 $m^3.s^{-1}$. Metal input into the Humber, particularly through direct discharge into the tidal waters, is comparatively recent (last 100 years), but significant mining and hence contamination has occurred since the 17th century in the vicinity of Restronguet Creek (Edwards, Freestone and Urquhart, 1987; Bryan and Gibbs, 1983).

ii) SAMPLING

a) Sites studied

The geographical location of the sites studied are shown in Figures 1,2 and 3 and are described in Table 1. The exact location of the Kingsbridge Avon is not shown in the Figures. The map reference of this site is MR 683 487 (O.S. Landranger map 202). The nomenclature for Restronguet Creek and Mylor Creek follows that of Bryan and Gibbs (1983) for ease of reference.

All sites were located around high water neaps other than Grimsby which was at approximately low water neaps. This station was

located further down the shore because the rocky nature of the upper shore restricted sampling and worms were less abundant.

b) Sampling methods

For studies on gametogenesis and population dynamics, worms were sampled by taking 5 cores (100 mm x 100 mm x 200 mm) at the following sites; Grimsby, Whitton, R1, R13 and Avon. Worms were also sampled at Hessle by digging under large rocks as the stony nature of the sediment precluded coring. The worms were sieved through 1000 μm and 500 μm mesh and allowed to recover in 17.5°/$_{oo}$ salinity water (sea-salt from Burton Engineering, Bridlington, Humberside) prior to preservation in a 1:1 mixture of 5% formalin and ethanol.

A small quantity of surface sediment was sampled and preserved in formalin/ethanol on site for screening of larval stages. One surface core (100 mm x 100 mm x 50 mm) was taken for quantification of juveniles and a similar core was taken for subsequent sediment metal level analyses. These cores were stored at -20 °C.

Extra worms were dug from all sites for use in toxicity experiments, breeding and electrophoretic studies. They were sieved through 1000 μm and 500 μm mesh and stored in aerated 17.5°/$_{oo}$ salinity water in glass aquaria at 12°C. To avoid entangling, glass tubing (5 mm diam. x 100 mm) was added to each tank. After one week the worms had evacuated their guts and approximately 10 worms were removed and immediately frozen for subsequent metal level analyses.

c) Sampling periodicity

The sampling dates are shown in Table 2 for the South West and Humber sites. The South West sites were visited approximately every 2-3 months during 1987 and 1988, whereas the Humber sites were visited more regularly, usually every 1-2 months. As the intended study of gametogenesis and population dynamics was just for these two years, the visits to both areas in 1989 were irregular and infrequent. Extra

trips to the Humber sites to dig worms for breeding or electrophoresis purposes are not shown in the table.

Figure 1

Map of England and Wales showing sampling areas.

Boxed area = Humber estuary

A = Kingsbridge Avon

R = Restronguet Creek and Mylor Creek

Figure 2

Map showing sampling sites on Restronguet Creek and Mylor Creek, Cornwall

FIGURE 1

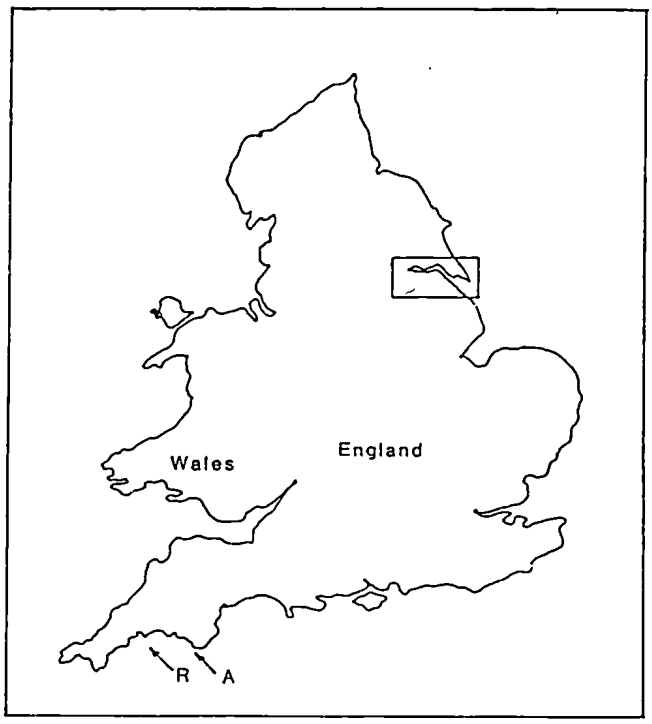


FIGURE 2

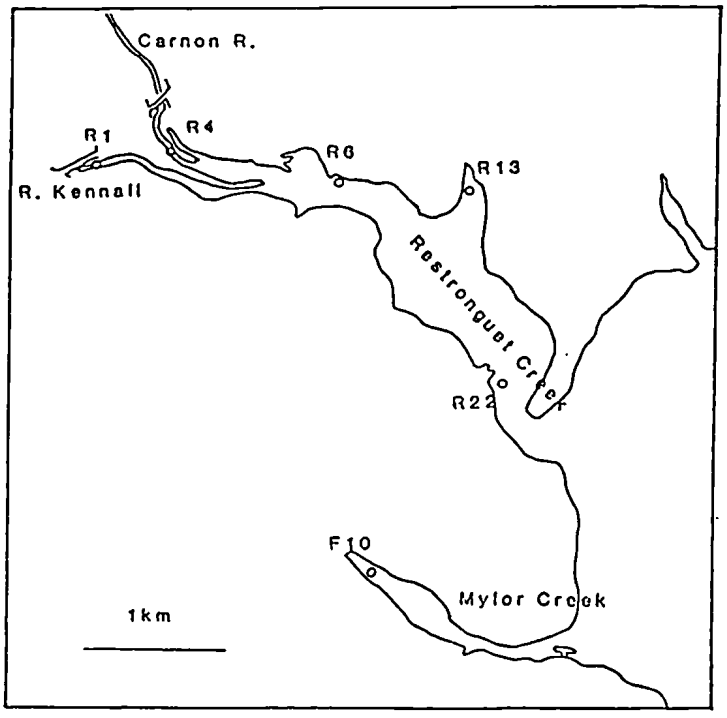


Figure 3

Map showing sampling sites on the Humber estuary, Humberside.

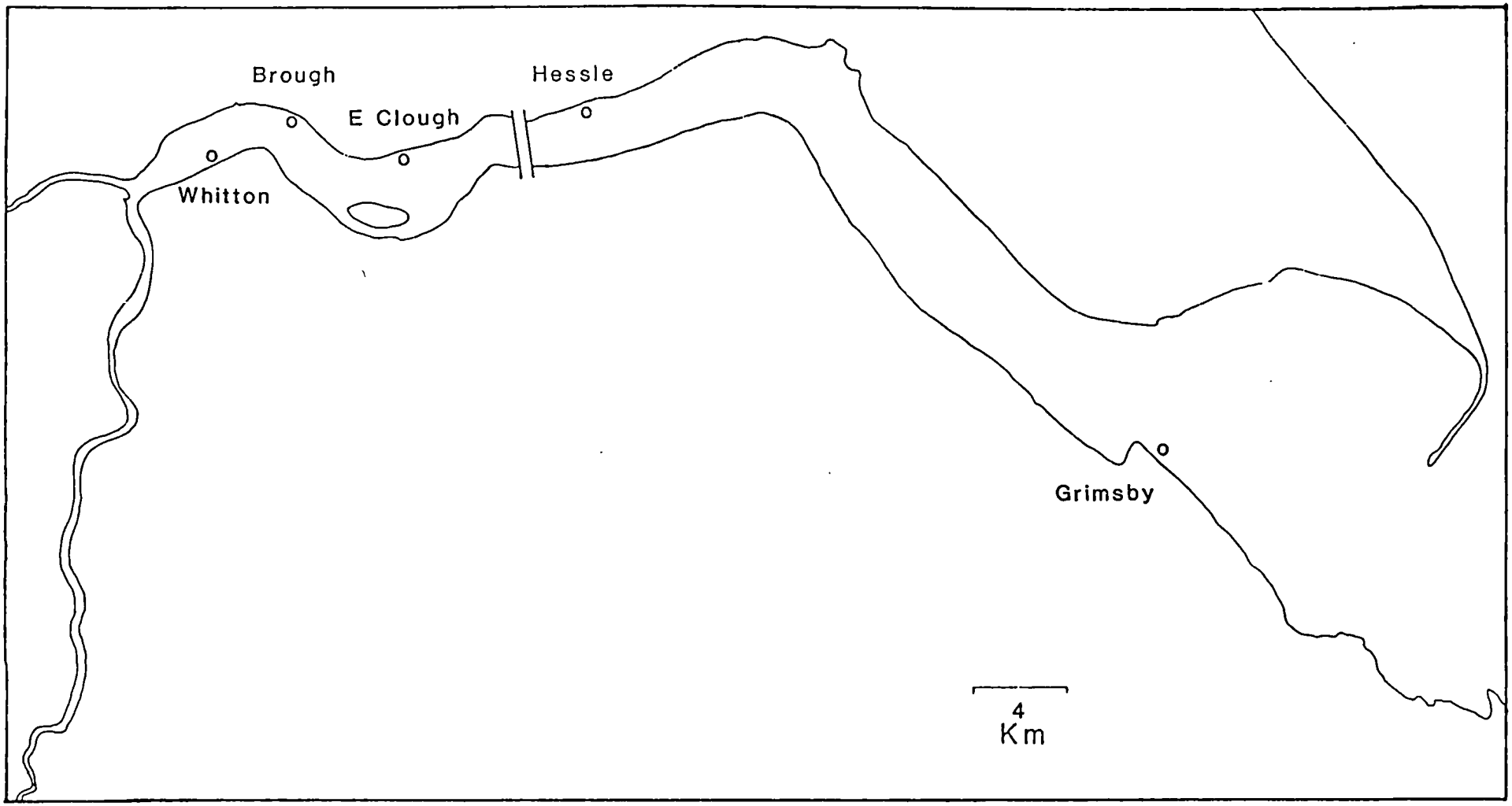

Brough
Hessle
E Clough
Whitton
Grimsby
4
Km

Table 1

Descriptions of sites studied. Salinities were obtained from Bryan and Gibbs (1983) for Restronguet Creek and the South West sites, and from Gameson (1976) for the Humber.

B = Breeding (specific and non-specific matings)

E = Electrophoresis (allele frequency analysis)

G = Gametogenesis and population dynamics

P = Paragnaths

T = Toxicity tests

HWN = High water neaps

LWN = Low water neaps

SITE	POSITION ON SHORE	APPROXIMATE SALINITY ($^{o}/_{oo}$)	SEDIMENT	STUDIES UNDERTAKEN
HUMBER SITES				
GRIMSBY	LWN	28	Muddy sand with small stones	B,E,G,P,T
HESSLE	HWN	18	Cobbles overlying mud	B,E,G,P
EAST CLOUGH	HWN	11	Cobbles overlying mud	B
BROUGH	HWN	9	Very soft mud	B,E,P,T
WHITTON	HWN	6	Soft mud	B,G,T
RESTRONGUET CREEK SITES				
R22	HWN	30	Muddy gravel	B,E,P,T
R13	HWN	30	Gravelly mud	B,E,G,P,T
R6	HWN	30	Soft mud	B,E,P,T
R4	HWN	25	Soft mud	E,T
R1	HWN	9	Organic rich mud	B,E,G,P,T
OTHER SOUTH WEST ENGLAND SITES				
F10	HWN	30	Soft mud with small stones	E,P,T
AVON	HWN	14	Sandy mud	B,E,G,P,T

Table 2

Sampling dates for all sites 1987-1989.

SITES	YEAR																																	
	1987												1988												1989									
	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A		
GRIMSBY			X		X		X	X		X			X	X	X	X	X		X			X		X	X	X			X		X			
HESSLE			X		X	X	X		X		X	X	X		X	X		X		X		X		X		X			X					
BROUGH			X		X	X	X		X		X	X	X		X	X		X		X		X		X	X				X					
WHITTON				X	X		X	X		X		X	X		X	X		X	X	X		X		X					X					
SW SITES			X	X		X	X		X		X			X			X			X		X						X			X			

CHAPTER 1

THE DISTRIBUTIONS OF METALS AND TOLERANCE TO COPPER AND ZINC

INTRODUCTION

Populations of N. diversicolor have been found that exhibit tolerance to copper and zinc (Bryan and Hummerstone, 1971; 1973). Copper tolerant populations were found in the heavily contaminated Restronguet Creek and Hayle estuaries where sediment copper levels were typically 700-3500 $\mu g.g^{-1}$. The 96-hour LC_{50} of copper citrate in 17.5°/oo salinity water for tolerant worms was 2.3 $mg.l^{-1}$ compared with 0.54 $mg.l^{-1}$ for non-tolerant worms (Bryan and Hummerstone, 1973).

Zinc tolerance was detected in Restronguet Creek and possibly Gannel populations, but not in the Hayle. The 96-hour LC_{50} of zinc sulphate for tolerant and non-tolerant animals were respectively 94 $mg.l^{-1}$ and 55 $mg.l^{-1}$ in 17.5°/oo salinity water (Bryan and Hummerstone, 1973).

Tolerance to silver has been demonstrated in worms from Restronguet Creek, the Hayle and the Gannel (with sediment silver levels of 4.6, 1.5 and 13.6 $\mu g.g^{-1}$ respectively). Surprisingly, animals taken from a site (East Looe) with higher sediment silver levels than the Hayle were found to be significantly less tolerant to silver. As the Restronguet Creek and Hayle animals were copper tolerant and the Gannel site contained large concentrations of lead in the sediments (>8000 $\mu g.g^{-1}$), it was suggested that tolerance to silver depends on the presence of tolerance to copper and perhaps lead (Bryan, 1976).

The very low solubility of lead in sea-water has restricted lead toxicity tests and consequently evidence for lead tolerance is weak (Bryan, 1976). There is no evidence that tolerance has developed to cadmium or any significant evidence for tolerance to arsenic (Bryan, 1974; 1976).

This study is primarily concerned with the two best studied examples of tolerance in N. diversicolor; copper and zinc. The first aim

of Chapter 1 is to determine if the level of tolerance is related to the degree of contamination (Luoma point (iv)).

The degree of tolerance to a toxicant has been shown to be related to environmental levels in plants and fish. Rocovich and West (1975) demonstrated that tolerance to arsenic in the grass Andropogon scoparius was related to the amount of arsenic in which the plant was growing. Similarly, the level of endrin resistance in six species of freshwater fish generally reflected the degree of contamination (Grant, 1976).

Although Bryan and co-workers have made extensive studies of sediment metal levels and tolerance in Restronguet Creek, there is little evidence that the degree of tolerance reflects the level of contamination. Copper and zinc toxicity tests were conducted on tolerant animals from the "Tullimaar" site (equivalent to R1), but not on animals from sites further down Restronguet Creek where contamination generally becomes less severe (Bryan and Gibbs, 1983).

To provide information on tolerance distribution, worms were sampled from a number of sites along Restronguet Creek and their relative tolerances determined using static toxicity tests. Toxicity test performances were then related to sediment metal levels, which were considered suitable indicators of the degree of contamination.

Additional studies were made on populations from the adjacent Mylor Creek which has high sediment metal levels (Bryan and Gibbs, 1983), the moderately contaminated Humber estuary (Bryan, 1984; Barnett and Ashcroft, 1989), and the uncontaminated Kingsbridge Avon (Bryan and Hummerstone, 1971; 1973).

Chapter 1 also considers whether tolerance to copper and zinc in N. diversicolor is due to specific physiological mechanisms, or through selection for vigorous strains (Luoma point (ii)).

Tolerances to heavy metals in plants are generally specific to individual metals, (Bradshaw, 1970). Copper tolerant populations of Silene vulgaris are not tolerant to zinc (and vice versa), and Agrostis tenuis has evolved separate mechanisms for tolerance to copper and lead. There is, however, a third tolerance mechanism in this species that appears to operate for both zinc and nickel (Gregory and Bradshaw, 1965). Tolerance to cadmium and mercury in the marine gastropod Cerithium scabridum and the shrimp Palaemon elegans is specific for each metal and also for their interactive effect (Ben-Shlomo and Nevo, 1988; Lavie and Nevo, 1986). In the freshwater isopod Asellus meridianus, Brown (1976,1978) found that copper tolerance may confer lead tolerance, but adaptation to lead was not accompanied by a tolerance to copper. This could again be explained by the mechanisms of tolerance being different for the two metals.

Bryan and Hummerstone have presented convincing evidence that tolerances to copper and zinc in Nereis diversicolor are the results of different processes. Zinc tolerance is partly based on decreased permeability to the metal, as the net uptake of ^{65}Zn in tolerant animals was 30-35% lower than non-tolerant animals. This tolerance mechanism is not specific to zinc as tolerant animals were also significantly less permeable to cadmium and manganese. Tolerant worms also possess a more efficient excretory system that can regulate the amount of zinc in the body (Bryan and Hummerstone, 1973; Bryan, 1976).

Tolerance to copper is also partly due to increased impermeability to the metal, but the difference is only apparent at high external concentrations. As copper concentrations in tolerant worms tend to reflect those of the sediments and can be extremely high ($>1400\ \mu g.g^{-1}$), there is little evidence for increased excretory capacity. Instead, tolerant worms are able to detoxify copper and store it within the body (Bryan and Hummerstone, 1971; Bryan and Gibbs, 1983).

Additional evidence that copper and zinc tolerance involve different mechanisms comes from the different localizations within the body. X-ray microprobe analysis has shown that zinc accumulates as soluble granules in the intestinal epithelial cells. In contrast, copper is immobilised as granules in tertiary lysosomes in the epithelial cells of the first segment, in the outer epidermis, oesophagus, stomach and nephridia, but not in the intestine. Both copper and zinc are associated with low molecular weight components and not metallothionein (Pirie, Liu Fayi and George, 1985).

There is, therefore, strong evidence for copper and zinc tolerances being distinct processes, and not the result of selection for vigorous strains. To provide incontrovertible evidence, the distribution of copper and zinc tolerances along Restronguet Creek were studied and mapped. It was reasoned that as zinc is less toxic than copper to N. diversicolor, the distributions of the tolerances would differ if discrete tolerance mechanisms were at work. The distributions would be the same if tolerance was due to enhanced vigour.

MATERIALS AND METHODS

i) METAL LEVELS

a) Sediment

Total sediment metal levels for copper, zinc, arsenic, tin and lead were measured by X-ray fluorescence spectrometry (XRF) using a Phillips PW1410 X-ray spectrometer.

Surface cores (see General Methods) were defrosted and dried overnight at 100°C. The sediments were initially disaggregated with a pestle and mortar (Grimsby and R13 sediment was sieved through 1 mm mesh to remove stones) and then finely ground using a tungsten carbide rotary mill. 7 g of finely ground sediment was required for pellet formation. After mixing with 10 drops of an aqueous binder the sediment was compressed in a stainless steel die at a pressure of 15 tons. The pressed pellets were dried at room temperature prior to XRF analysis.

Calibrations were based on a series of international silicate rock standards. Too few reference standards were available for arsenic, so two mud samples spiked with known amounts of arsenic in solution were employed (Middleton and Grant. Submitted). The calibration lines were suitable for Humber and Avon sediment but the exceptionally high metal levels in Restronguet Creek (Bryan and Gibbs, 1983) necessitated recalibration. Abiotic Hessle silt was spiked with, tin, arsenic, copper and zinc oxides to final concentrations of approximately 40 000 $\mu g.g^{-1}$ for each metal. This was mixed with unspiked Hessle sediment to give a series of two-fold dilutions down to a nominal 625 $\mu g.g^{-1}$. Satisfactory calibrations were obtained for all four metals using these standards.

To check for temporal variations in sediment metal levels, two samples were examined from each site. Samples collected in summer 1988 were compared with samples collected in summer 1989.

b) Worms

Total metal body burdens for copper and zinc were measured by acid digest and atomic absorption spectrophotometry.

The frozen worms (see General Methods) from each site were dried in conical flasks at 100°C and the dry weights determined. 10 ml of ANALAR nitric acid were added to each sample and a glass ball was placed on the flask. The digestion proceeded overnight on a warm hot-plate. The ball was removed and the acid was evaporated off until the sample was near dryness. 2 ml of 50% hydrochloric acid were added and also evaporated. Finally, hydrochloric acid and distilled water were added to produce a 0.1 N solution. This was filtered through Whatman filter paper (No.1) (Bryan and Hummerstone, 1971). Known concentrations of copper (as copper sulphate) and zinc (as zinc sulphate) were employed as reference standards. To correct for any digestion related artefacts, the standards were also digested as described. A Phillips SP9 atomic absorption spectrophotometer with a multi-element bulb measured copper and zinc metal levels, which were expressed as $\mu g.g^{-1}$ dry weight.

Two samples collected two years apart (spring 1987 and spring 1989) were examined from each South West site to identify any temporal variaton. Single samples (summer 1989) were examined from the Humber sites.

ii) TOXICITY EXPERIMENTS

Both zinc and copper are more toxic to N. diversicolor at extreme salinities and high temperatures (McLusky, Bryant and Campbell, 1986). Jones et al (1976) demonstrated that intermediate salinities of 10-17.5°/$_{oo}$ favoured survival of worms from both high (20.5-30.4°/$_{oo}$) and low (7.0-15.0°/$_{oo}$) salinity populations in low copper concentrations. As most of the sites studied in this work had average salinities in the

range 7.0-30.0$^{o}/_{oo}$, a standard experimental salinity of 17.5$^{o}/_{oo}$ and a low temperature of 12°C were used as a reasonable compromise (Bryan and Hummerstone, 1971; Bryan, 1976).

Unlike Bryan and Hummerstone (1971;1973) and Jones et al (1976), body concentrations were not measured after toxicity tests. Both metals caused considerable tissue damage before actually killing the animals (the rear segments disintegrated). It was felt that a considerable proportion of the metal found in the body may simply have entered through passive diffusion into dead tissue rather than been taken up by living tissue. Similarly, metal may have been lost through tissue disintegration.

a) Field-derived worms

Worms selected for toxicity experiments were washed and acclimatized in 17.5$^{o}/_{oo}$ salinity water at 12°C for approximately one week. Only young healthy worms (approximately 30 mm long) were used in the toxicity tests, damaged and green worms which may have been approaching spawning were rejected. The static toxicity tests were conducted in glass crystallizing dishes (140 mm diam. x 70 mm) with 4-5 glass rods added to stop the worms entangling. Groups of 10 worms were placed in 500 ml of fully oxygenated 17.5$^{o}/_{oo}$ salinity water adjusted to a pH of approximately 7.8, with the temperature maintained at 12°C. Copper was added to the water as copper (II) sulphate from a stock solution of 1000 mg.l^{-1} to make final concentrations of 0.5, 0.7 and 1.0 mg.l^{-1}. Zinc was added as zinc (II) sulphate from a stock solution of 40 000 mg.l^{-1} to make a final concentration of 40 mg.l^{-1}. 10 worms from each site were kept in uncontaminated sea-water as controls. The worms were checked daily; dead animals were removed and frozen at -20°C. Animals were considered dead when they failed to respond to mechanical stimulation. The water was changed daily. The experiment was replicated a

number of times so that a total of 40-100 worms from each site were tested.

The elapsed time to death for an individual was considered to be the time mid-way between the worm being removed and the previous examination time. The data were transformed (reciprocal for copper toxicity tests and logarithmic for zinc toxicity tests) to give approximate homogeneity of variances and symmetrical distributions for each sample. As the sample mean is the best estimator of the sample median for normally distributed data, LT_{50} values were then estimated by mean time to death. At lower copper concentrations (0.5 - 0.7 $mg.l^{-1}$), some tolerant worms survived the course of the experiment (> 600 hours). These animals were considered to represent censored observations and parameters were estimated by the method of Wolynetz (1979) using a procedure from the GLIM statistics package macro library.

b) Laboratory-bred worms

Animals from specific and non-specific matings were tested for copper tolerance alone in the same manner. The challenge concentration used was 0.5 $mg.l^{-1}$ $CuSO_4$. Three or four replicates were made for non-specific matings but usually there were too few worms available for replicates of specific matings.

RESULTS

i) METAL LEVELS

a) Sediment levels

Surface sediment metal concentrations at the South West sites are summarised in Table 1.1.

For all five metals measured, concentrations were highest in Restronguet Creek (sites R1-R22), lowest in the Avon and intermediate in Mylor Creek (F10). Levels in Restronguet Creek typically were a factor of 40-50 higher than the Avon and 2-4 times higher than Mylor Creek. Neither the Avon nor Mylor Creek directly receive high freshwater inputs of metals (Bryan and Gibbs, 1983) but Mylor Creek sediments were notably metalliferous. Mylor Creek is 2 km south of Restronguet Creek and it is from here the contaminating sediments originate. Hosking and Obial (1966) have previously noted the tendency of contaminated water to persist between the two Creeks with little eastward migration.

Within Restronguet Creek the relative abundances of the metals were Zn = Sn > Cu > As > Pb. Generally there was a decline in metal levels moving down the estuary. R1 and R4 tended to have the highest concentrations, R22 the lowest and R6/R13 intermediary levels. There were exceptions. The highest lead concentrations were found at R13 and copper/zinc concentrations at R6 and R13 were similar to the two upstream sites.

The distributions are explained through R1 and R4 being the sites nearest to the contaminating Carnon River as it enters the estuary. As the freshwater mixes with sea-water, the pH and salinity increase causing iron to precipitate as the hydrous oxide. Copper, arsenic and lead are associated with this flocculation process and as tin enters the estuary in particulate form they all are quickly deposited in the sediment. Very little zinc is actually deposited (approximately 3%; Bryan and Gibbs,

1983) and as the dissolved concentration is linearly related to salinity, the deposition is more even throughout the estuary.

At each site sediment metal concentrations were rather similar in 1988 and 1989. The only notable exception to this was the apparent doubling of tin concentrations at R13. In view of the stability of tin concentrations at other sites, this is probably due to local sediment patchiness in metal content rather than temporal change. Comparison of sediment metal levels measured in 1921 and 1979 indicate long-term stability of metal concentrations in sediments, with similar levels of copper, zinc and iron being found (Bryan et al., 1987).

Data from Bryan and Gibbs (1983) have been included in Table 1.1. These concentrations were obtained by nitric acid digestion of the <100 μm fraction of surface sediment (fusion was used for tin). Direct comparisons between these values and the XRF derived values should be made with caution because of the different methods used. XRF analysis gives a true total of metal in the sediment whereas nitric acid digests always leave some metal unextracted. The acid digest figures are useful, however, because they indicate the amount of 'bioavailable' metal in a sample (Bryan and Gibbs 1983). Unexpectedly low concentrations were recorded for copper, zinc, lead and arsenic at R1. These values are misleading as a large proportion of the metal was associated with >100 μm particles sieved out prior to the digest. Unsieved sediment digests gave values of approximately 3200 $\mu g.g^{-1}$ copper, 2290 $\mu g.g^{-1}$ zinc and 330 $\mu g.g^{-1}$ lead (Bryan and Gibbs, 1983).

Surface sediment metal concentrations at the Humber sites are summarised in Table 1.2.

The relative abundances of the metals in the Humber were Zn > Pb > As > Cu. Slightly higher values were recorded at the upstream sties, with Brough having the highest levels. Two factors probably contribute to this

distribution; the proximity of a metal smelting works at East Clough (3 km east of Brough) and the tendency for metals to associate with fine sediments (Grant and Middleton, Submitted). When particle size effects are compensated for by normalising metal concentrations as ratios to an element associated with clay minerals (aluminium or rubidium), the metal levels are rather uniform (Grant and Middleton, Submitted). There was little variation between the two sampling times. Metal concentrations in the Humber ranged from 20-73 $\mu g.g^{-1}$ copper, 40-148 $\mu g.g^{-1}$ lead, 101-355 $\mu g.g^{-1}$ zinc and 32-139 $\mu g.g^{-1}$ arsenic. These values are similar to those reported by Jaffe and Walters (1977) for south bank Humber sites. In their study, metal concentrations ranged from 51-160 $\mu g.g^{-1}$ copper; 28-224 $\mu g.g^{-1}$ lead and 53-519 $\mu g.g^{-1}$ zinc.

Metal levels in the Humber were higher than those in the uncontaminated Avon estuary (Table 1.1). Copper, lead and zinc concentrations were higher by a factor of 2-3, arsenic by a factor of 10. Humber levels were considerably lower than Restronguet Creek and Mylor Creek concentrations for all metals.

b) Worm metal concentrations

Copper and zinc concentrations in worms sampled from the South West sites are shown in Table 1.3. R4 and R6 were not visited in 1987 and R22 in 1989. Where possible the missing values were substituted by data from spring 1988.

Copper levels in 1989 were higher than in 1987. The differences were particularly pronounced at F10 (x7.2) and R1 (x2.6). Body burdens were generally lower than, but of the same order of magnitude, as those obtained by Bryan and Gibbs (1983) in 1976-78 . The results are comparable because the same digestion procedures were used (Bryan and Hummerstone, 1971). These indicate considerable temporal variation in body copper concentrations at the sites studied.

Table 1.3 also demonstrates considerable spatial variation in body copper concentrations. In 1987 and 1989 the highest concentrations (410 and 1061 $\mu g.g^{-1}$) were recorded in R1 worms. This pattern was in agreement with Bryan and Gibbs' observations for 1976-1978. In 1987, body burden steadily decreased as one moved down Restronguet Creek and the lowest concentrations were found at F10 and the Avon. In 1989 the lowest body concentrations were again found in Avon worms but there was no particular relationship between body burden and position on the estuary for the intermediate Restronguet Creek sites.

Zinc concentrations were reasonably constant from year to year as values for 1987 were very similar to those for 1989 and 1976-78. There was also little spatial variation. Levels in Restronguet Creek animals were not significantly higher than Avon levels. Concentrations within Restronguet Creek also appeared to be relatively uniform.

Copper and zinc concentrations in Humber worms are shown in Table 1.4. Concentrations of both metals were fairly uniform throughout the estuary and neither were substantially elevated above Avon values.

ii) TOLERANCE DISTRIBUTION

a) Copper

The results of the copper toxicity tests are illustrated in Figure 1.1.

Originally, the challenge concentration was 0.5 $mg.l^{-1}$ but as the more tolerant worms could apparently survive this level indefinitely (i.e. were unstressed after 600 hours), it was increased to 0.7 $mg.l^{-1}$ and 1.0 $mg.l^{-1}$. No control animals died during the experiment. The graph clearly demonstrates a steady decline in tolerance to copper as one moves away from the contaminating source at the head of Restronguet Creek. The most tolerant worms were found at the upstream R1 site and the least

tolerant worms were found at R22 near the mouth. The R22 worms show similar LT_{50} values to Avon worms indicating an almost complete absence of copper tolerance just 3 km from the contaminating source. It was noted that fully tolerant animals (surviving >600 hours in 0.7 $mg.l^{-1}$) were found throughout the estuary. However, the frequency of these animals declined as one moved towards the estuary mouth and they were very rare (approximately 2% of the population) at site R22. The LT_{50} ratio between R1 and Avon worms was approximately 8. This compares favourably with the LT_{50} ratio of approximately 4 reported by Bryan (1976).

Animals from the adjacent Mylor Creek (site F10) were essentially non-tolerant despite living in sediment containing copper levels of more than 1400 $\mu g.g^{-1}$. No individuals survived longer than 216 hours in 0.5 $mg.l^{-1}$ copper compared with the 190 hour maximum recorded with Avon worms. Animals from the Humber also showed no evidence of tolerance.

b) Zinc

The decline in tolerance to zinc moving down the estuary was more punctuated (Figure 1.2). The most tolerant animals were again found at R1 but they were not significantly more tolerant than R4 worms (Scheffe multiple comparison test). The downstream sites R6 and R13 exhibited no zinc tolerance as their LT_{50} values were not significantly different to that of Avon worms. The ratio of R1 and Avon LT_{50} values was approximately 2. This agreed with Bryan's (1976) LT_{50} ratio of 1.7.

There was no evidence for zinc tolerance in the Humber.

DISCUSSION

XRF analysis of sediment metal levels rank the estuaries in order of contamination; Restronguet Creek > Humber > Avon. This is in agreement with Bryan (1984) when he compared his data for the South West (Bryan, Langston and Hummerstone, 1980) with Jaffe and Walters (1977) data for the Humber.

Levels in the Avon are similar to concentrations found in 'average' shale (Krauskopf, 1967) and are typical of a clean estuary (Bryan, 1984). Humber sediment concentrations are higher than these 'background' levels and are indicative of significant metal contamination. The concentrations of copper, zinc, lead and arsenic are typical of an estuary polluted by industrial and domestic sewage (Jaffe and Walters, 1977). Hamilton et al (1979) found similar levels of these metals in the Severn estuary. The Humber is 5-10 times less contaminated than the Rhine (Banat, Forstner and Muller, 1972) and has lower metal levels than either the Conway estuary (Elderfield, Thornton and Webb, 1971) or the River Tawe (Bloxam et al, 1972) which receive substantial metalliferous waste from mine drainage and smelting industries respectively. In the short term, Humber sediment levels appear to be stable as there was little variation between samples taken one year apart. In the long-term levels have been shown to be steadily increasing (Grant and Middleton, Submitted). A sample of Scrobicularia clay from the ancient bed of the estuary (4-6000 b.p.) was used as an indicator of background levels (Middleton and Grant, Submitted). Significant anthropogenic enrichment of sediment levels has occurred for many metals notably arsenic (x6), lead (x5), copper (x5) and zinc (x4). In the cases of arsenic, copper and zinc the majority of the enrichment is probably relatively recent (i.e. within the last 100 years) (Middleton and Grant, Submitted).

Metal analysis of Restronguet Creek sediment confirms it as one of the most highly contaminated estuaries in Britain (Bryan, 1984). Unlike

the Humber, continuing metal input is not the result of current industrial activities, but rather is a legacy of the past. Extensive copper and tin mining ceased by the beginning of this century but drainage of old mines results in a continuous influx of metals into the estuary (Bryan et al, 1987). Sediment concentrations have been shown to be temporarily stable both in the short-term (this work) and the long-term (Bryan et al, 1987).

Relating copper and zinc concentrations in native N. diversicolor to total sediment concentrations provides little information on the extent of ecological impact of the metals at a site. Ideally, biological indicators should be good accumulators of metals and reflect changes in environmental availability (Bryan, 1984). Copper levels in Restronguet Creek worms showed considerable temporal variation at all sites (e.g. x7.2 at F10 and x2.6 at R1). Although body burden has been shown to be roughly related to sediment copper levels (Bryan, 1971; Luoma and Bryan, 1982) temporal fluctuations in sediment levels were minimal (Table 1.1) and could not possibly account for all the observed variation.

A number of factors are known to cause variations in metal concentrations at a site in marine organisms (Bryan, 1984). In mussels, size (Boyden, 1977), sex (Watling and Watling, 1976), reproductive state (Lowe and Moore, 1979) and season (Majori et al, 1978) are all modifiying variables. Howard and Brown (1983) identified worm size and the reproductive cycle as sources of temporal variation in copper levels in N. diversicolor in the Tees estuary. They speculated that additional variation may be due to fluctuations in copper concentration and speciation in the sea-water. Short-term changes on a daily or tidal basis may have a large effect on the biota without influencing total sediment concentrations (Howard and Brown, 1983).

Both worm size and reproductive state may have been contributory factors in the variable worm copper content observed in this work. The dry weight of ten F10 worms sampled in 1987 was 0.488 g compared with 0.297 g for 1989. Smaller worms have been shown to have higher copper concentrations than larger worms (Howard and Brown, 1983) which may partially explain the differences in body concentrations between the two years.

The reproductive condition of the two samples may also have differed. Both were collected in spring during the period of maximum oocyte growth in the South West (Chapter 4). Consequently, even the small differences between the corresponding sampling dates (June 1987 and April 1989) may have resulted in large differences in mean oocyte diameter. As oocytes have been shown to have significantly lower copper concentrations than somatic tissue (Howard and Brown, 1983), they effectively dilute whole body copper levels and therefore may cause differing sample concentrations.

A further source of temporal variation may occur through age effects. Studies on population dynamics at R1 demonstrated how mean adult worm age at a site can vary from year to year (Chapter 4). In spring 1987 all R1 worms were approximately 8-9 months old. In spring 1989, the adult population was a mixture of 8 and 20 month old worms. If body burden is in anyway related to period of exposure, this may be a significant source of intra-site temporal variation.

Inter-site comparisons of body copper concentrations are complicated by further environmental factors. Despite site R4 being adjacent to the contaminating Carnon River and having similar sediment copper levels to site R1 (3246 $\mu g.g^{-1}$ and 3539 $\mu g.g^{-1}$ in 1989), there was considerably less copper in the R4 worms (Table 1.3). The interstitial salinity at R1 is approximately $9^{o}/_{oo}$ compared with $25^{o}/_{oo}$ at R4. It is probable that the combination of high sediment levels and low salinity

results in R1 worms having the highest copper concentrations in the estuary (see Bryan and Hummerstone, 1971).

Whole body zinc concentrations demonstrated little temporal and spatial variation. This reflects the ability of N. diversicolor to effectively regulate concentrations of zinc in its soft tissues (Bryan and Hummerstone, 1973).

Small variations in body burden may have occurred as a result of variable worm size (concentrations are highest in smaller worms) or through periods of rapid growth (Howard and Brown, 1983). Up to 50% of the total body burden of zinc is retained in the jaws (Bryan and Gibbs, 1980) and during periods of accelerated growth there will be a demand for the metal as the jaws are laid down. This effect will be particularly pronounced in small worms where small increases in zinc content may result in large increases in total body concentration (Howard and Brown, 1983).

The limitations of N. diversicolor as an indicator organism have been discussed by other workers (Howard and Brown, 1983; Bryan, Langston and Hummerstone, 1980). Concentrations in the worm do tend to reflect changes in the availability of certain metals (e.g. silver, cadmium, copper and lead) (Bryan and Hummerstone, 1971; 1973; 1977) but in the case of copper, concentrations are also dependent to some extent on the size of the worm, its reproductive state and the interstitial salinity. As zinc concentrations are regulated and unrelated to environmental levels (Bryan and Hummerstone, 1973), it is understandable why Phillips (1980) considered N. diversicolor "far from an ideal indicator", at least as regards these two metals.

Assessing the sphere of influence of particular metals on the biota through the distribution of tolerance requires the extensive use of toxicity tests ('bioassays'). The validity of using small, static volumes

of test solutions has been questioned on the grounds that the animals may alter their environmental conditions. The accumulation of waste products, depletion of dissolved oxygen and alteration of pH may all have significant effects (Connor and Wilson, 1972; Pagenkopf, Russo and Thurston, 1974). Extrapolating toxicity test results directly to the field (principally to establish environmental quality standards) is also an area of some concern as it is extremely difficult reproducing field conditions in the laboratory (Lee, 1973). In this work, no extrapolation to the field was required as the only interest was in the relative performances of various populations. All test animals were therefore exposed to the metals under identical experimental conditions and optimal survival conditions (low temperature and mid-salinity; Jones, Jones and Radlett, 1976; Fernandez, 1983). To guarantee experimental reproducibility, artificial sea-water was employed as the test medium (Lee, 1973).

Examining the distribution of tolerance would appear to give a much better indication of where copper and zinc are exerting the greatest ecological impact. The most tolerant worms to both metals (and hence the greatest impact) were found at the head of Restronguet Creek at site R1. Copper tolerance gradually declined moving down the estuary until at site R22 the worms were essentially non-tolerant. This indicates that copper is no longer having an ecological impact at the mouth of the estuary just 3 km from the contaminating source. Similarly, copper must not exert any significant detrimental effect at F10 in Mylor Creek despite sediment levels being over 1400 $\mu g.g^{-1}$.

Unlike the smooth spatial cline in tolerance to copper, zinc tolerance in Restronguet Creek was more disjunct. R1 worms were not significantly more tolerant than R4 worms but tolerance disappeared by site R6. This indicates that zinc exerts its greatest ecological impact

at the two upstream sites but exerts negligible impact just 1 km downstream from the contaminating source.

The differences between the two clines indicate that copper is exerting the greater effect over the estuary as a whole. Populations above R22 but below R4 require an element of copper tolerance in order to survive, but not zinc tolerance. This conclusion is supported by work on Fucus vesiculosus as specimens of the algae sampled from the creek were significantly tolerant of copper but not of zinc (Bryan, 1984). This is not entirely surprising as although copper and zinc sediment levels are approximately the same at each site (Table 1.1), copper is the more toxic metal to most marine organisms (Bryan, 1984).

Generally, the degree of tolerance was related to environmental levels, i.e. the most tolerant animals were found at the most contaminated sites and the least tolerant at the least contaminated sites. However, a number of factors can substantially affect the relationship between metal concentration and toxicity in the field. For example.

i) Form of the metal in the environment; this governs its bioavailability.

ii) Environmental factors (temperature, salinity, pH, dissolved oxygen); these can both alter the speciation of the metal and the physiological stress on the organism.

iii) Condition of the organism (sex, age, size, nutritional state, reproductive state).

iv) Presence of other metals; they can both increase or decrease toxicity (adapted from Bryan, 1984).

Salinity would appear to have a marked effect on copper toxicity and hence tolerance at the upstream sites. As mentioned earlier, R1 and R4 animals inhabited sediment containing similar concentrations of metals but at different interstitial salinities ($9^{o}/_{oo}$ and $25^{o}/_{oo}$). Not only did

R1 worms contain more copper than R4 worms but they were also significantly more tolerant. Copper has been shown to be more toxic to N. diversicolor at low salinities (Jones, Jones and Radlett, 1976) which would explain the difference in relative tolerances.

Factors other than copper concentration and salinity must be responsible for determining the upstream limit of N. diversicolor on the Carnon River (site R4). Worms at this site withstand similar copper levels to R1, but at a higher salinity, and contain only 281 $\mu g.g^{-1}$ copper. The limiting factor may be zinc contamination or other physical parameters such as water, pH or sediment texture. When sediment samples from R4 were compared with sediment from R3 (upstream, no worms) there were considerable differences in water and organic content. R3 sediment had approximately half the water content (measured by weight loss on drying) and half the carbon content (measured by dichromate titration) of R4 sediment. This change corresponded with a visible discontinuity between typical estuarine sediment and hard red mud. Sediment/worm transplant experiments would confirm whether compacted sediment was indeed responsible for determining the upstream limit of worms on the Carnon River.

The main aim of this chapter is to determine if the degree of metal tolerance in N. diversicolor reflects the extent of metal contamination (Luoma point (iv)). For biomonitoring purposes this would be useful as estimates can then be made of the levels of environmental contamination required to cause a significant ecological impact on the species.

Rocovich and West (1975) demonstrated that arsenic tolerance in the plant Andropogon scoparius was related to soil arsenic concentration. Using their data one could assume that soil arsenic concentrations below approximately 100 $\mu g.g^{-1}$ would not have an ecological impact as the plants grown in these concentrations did not exhibit arsenic tolerance.

Unfortunately such simple extrapolations cannot be so easily made as plants grown under different environmental conditions would almost certainly have different 'threshold' concentrations.

Similarly, although copper and zinc tolerance in N. diversicolor was roughly related to sediment levels, it would not be possible to state at which concentration the metals were no longer having an effect. Worms from R22 and F10 were essentially copper non-tolerant in sediment containing approximately 1800 $\mu g.g^{-1}$ and 1400 $\mu g.g^{-1}$ copper respectively. It would, however, be incorrect to state that sediment copper levels below 1800 $\mu g.g^{-1}$ do not require copper tolerance as Bryan (1976) found copper tolerant animals from the Hayle estuary living in just 700 $\mu g.g^{-1}$ copper.

The dramatically different distributions of copper and zinc tolerance provide further evidence that metal resistance is not due to selection for vigorous strains (Luoma point (ii)). The distributions can only be explained through the action of different physiological mechanisms. Bryan and Hummerstone (1973) determined that zinc tolerance was partly based on a decreased permeability to the metal and partly on a more efficient excretory system. Copper tolerance is also partly due to increased impermeability but rather than excess metal being excreted, it is detoxified and stored in the epidermis, nephridia and other tissues (Bryan and Hummerstone, 1971; Pirie, Liu Fayi and George, 1985).

The main attraction of tolerance distribution as a biomonitoring method is the specificity by which impacts can be related to pollutants. Although tolerance mechanisms do generally appear to be specific to individual metals in land plants (Bradshaw, 1970) there are examples of co-tolerance in algae and metazoans. Stokes, Hutchinson and Krauter (1973) discovered that cells of the planktonic alga Scenedesmus sampled from lakes polluted by copper and nickel were significantly tolerant of

both metals and also silver. As silver was found in very low concentrations in the water the authors proposed a common tolerance mechanism. Tolerance to chromium in crayfish (Austropotamobius pallipes) sampled from high chromium/low lead waters was accompanied by lead tolerance, however, lead tolerance did not confer chromium tolerance (Vareille-Morel and Chaisemartin, 1982). A similar effect was noted by Brown (1976,1978) who found copper tolerance in the freshwater isopod Asellus meridianus was accompanied by lead tolerance, but not vice versa.

Co-tolerance is also suspected of occurring in N. diversicolor. Copper tolerant worms from Restronguet Creek and the Hayle estuary and high-lead animals from the Gannel were all tolerant of silver. As sediment silver levels were relatively low in all three estuaries, it was suggested that tolerance to silver depends on the presence of tolerance to copper and perhaps lead (Bryan, 1976). Similarly, the increased impermeability of zinc tolerant animals is not a specific mechanism as they are also less permeable to cadmium and manganese (Bryan and Hummerstone, 1973). Such co-tolerances are not entirely surprising as copper and silver are chemically very similar, as are cadmium and zinc.

A certain degree of caution must therefore be applied when directly attributing ecological impact to a specific metal through studying metal tolerance. Copper, zinc and perhaps lead tolerances do seem to have developed separately and therefore are excellent indicators of impact by those metals. Tolerance to manganese, cadmium and silver must be viewed with considerable scepticism before directly attributing their presence to those metals and not to others which share the tolerance mechanisms.

To summarise the salient points of tolerance distribution with respect to its biomonitoring value:

i) Copper and zinc tolerances are roughly proportional to environmental levels, but the pressure to adapt at a site is dependant on

a number of other factors including salinity, bioavailability, etc. It is therefore not possible to estimate critical environmental levels where metals begin to exert significant ecological impacts (Luoma point (iv).

ii) Copper and zinc tolerances are based on different physiological mechanisms and are not due to selection for vigorous strains (Luoma point (ii)).

Table 1.1

Surface sediment metal concentrations ($\mu g.g^{-1}$) for South West sites in 1988 and 1989.

* = acid digest data from Bryan and Gibbs (1983)
\- = no data

METAL	YEAR	SITES						
		R1	R4	R6	R13	R22	F10	AVON
Cu	1988	3432	2790	3214	2871	1967	1425	65
	1989	3539	3246	3352	3503	1836	1459	21
	1976-80*	1733	-	2540	2170	1785	1117	19
Zn	1988	4688	5014	4630	3950	2930	1262	137
	1989	5063	4232	5003	4526	2816	1286	132
	1976-80*	1587	-	3515	3000	1978	980	98
Sn	1988	4794	4637	3296	2678	2379	2326	100
	1989	4435	5156	3913	4950	2070	2024	-
	1976-80*	2672	-	1730	1350	-	-	28
Pb	1988	267	371	244	524	169	164	30
	1989	236	361	244	558	166	167	36
	1976-80*	204	-	290	396	198	179	39
As	1988	2238	4050	1699	1263	568	250	11
	1989	1861	3596	1664	1305	527	253	20
	1976-80*	1076	-	2520	1600	-	-	13

Table 1.2

Surface sediment metal concentrations ($\mu g.g^{-1}$) at Humber sites in 1988 and 1989. Tin concentrations were not measured.

METAL	YEAR	SITES			
		GRIMSBY	HESSLE	BROUGH	WHITTON
Cu	1988	20	51	73	64
	1989	43	38	56	41
Zn	1988	101	252	355	317
	1989	291	188	308	217
Pb	1988	40	94	148	127
	1989	99	72	118	81
As	1988	32	89	139	109
	1989	58	56	102	61

Addendum: Correlation coefficients for all calibration curves; Pearson's $r > 0.999$. Each concentration is a mean value of duplicate samples. Variation between duplicates did not exceed 7% of the mean.

Table 1.3

Metal concentrations ($\mu g.g^{-1}$ dry weight) in worms from South West sites in 1987 and 1989. Total dry weight of worms analysed in italics (mg).

* = data from Bryan and Gibbs (1983)
\- = no data
\+ = 1988 sample

		SITES						
METAL	YEAR	R1	R4	R6	R13	R22	F10	AVON
Cu	1987	410	-	477+	142	97	23	29
		226	-	*113*	*196*	*168*	*488*	*136*
	1989	1061	281	286	338	462+	166	34
		274	*315*	*130*	*258*	*111*	*297*	*187*
	1976-78*	1430	630	832	932	271	298	19
		-	-	-	-	-	-	-
Zn	1987	254	-	232+	122	147	128	221
		226	-	*113*	*196*	*168*	*488*	*136*
	1989	226	326	278	220	432+	298	199
		274	*315*	*130*	*258*	*111*	*297*	*187*
	1976-78*	262	405	318	302	146	208	197
		-	-	-	-	-	-	-

Table 1.4

Metal concentrations ($\mu g.g^{-1}$ dry weight) in worms from Humber sites in 1989. Total dry weight of worms in italics (mg).

		SITES			
METAL	YEAR	GRIMSBY	HESSLE	BROUGH	WHITTON
Cu	1989	47	35	51	28
		261	*182*	*161*	*140*
Zn	1989	172	204	220	189
		261	*182*	*161*	*140*

Figure 1.1

LT_{50} in hours for populations of N. diversicolor challenged with copper.

Bars represent 95% confidence limits.

+ = 1.0 $mg.l^{-1}$

X = 0.7 $mg.l^{-1}$

O = 0.5 $mg.l^{-1}$

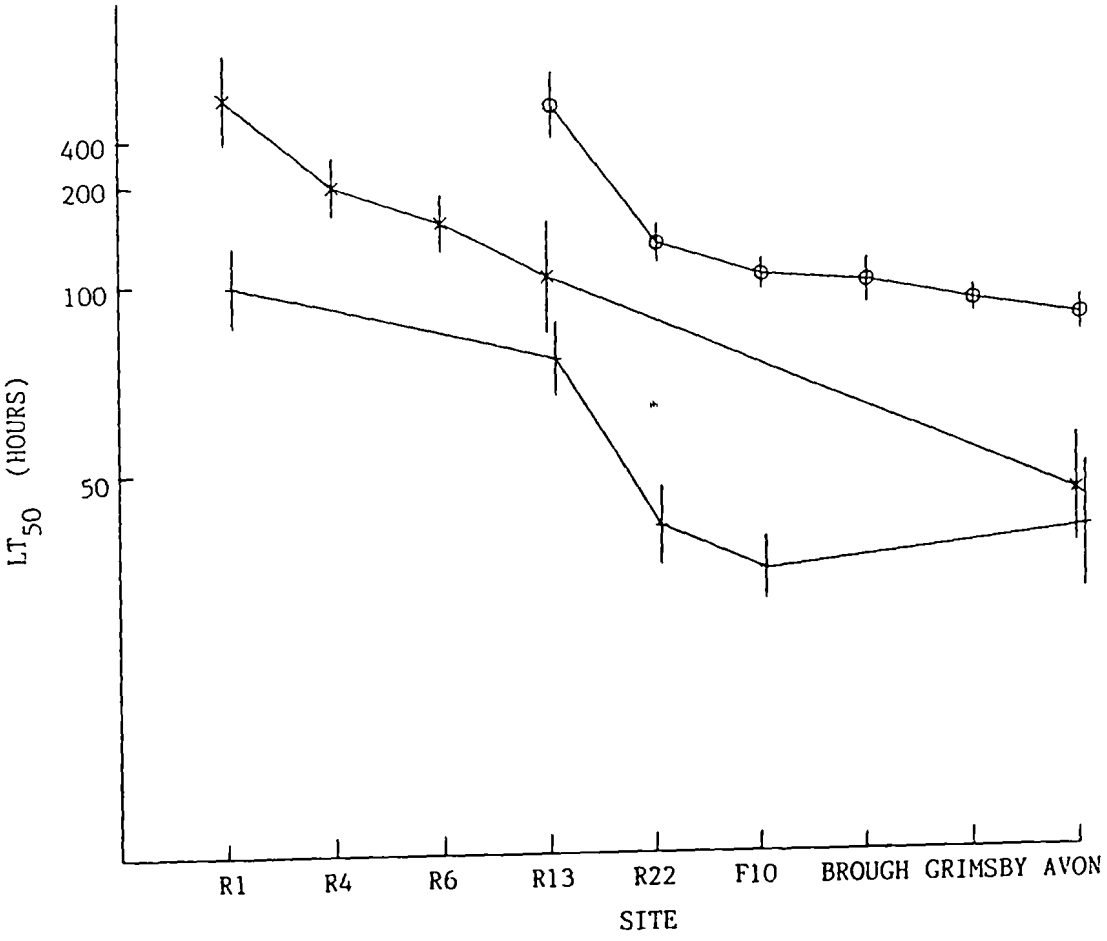
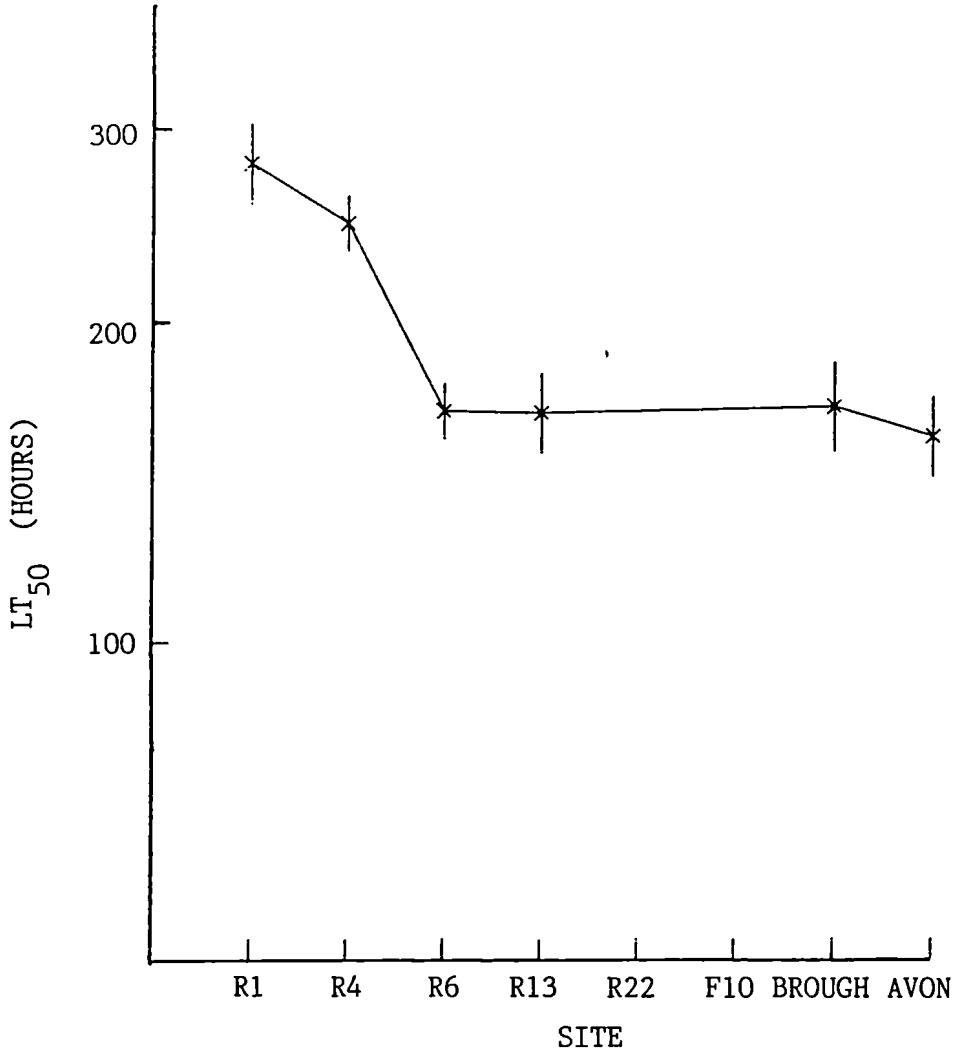

400
200
100
50
LT_{50} (HOURS)
R1
R4
R6
R13
R22
F10
BROUGH
GRIMSBY
AVON
SITE

Figure 1.2

LT_{50} in hours for populations of N. diversicolor challenged with zinc.

Bars represent 95% confidence limits.

X = 40 $mg.l^{-1}$


300
200
100
LT_{50} (HOURS)
R1
R4
R6
R13
R22
F10
BROUGH
AVON
SITE

CHAPTER 2

GENETIC STUDIES OF COPPER AND ZINC TOLERANCE

INTRODUCTION

In an attempt to determine whether copper tolerance in N. diversicolor had a genetic or induced basis, Bryan and Hummerstone (1971) exposed animals to unfamiliar sediments. Tolerant animals (from R1) were placed in low-copper sediment from the Plym estuary, and non-tolerant Plym animals were transferred to high-copper sediment. After a period of exposure ranging from 45-76 days, the worms were removed from the sediment and challenged with 1.0 $mg.l^{-1}$ copper citrate. Pre-exposure to high-copper sediment failed to induce tolerance in the non-tolerant Plym worms (LT_{50} = 17.3 hours). Similarly, pre-exposure to clean sediment failed to diminish tolerance in the R1 worms (LT_{50} = 163 hours). The authors therefore concluded that they were probably dealing with a factor under genetic control (Bryan and Hummerstone, 1971).

Despite criticism that the challenge concentration was too high and could have masked any induced effect (Pesch and Hoffman, 1982), the experiments nevertheless demonstrated that copper tolerance was neither readily gained nor lost. Similar induction experiments were not used to investigate zinc tolerance. However, Bryan and Hummerstone (1973) still considered it likely that zinc tolerance was due to genetic adaptation.

Chapter 2 investigates the factors responsible for copper and zinc tolerance in N. diversicolor in more detail. The main objective is to determine if the tolerances are due to selection for resistant genotypes as suggested by Bryan and co-workers ("adaptation"), or if they could be explained by physiological acclimation through earlier exposure to sub-lethal levels ("acclimation"). Confirmation of the former case would satisfy Luoma point (i).

Heavy metal tolerance in land plants invariably appears to be due to selection for resistant genotypes and cannot be induced over the

plants' life-time (Bradshaw, 1970). However, tolerance in aquatic organisms has been attributed to both adaptation and acclimation (reviewed in Klerks and Weis, 1987).

Metal tolerance in bacteria appears to be predominantly due to genetic adaptation. Timoney and Port (1982) found cadmium and zinc resistant bacteria in a population sampled from a dump site for metal-laden dredge spoils. The elevated levels of these metals at the site and the bimodality in the distribution of minimal inhibitory concentration within the population pointed to locally evolved adaptation having occurred. Similarly, mercury resistance in strains of Pseudomonas, Vibrio, Aeromonas and Bacillus were found to have a genetic basis (Nelson and Colwell, 1975).

Most examples of metal tolerance in algae are also explained through adaptation. Populations of the marine fouling alga Ectocarpus siliculosus taken from ships treated with copper based anti-fouling paints were a factor of ten more tolerant of copper than a control population. As the experiments were performed on cultures grown under clean conditions the basis of resistance must be genetic (Russell and Morris, 1970). In contrast, copper tolerant isolates of Scenedesmus lost their ability to grow at high copper concentrations after 10 generations, implying acclimation was responsible for tolerance in the field (Stokes, Hutchinson and Krauter, 1973; Stokes and Dreier, 1981).

Annelids have also demonstrated tolerance through both adaptation and acclimation. Populations of the freshwater oligochaete Limnodrilus hoffmeisteri sampled from high cadmium sediment exhibited tolerance to the metal. This tolerance was conferred to second generation offspring raised in a clean environment (Klerks and Levinton, 1987). However, Pesch and Hoffman (1982) successfully acclimated specimens of Neanthes arenaceodentata to survive longer in high copper conditions through pre-exposure to sub-lethal levels.

Nevo, Ben-Shlomo and Lavie (1984) demonstrated adaptation in a mollusc and a crustacean. Mercury selected for specific allozyme genotypes in the gastropod Monodonta turbinata and the shrimp Palaemons elegans. Brown (1976) also found a genetic basis for lead tolerance in the isopod Asellus meridianus, as growth in second generation animals was not inhibited by high lead concentrations.

Other crustaceans can become acclimated to heavy metals. Induction is believed to be responsible for lead tolerance in Asellus aquaticus (Fraser, Parkin and Verspoor, 1978; Fraser, 1980), copper tolerance in the copepod Tisbe holothuriae (Moraitou-Apostolopolou et al, 1983), and lead and chromium tolerance in the crayfish Austropotamobius pallipes (Vareille-Morel and Chaisemartin, 1982).

In their comprehensive review, Klerks and Weis (1987) concluded that only three studies positively showed adaptation to have occurred in aquatic metazoans; Asellus meridianus (Brown, 1976), Limnodrilus hoffmeisteri (Klerks and Levinton,1987) and Monodonta turbinata / Palaemons elegans (Nevo, Ben-Shlomo and Lavie, 1984). Of these, only the latter demonstrates adaptation in marine metazoans. Confirmation that adaptation is responsible for copper and zinc tolerance in N. diversicolor would only be the second such example.

In this chapter, the bases of tolerance were investigated through induction experiments and by testing the progeny of non-specific intra-site matings for tolerance. Specific matings between individuals from various sites were also performed to supplement results and to provide information on the mode of inheritance of tolerance. Similar studies on plants have been useful in determining the dominance and polygenic control of certain metal tolerances (Gartside and McNeilly, 1974; Urquhart, 1971).

MATERIALS AND METHODS

i) INDUCTION EXPERIMENTS

To examine if tolerance to copper could be induced in N. diversicolor, adult worms were exposed to sub-lethal levels of copper for a period of 30 days prior to being challenged with a lethal dose (Pesch and Hoffman, 1982).

Induction: 80 Grimsby and 80 Whitton worms were acclimatized for 1 week in 17.5°/$_{oo}$ salinity water at 12°C. 20 worms from each site were then exposed to copper levels of 0.01, 0.02 and 0.03 mg.l^{-1} $CuSO_4$ in glass crystallizing dishes at 12°C. The remaining 20 worms from each site were kept in clean sea-water as controls. The 17.5°/$_{oo}$ salinity water and copper solution was changed every three days and the pH was maintained at approximately 7.8. The worms were fed on powdered commercial rat food every three days, 24 hours prior to the water change. Dead or damaged worms were removed from the dishes when necessary.

Challenge: After 30 days, 10 worms from each concentration were exposed to a challenge concentration of 0.3 mg.l^{-1} $CuSO_4$ and the other 10 remained in uncontaminated sea-water as controls. The animals were checked daily for response to mechanical stimulation; dead animals were removed and frozen. The water and copper solution were replaced daily. The experiment was replicated.

ii) LABORATORY BREEDING OF WORMS

a) Non-specific matings

Following Bogucki (1962), approximateley 20 ripe adults from the heavily contaminated site R1 (Bryan and Gibbs, 1983), R13 and Whitton were placed in tanks of 17.5°/$_{oo}$ salinity water, containing clean sand to a depth of 50-100 mm, and aerated at 20°C. Within a few weeks young worms (20 mm) could be removed by disturbing the sand and sieving the water through 250 µm mesh.

b) Specific matings

Fertile males could easily be identified. A small amount of coelomic fluid was extracted by puncturing the posterior segments with a fine capillary tube and gently sucking through an aspirator (Smith, 1964). The sperm was mixed with a drop of 17.5°/$_{oo}$ salinity water and viewed at x200 magnification. N. diversicolor spermatozoa appear to have two motile phases in the coelom; when 4 spermatozoa are linked at the head forming a 'tetrad', and after a period of dormancy as individual spermatozoa. Only in the latter motile phase are sperm capable of fertilisation.

The shape and size of oocytes were reasonable indicators of the state of maturity of females. Coelomic samples were taken (as described above) and observed at x50 magnification. Provided the oocytes were spherical, over 200 µm in diameter and contained large fatty dropules typical of mature nereid oocytes (Dales, 1950), there was a good chance of fertilisation (personal observations). These morphological criteria were not ideal indicators, as cleavage was often abnormal and, more often than not, larval development subsequently failed.

Following selection for fertilisation each female was placed on a clean petri dish and washed with 17.5°/$_{oo}$ salinity water. The coelomic cavity was cut open and the released oocytes were pipetted into a small crystallizing dish (60 mm diam. x 30 mm) containing 17.5°/$_{oo}$ salinity water at 12°C and pH 7.6-8.6. The rear segments of the male were punctured and approximately 20 µl of sperm were inoculated into the dish and stirred. The larvae were incubated at 12°C and the water was changed daily.

Under these conditions, larvae developed at the following rate:

Larval stage	Hours after fertilisation
Cleavage	6
Monotrochophore	72
Telotrochophore	120
Muscular contractions	168
Protruding chaetae	192
Eyespots	240
3-segments	264

Larval development does proceed at other combinations of temperature and salinity (Ozoh, 1986) but from personal experience maximal larval yield was attained at low temperature and medium salinity. The pH of the water was the most critical factor in larval development as larvae would not develop correctly if the pH was below 7.0. Reduced pH is known to have detrimental effects on a variety of marine organisms (Knutzen, 1981), including reduced survival and growth of Crassostrea virginica larvae (Calabrese and Davis, 1966).

After 264 hours the larvae were transferred to larger crystallizing dishes (140 mm diam. x 70 mm) containing a thin layer of acid washed sand (250-500 μm) and 17.5$^{o}/_{oo}$ salinity water (pH 7.6-8.6). They were then maintained at room temperature to accelerate growth. The water was continually aerated and the larvae fed on powdered commercial rat food. After a month small worms were visible to the naked eye and they were large enough for toxicity experiments (30 mm) within 3 months.

c) Nomenclature

Each parent used in a specific mating was registered using the following classification scheme; an initial letter (or letters) designating the worm's origin followed by a subscript number.

Origin	Letter	Origin	Letter
Grimsby	G	R1	P
Brough	BR	R6	R
Hessle	H	R13	PP
East Clough	C	R22	PD
Avon	A		

The larvae generated from a cross between, for example, $H_3♂$ and $A_{15}♀$ were labelled H_3A_{15} (male parent first). This form of nomenclature was useful when studying the mode of inheritance of tolerance and paragnath patterns.

d) Toxicity tests

Worms of 10-20 mm (non-specific matings) and 30 mm (specific matings) were used in the toxicity tests. To enable comparisons to be made between laboratory-bred worms and field-derived worms, a parallel set of toxicity experiments were established using similarly sized animals from Whitton and R1. The toxicity tests were conducted as described in the previous chapter.

RESULTS

i) INDUCTION EXPERIMENTS

Two identical experiments were run but the data were treated separately. The LT_{50} values for both sites and experiments are summarised in Table 2.1. No control animals died in either of the studies.

In both experiments, Grimsby and Whitton animals with no pre-exposure to copper survived longer than pre-exposed animals. In experiment one, Whitton worms demonstrated a marked reduction in survival time as the induction concentration increased. No such pattern was observed in experiment two.

The log-transformed data from each experiment were analysed by two-way analysis of variance. In experiment one (Table 2.2), induction concentration was found to significantly affect mortality ($p < 0.01$) as was the interaction of induction concentration and site ($p < 0.05$). When the data was partitioned by site and then tested by one-way analysis of variance (Table 2.3), the significance was apportioned to the effect of induction concentration at Whitton ($p < 0.01$), principally due to the relatively rapid mortality of animals pre-exposed to 0.03 $mg.l^{-1}$.

No significant differences were found in experiment two log-transformed data by two-way analysis of variance ($p > 0.05$. Results not shown).

These experiments demonstrated that pre-exposure concentrations of 0.01 - 0.03 $mg.l^{-1}$ over a period of 30 days failed to induce copper tolerance in *N. diversicolor*. In experiment one, quite the opposite effect was evident as pre-exposed worms from Whitton were more susceptible to the toxic effects of the metal than unexposed worms.

ii) BREEDING EXPERIMENTS

a) Non-specific matings

When conducting non-specific and specific matings with

N. diversicolor a number of assumptions had to be made with respect to the tolerance of the parent worms (which obviously could not be tested). All R1 worms were considered fully tolerant and all Avon/Humber worms were assumed to be non-tolerant. These assumptions were based on the results of the field toxicity tests conducted in the previous chapter. As worms from R6, R13 and R22 exhibited a whole range of tolerances, parental tolerance had to be considered indeterminate.

The relative performances of laboratory-bred and field-derived worms in copper and zinc toxicity tests are summarised in Tables 2.4 and 2.5.

For both metals, the offspring of R1 parents bred in the laboratory were significantly more tolerant than the offspring of Whitton parents. For copper it was by a factor of 10 (one way ANOVA: $F_{1,48} = 460$; $p < 0.0001$) and for a zinc by a factor of 1.4 ($F_{1,58} = 17.4$; $p < 0.001$).

These results, combined with the inability to induce tolerance indicate that both copper and zinc tolerance have large heritable components.

In a separate experiment, the relative performances of F_1 animals from R1 parents and F_1 animals from R13 parents were compared in copper and zinc toxicity tests. No field-derived R13 worms were available for comparison.

Laboratory-bred R13 worms generated a copper LT_{50} of 287 hours (208-366 hours; 95% confidence limits). This value was approximately mid-way between those obtained for R1 and Whitton F_1 worms (c.f. Table 2.4).

In the zinc toxicity tests, F_1 animals from R1 parents produced an LT_{50} value of 241 hours compared with 156 hours for R13 generated worms. The difference between R1 and Whitton zinc LT_{50} values ($\log_e$ transformed) was 0.1566 (S.E. = 0.0266) for the first experiment (Table 2.5). The difference between R1 and R13 $\log_e$ transformed LT_{50} values was 0.1796 (S.E. = 0.018) for the second experiment. Therefore the difference

between R13 and R1 LT_{50} values was greater than the difference between Whitton and R1 LT_{50} values. Given that, and the difference between the two experiments being just 0.023 (the same order of magnitude as the standard error) there is little evidence that R13 F_1 worms were more tolerant than Whitton F_1 worms.

b) Specific matings

Producing large numbers of offspring from specific crosses proved problematical. Some crosses produced over 30 individuals, others solitary animals.

The limited numbers of F_1 worms dictated that the mode of inheritance of tolerance could only be studied for one metal. Copper was selected because of the substantially higher LT_{50} ratio between tolerant and non-tolerant animals demonstrated in the previous section.

Table 2.6 compares values of LT_{50} for 11 specific matings exposed to 0.5 $mg.l^{-1}$ copper. 122 individuals were examined in total.

Most crosses had at least one parent of indeterminate tolerance. Two crosses, H_3A_{15} and G_2G_{11}, were the products of two non-tolerant parents. They generated LT_{50} values of approximately 138-165 hours which were comparable to those produced by similarly sized field-derived worms (Figure 1.1). Two other crosses, P_5BR_2 and GPD_1P_{32} (the male was a product of cross G_1PD_3) were the product of a tolerant and non-tolerant parent. Only 15 worms were examined and they generated LT_{50} values of approximately 210-217 hours. Unfortunately, no worms were successfully bred from a tolerant x tolerant cross for a direct comparison. However, these values are considerably lower than the LT_{50} for the smaller worms generated from the non-specific tolerant x tolerant matings (495 hours. See Table 2.4).

The eleven crosses were found to be significantly different in median lethal times by analysis of variance on log-transformed data ($F_{10,111} = 3.95$; $p < 0.001$). This indicates that some crosses are more

tolerant than others. However, pairwise comparisons showed no two specific matings to be significantly different at the 0.05 level (Scheffe multiple range test).

Only one worm survived the course of the experiment (> 528 hours) and was obviously fully tolerant (from cross GPD_1P_{32}).

DISCUSSION

The failure to induce tolerance in N. diversicolor through preliminary exposure to sub-lethal levels of copper supports the findings of Bryan and Hummerstone (1971). They were unable to induce tolerance in non-tolerant worms through exposure to high-copper sediments from Restronguet Creek for 76 days. The concern expressed by Pesch and Hoffman (1982) that Bryan's challenge concentration (1.0 $mg.l^{-1}$) was too high to distinguish induced changes does not appear valid. The lower challenge concentration used in this study (0.3 $mg.l^{-1}$) also failed to demonstrate successful acclimation. If anything, long-term exposure of non-tolerant N. diversicolor to low copper concentrations may have diminished the animals ability to cope with the toxic effects of the metal. In both induction experiments the control animals fared better than the pre-exposed animals, and in experiment one the performance of Whitton worms was inversely proportional to the induction concentration (Table 2.1).

Two other attempts to induce metal tolerance in annelids have also been unsuccessful. Oshida et al (1976) reported no differences in LC_{50} values for F_1 and F_2 generation Neanthes arenaceodentata raised in hexavalent chromium (0.0125, 0.025 and 0.05 $mg.l^{-1}$). Similarly, Saliba and Ahsanullah (1973) were unable to acclimate the polychaete Ophryotrocha labronica to copper. Only Pesch and Hoffman (1982) have successfully induced metal tolerance in marine worms. Neanthes arenaceodentata pre-exposed to 0.028 $mg.l^{-1}$ copper were significantly more tolerant than control worms or animals pre-exposed to 0.01 and 0.016 $mg.l^{-1}$ copper. Interestingly, the control worms performed better than animals pre-exposed to the two lower concentrations. The authors therefore concluded that a minimum threshold concentration of between 0.028 and 0.016 $mg.l^{-1}$ copper existed, below which no adaptation occurred.

Pesch and Hoffman also speculated whether the mechanism in the induced tolerance may have involved metallothioneins. No metallothioneins have been found in polychaetes as yet but similar proteins are known to be involved in metal-binding in mussels (Noel-Lambot, 1975; Pavicic et al, 1987), oysters (Imber, Thompson and Ward, 1987), limpets (Howard and Nickless, 1974) and teleosts (McCarter and Roch, 1983). Pavicic et al (1987) demonstrated that adult Mytilus galloprovincialis responded to elevated concentrations of cadmium by increasing synthesis of metal-binding proteins. Similarly, McCarter and Roch (1983) found that levels of metallothionein in salmon were highly correlated with 168 hour LC_{50} values for copper. If inducible tolerance is usually based to some extent on increased production of metallothionein/metal-binding proteins, it may explain why copper tolerance could not be induced in N. diversicolor. X-ray microprobe analysis demonstrated that copper (and zinc) in the cytosol fraction was predominantly associated with low molecular weight components but not metallothionein (Pirie, Liu Fayi and George, 1985). The authors concluded that "tolerance is probably due to increased deposition of copper in tertiary lysosomes and not the presence of a significant concentration of cytosolic metallothionein".

The offspring generated by R1 parents through non-specific matings demonstrated that both copper and zinc tolerance have significant heritable components. The only other examples of adaptation to heavy metals in marine metazoans are allozyme genotype selection by mercury in the gastropod Monodonta turbinata and the shrimp Palaemons elegans (Nevo, Ben-Shlomo and Lavie, 1984). Adaptation has been shown to occur in two fresh-water species. Brown (1976) determined a genetic basis for lead tolerance in the isopod Asellus meridianus and cadmium/nickel tolerance in the oligochaete Limnodrilus hoffmeisteri was also found to be genetically determined (Klerks and Levinton, 1987).

The latter two studies confirmed that tolerance was retained in the F_2 generation and therefore maternal effects (e.g. egg cytoplasmic transmission) were not responsible for their inheritance. Levinton (1980) emphasised the importance of testing the second generation, "otherwise the conceivably different environmental and trophic conditions experienced by the parent can influence physiological performance of the offspring". Bayne, Gabbott and Widdows (1975) demonstrated such maternal effects in Mytilus edulis as the offspring of starved parents grew more slowly than mussels spawned from fed parents. Unfortunately, limited time and the difficulties of artificial breeding prevented the production of F_2 animals in this work. However, the worms tested were reasonably large (20 mm) and it is unlikely maternal effects would persist in animals of that size. Additional evidence for negligible maternal effects were provided by specific matings (see later).

For both metals, median survival times in laboratory-bred animals were lower than those for the corresponding field-derived animals. For copper this may in part be a size-related effect. Both the non-tolerant and tolerant field-derived animals did not perform as well as the larger animals (30 mm) used to produce Figure 1.1. For zinc there were no size related effects as the smaller animals produced similar LT_{50} values to those in Figure 1.2. The reduction in tolerance was possibly due to a relaxation of selection pressure when culturing worms in clean sediment for one generation. Lack of predators, abundant food and limited competition may have enabled less fit individuals to survive for use in the toxicity tests (Klerks and Levinton, 1987). There was a similar decrease in tolerance in F_2 Limnodrilus hoffmeisteri (Klerks and Levinton, 1987) but not in F_2 Asellus meridianus (Brown, 1976).

The performance of laboratory-bred F_1 animals from R13 parents conformed to the expectations suggested by copper and zinc tolerance distribution in Restronguet Creek. There was no detectable zinc tolerance at R13 (Figure 1.2) and as expected there were no zinc tolerant F_1 animals. Copper tolerance at R13 was found to be intermediate in value both in the field (Figure 1.1) and in worms bred in the laboratory. A visual examination of mortality in the copper toxicity tests suggests that R13 F_1 deaths were equally distributed throughout the course of the experiment (i.e. there was no apparent modality in deaths). Non-specific matings of R13 adults therefore produce the whole range of copper tolerances, from non-tolerant worms to fully tolerant worms surviving the course of the experiment. There is no indication that selection acts strongly against the low tolerance genotypes at R13 as the whole tolerance range was also found in worms collected from the field.

Specific matings between parents of known tolerance are useful in determining the mode of inheritance of genetically determined tolerance. Most such studies have been conducted on metal tolerance in higher plants. Gartside and McNeilly (1974) demonstrated that zinc tolerance in Agrostis tenuis was under polygenic control as was lead tolerance in Festuca ovina (Urquhart, 1971; Wilkins, 1960). The dominance of the traits appeared to be variable and probably depended on complex interactions between loci (Macnair, 1979). Specific matings have also determined that copper resistance in the protozoan Paramecium aurelia was due to a single recessive allele (Nyberg, 1975).

Despite generating approximately 150 individuals from eleven different crosses, little can be deduced of the mode of inheritance of copper tolerance in N. diversicolor. The main problems encountered were lack of suitable crosses (e.g. tolerant x tolerant) and the limited number of worms generated by some matings.

The absence of significant tolerance from tolerant x non-tolerant crosses indicates that copper tolerance is probably determined by a recessive allele (or alleles) at one or more loci and not by a dominant allele at a single locus. The production of a solitary fully tolerant animal from cross GPD_1P_{32} would appear to support this hypothesis. Its presence could be explained by assuming tolerance is determined by a single locus, the recessive allele for tolerance (t) on the paternal side having come from the R22 (PD) 'grandmother'. This would not show in the next generation (Cross G_1PD_3 = (Tt) non-tolerant. Table 2.6) but the allele must have then segregated with the tolerant allele (t) from the R1 mother (P_{32}) to produce a fully tolerant animal (tt). If this genetic system was responsible for tolerance it requires that all individuals are either fully tolerant (tt) or completely non-tolerant (Tt or TT). The cline in copper tolerance would then have to be explained through each population containing varying ratios of tolerant and non-tolerant worms. As mentioned earlier, there was no apparent bimodal mortality in worms from R22, R13 or R6 as there were intermediate tolerances. It therefore seems very likely copper tolerance is actually under polygenic control.

Considerably more crosses and animals would be required to accurately determine the mode of inheritance. Unfortunately N. diversicolor is not a particularly amenable animal for use in genetic studies, as it has a biased sex-ratio, is monotelic, has differing male and female development rates and differing breeding times in different populations (c.f. Smith, 1964).

The specific matings were useful, however, for finally eliminating maternal effects as a factor in copper tolerance. Although one animal from the GDP_1P_{32} cross was fully tolerant its sibs all perished in the same LT_{50} range as non-tolerant x non-tolerant worms. The tolerant mother therefore failed to confer any detectable tolerance to the remaining offspring.

To summarise the important points of this chapter with respect to the biomonitoring value of tolerance distribution:

i) Copper tolerance cannot be induced over an individual's lifetime.

ii) There are significant heritable components in both copper and zinc tolerance which satisfies Luoma point (i).

Table 2.1

LT_{50} in hours for Grimsby and Whitton worms challenged by 0.3 $mg.l^{-1}$ copper. This followed a 30 day induction period exposed to 0.00, 0.01, 0.02 and 0.03 $mg.l^{-1}$ copper.

a) Experiment one; b) Experiment two.

		INDUCTION CONCENTRATIONS ($mg.l^{-1}$)			
SITE OF ORIGIN		0.00	0.01	0.02	0.03
a)	GRIMSBY	226	170	213	186
	WHITTON	229	196	185	123
b)	GRIMSBY	233	218	219	226
	WHITTON	233	226	209	206

Table 2.2

Two-way analysis of variance of log-transformed times to death from experiment one.

a)

SOURCE OF VARIATION	DF	SS	MS	F-RATIO	
SITE	1	0.1927	0.1927	1.97	NS P>0.05
CONCENTRATION	3	1.7345	0.5782	5.90	** P<0.01
INTERACTION	3	0.8779	0.2926	2.99	* p<0.05
ERROR	72	7.0529	0.0980		
TOTAL	79	9.8580			

Table 2.3

One-way analysis of variance of log-transformed times to death from experiment one by site: a) Whitton b) Grimsby.

a)

SOURCE OF VARIATON	DF	SS	MS	F-RATIO
CONCENTRATION	3	2.112	0.7037	7.09 ** P<0.01
ERROR	36	3.5748	0.0993	
TOTAL	39	5.6861		

b)

SOURCE OF VARIATION	DF	SS	MS	F-RATIO
CONCENTRATION	3	0.5011	0.1670	1.73 NS P>0.05
ERROR	36	3.4780	0.0966	
TOTAL	39	3.9792		

Table 2.4

LT_{50} in hours (and 95% confidence limits) for laboratory-bred and field-derived worms in 0.5 $mg.l^{-1}$ copper.

* Non-tolerant laboratory-bred worms were offspring of Whitton animals. Field-derived worms were from the Avon.

	NON-TOLERANT*	R1
LABORATORY-BRED	47 (39-58)	495 (339-723)
FIELD-DERIVED	70 (56-87)	1407 (1029-1922)

Table 2.5

LT_{50} in hours (and 95% confidence limits) for laboratory-bred and field-derived worms in 40 $mg.l^{-1}$ zinc.

* Non-tolerant laboratory-bred worms were offspring of Whitton animals. Field-derived worms were from the Avon

	NON-TOLERANT*	R1
LABORATORY-BRED	133 (119-148)	190 (165-219)
FIELD-DERIVED	156 (142-170)	282 (259-308)

Table 2.6

LT_{50} in hours for offspring of specific crosses exposed to 0.5 $mg.l^{-1}$ copper.

CROSS	NUMBER EXAMINED	LT_{50}	95% CONFIDENCE LIMITS
G_1PD_3	20	165	149-183
R_1G_4	20	157	144-172
G_2G_{11}	10	138	115-165
R_2C_5	4	207	136-314
R_2C_4	2	180	-
R_2H_{11}	1	207	-
PP_3A_{15}	10	140	122-159
PP_4C_4	10	155	130-185
H_3A_{15}	30	165	148-184
P_5BR_2	5	210	157-281
GPD_1P_{32}	10	227	169-305

CHAPTER 3

THE MAINTENANCE OF TOLERANCE CLINES

INTRODUCTION

The previous chapters have shown copper and zinc tolerances in N. diversicolor to have heritable components, and have demonstrated the existence of clines in the magnitude of tolerance down the length of Restronguet Creek. Tolerance clines are also found in populations of metal resistant plants and these appear to be maintained through a balance between the opposing forces of selection and gene flow (Jain and Bradshaw, 1966; McNeilly, 1968).

McNeilly (1968) demonstrated that clines of copper tolerance in Agrostis tenuis were maintained despite the extensive diluting effects of high gene flow. Natural selection was found to be responsible for the differentiation. On toxic soils, selection strongly favoured tolerant genotypes as non-tolerant plants were incapable of surviving the high metal levels. On normal soils there was weak selection for non-tolerant plants, which were found to be competitively superior in interference experiments. Similar properties have been reported in other plant species, including Anthoxanthum odoratum and Plantago lanceolata (Cook, Lefebvre and McNeilly, 1972).

Chapter 3 will attempt to determine whether the tolerance clines found in Restronguet Creek are also maintained through natural selection overriding migration. To this end, both gene flow and the selective benefit/cost of tolerance will be investigated.

Unfortunately, population genetics has been largely untouched in polychaetes and there is very little information available on gene flow in this class (Nevo, 1978). Drift tube studies have been used to estimate the degree of larval dispersion of the intertidal spionid polychaete Pseudopolydora paucibranchiata both within and between bays (Levin, 1983). The results indicated that most larvae were retained within a bay through sluggish surface water movements. Those larvae that

did escape to the open sea could be transported large distances (150 km) during their two week pelagic period. There is, therefore, potential for limited gene-flow between populations in isolated bays through larval exchange. Rice and Simon (1980) have studied gene flow in Polydora ligni through allele frequency analysis and found considerable genetic differentiation within a large bay area (populations were over 8 miles apart). Preliminary work by Mustaquim (1988a) indicates that similar studies are possible with N. diversicolor. Six énzyme systems (esterase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, leucine aminopeptidase, malate dehygrogenase and phosphoglucose isomerase) were examined electrophoretically for polymorphisms. Esterase demonstrated considerable variation but the sample sizes were too small for the banding patterns to be readily explained by a genetic system. The other enzyme systems all appeared to be monomorphic.

There is morphological and perhaps physiological evidence that separate races of N. diversicolor may exist. Smith (1964) demonstrated that 'salinity bottlenecks' in cleavage and early development varied with location in the Baltic. At Tvarminne (Finland), the optimal salinities for larval development were 5-15$^{o}/_{oo}$ compared with 9-27$^{o}/_{oo}$ at Kristineberg (Sweden). There is, however, no genetic evidence that these are 'physiological races'. They may purely be populations conditioned by different environmental pressures during the individuals life-time.

A variety of workers have studied the number of paragnaths (chitinous denticles arranged in groups on the proboscis) on worms taken from various locations (Barnes, 1978; Barnes and Head, 1977; Muus, 1967a; Muus, 1967b; Khlebovich, Komendantov and Yakovishina, 1984). The numbers of paragnaths per group varied from site to site showing no obvious correlations with either sediment type or salinity (Barnes, 1978).

In this chapter, both paragnath variation and allele frequency variation were investigated as a means of determining the degree of gene flow between populations. If it could be demonstrated that paragnath numbers are genetically determined, the interpopulation differences in paragnath numbers would support the idea of localised races and therefore restricted gene flow between populations. Similarly, differences in allele frequencies between populations would demonstrate relative genetic isolation provided the polymorphisms studied are selectively neutral (Ferguson, 1980). A common method for quantifying the degree of genetic divergence between two populations is through the statistics of Nei (1972). Pairwise comparisons are made between the mean genetic identity (*I*) and mean genetic distances (*D*) for each population. The nearer *I* approaches 1.0 or *D* approaches zero, the more closely related the populations. Alternatively, Slatkin (1985) has proposed a method of directly estimating the degree of gene flow between populations through studying the spatial distribution of rare alleles. The greater the estimate of *Nm* (average number of migrants exchanged between local populations) calculated from the average frequency of private alleles, the greater the gene flow. Both approaches require that a large number of loci are sampled for accurate estimates of mean genetic distance (*D*) and *Nm* (Nei and Roychoudhury, 1974; Slatkin, 1985).

The contribution of natural selection to the distribution of tolerance will also be considered. Work in this thesis (Chapter 1) and by Bryan and Hummerstone (1971) has shown that highly contaminated sediments strongly select against non-tolerant genotypes. Competition experiments would determine whether tolerant animals are, like tolerant plants, competitively inferior in uncontaminated conditions. Interference experiments have been performed between N. diversicolor, N. virens and

N. succinea in the laboratory (Kristensen, 1988) but similar experiments have not been performed between genotypes of one species of worm. Confirmation that there is a cost associated with tolerance in N. diversicolor would not only partially explain its distribution but would also satisfy Luoma point (iii) that resistance lowers the overall fitness of the population.

MATERIALS AND METHODS

i) PARAGNATH PATTERNS

a) Populations

Between 17 and 64 preserved worms from each of the following sites were examined; Grimsby, Hessle, Brough, Whitton, R1, R6, R13, R22, Avon and F10. Immersion in formalin/ethanol normally causes the worm to die with the proboscis everted. If the proboscis was retracted, pressure was exerted with forceps 5 or 6 segments behind the head to evert the proboscis. Under x10 - x50 magnification, the numbers of paragnaths in each group were counted, scar-marks indicating recent loss of paragnaths were scored as if the paragnath was present. Designation of groups follows that of Barnes and Head (1977) (Figure 3.A).

b) Specific matings

Worms produced from specific matings were examined for paragnath patterns following death through narcotization in 95% ethanol or after toxicity experiments (both copper and zinc poisoning cause proboscis evertion).

There is strong bilateral symmetry in groups II, IV and VI (Barnes and Head, 1977). In view of this, only the left-hand groups were counted.

c) Experiments with sediment

The influence of sediment type on paragnath pattern was examined. Three-setigerous larvae from cross H_4G_{35} were divided between identical crystallising dishes, one containing the 63 μm fraction of Hessle silt rendered abiotic by heating at 100°C, the other containing acid washed sand with grain sizes >250 μm. As a control, larvae from cross A_7A_{26} were divided between three dishes of sand. All dishes contained 17.5°/$_{oo}$ salinity water, were aerated and incubated at room temperature. The worms were fed on powdered commercial rat food and paragnaths were counted after 4 months when the worms were 20-30 mm long.

ii) ELECTROPHORESIS

Preliminary work was carried out at the School of Biological Sciences, University College, Swansea. The intention of this was to find as many enzyme systems in N. diversicolor that exhibited protein polymorphism as possible. Enzyme systems screened were; arginine phosphokinase (APK), creatine kinase (CK), esterase (EST), esterase-D (EST-D), hexokinase (HK), isocitrate dehydrogenase (IDH), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), malic enzyme (ME), peptidase (PEP), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PGDH), phosphoglucose isomerase (PGI) and superoxide dismutase (SOD). These screening experiments were all conducted on horizontal starch gels. Following this work and a review of recent literature, two polymorphic enzyme systems were considered suitable for information on population genetics; lactate dehydrogenase (LDH) and esterase (EST). Detailed study of these loci were carried out at Hull using horizontal polyacrylamide gel electrophoresis.

STARCH GEL ELECTROPHORESIS

a) Gel preparation

11.7% starch gels were prepared 24 hours in advance of use. 27 g of hydrolysed starch (Connaught) was mixed with 230 ml of diluted gel buffer (Table A.1, Appendix) in a 500 ml conical flask. The flask was heated with an erratic action until the solution became stiff and translucent. After degassing with a vacuum pump for 30 seconds the starch was poured evenly over a clean glass plate within a perspex frame (155 mm x 185 mm x 5 mm internal dimensions). A second glass plate was placed on top of the frame, taking care that no air bubbles were formed.

b) Tissue preparation and gel loading

Tissue samples from 9 worms were tested for polymorphic enzyme systems; 3 from Grimsby, 3 from Barton and 3 from Whitton. The

posterior 4-5 segments were removed from large animals (approximately 10 from small animals), washed in two drops of distilled water and macerated with a glass rod in microfuge tubes. The samples were spun for 5 minutes at high speed in a microfuge. Filter paper sample wicks (Whatman No.3, 6 mm x 3 mm) were placed in the tubes and allowed to soak for approximately 30 minutes. The tubes were stored on ice at 4°C. In their electrophoretic studies of N. diversicolor, Mustaquim (1988a) macerated whole worms and Jones (1970) homogenised the anterior segments, including the prostomium. It would be advantageous, however, to use the posterior segments as animals can then be non-lethally genotyped and still be available for crossing experiments. As there were no apparent differences in LDH banding patterns between anterior, mid- and posterior sections of worms (personal observations), all tissue samples were prepared from the posterior segments.

A glass plate was carefully removed from the gel mould and a slit was cut along the length of the gel, forming a stub approximately 20 mm wide. The wicks were removed from the tubes, blotted dry and placed vertically in the slit with forceps. The gel was placed in the electrophoresis tank (Shandon) with the sample wicks at the cathode. The electric field was applied by lint wicks pre-soaked in electrode buffer (Table A.1, Appendix). The gel was covered with cling-film to avoid dehydration and an ice-box was placed on top of the gel to minimise heating effects.

c) Electrophoresis and staining

A Heath-Schlumberger power-pack applied a constant current of 60 mA for Tris/citric acid/EDTA and Tris/citric acid buffers, and 50 mA for boric acid/NaOH buffer (Table A.1, Appendix). The gels were run for approximately 4 hours at 4°C.

The gels were sliced with a gel slicer into 3 slabs and stained. The stains were made up fresh on the day and the staining procedures are described in the Appendix.

POLYACRYLAMIDE GEL ELECTROPHORESIS

a) Gel preparation

6.3% polyacrylamide gels were prepared the day before use. 10 ml of acrylamide solution (6.1 g acrylamide, 0.2 g N,N'-methylene-bis-acrylamide, 25 ml distilled water) was added to 5 ml of electrode buffer (Table A.2, Appendix), 5 ml of 0.28% TEMED, and 20 ml of 0.14% ammonium persulphate. The solution was mixed thoroughly and poured into the gel mould.

The mould was constructed from 4 strips of perspex (2 x 150 mm x 10 mm x 1.5 mm and 2 x 100 mm x 10 mm x 1.5 mm) bonded to a perspex plate with paraffin jelly. All surfaces had been washed thoroughly and cleaned with 75% ethanol. After the acrylamide had been poured into the mould, a clean glass plate was carefully placed on top ensuring no air bubbles were formed. The gel was left to polymerise at room temperature for 2 hours.

The perspex plate was lifted from the mould leaving the gel preferentially bound to the glass plate. The gel was covered in cling-film to avoid dehydration and stored at 4°C.

b) Tissue preparation and gel loading

Tissue preparation and gel loading were as previously described for starch gel electrophoresis. The filter paper wicks were inserted horizontally into the slit, allowing approximately 20 samples to be loaded onto one gel. Approximately 100 worms were examined from each of the following sites; Grimsby, Brough, Hessle, R1, R4, R6, R13, R22, F10 and Avon.

c) Electrophoresis and staining

A purpose-built flat-bed electrophoresis tank was used in conjunction with a Shandon Vokam 2761 power-pack. A constant current of 60 mA was applied for Tris/citric acid buffer (LDH) and 16 mA for Tris/glycine buffer (EST). The gels were run for 45 minutes at 4°C. The staining procedures are described in the Appendix. Staining reactions were stopped by rinsing the gels in distilled water, they were then illuminated on a light table and scored. Gels were photographed for permanent records.

iii) COMPETITION EXPERIMENTS

The relative fitness of tolerant and non-tolerant worms were studied using resource competition experiments in laboratory tanks. The resources examined were food and space. The quantity of food in a tank remained constant but the experimental densities were varied. Consequently resources in the form of food and available space per worm decreased with increasing densities. Relative performance was measured by the change in wet weight for each genotype.

75 worms from R1 and Avon were acclimated for one week in 17.5°/$_{oo}$ salinity water at 12°C. Only young healthy animals were selected for the experiments. 5 similarly sized animals from each site were blotted dry, weighed and the total initial wet weights recorded.

The 10 worms were placed in a glass crystallising dish (140 mm diameter x 70 mm) containing 25 mm of builder's sand and 17.5°/$_{oo}$ salinity water. This produced a density of 650 m^{-2}. The same procedure followed for 20, 30, 40 and 50 worms producing densities of 1300 m^{-2}, 1950 m^{-2}, 2600 m^{-2} and 3250 m^{-2}.

The tanks were aerated and maintained at 12°C. Every 7 days 300 mg of pre-soaked powdered commercial rat food was added to each tank.

Dead or dying worms and unattached tails were removed daily to ensure they were not ingested. The number of worms removed from each tank was recorded.

After 30 days the worms were carefully sieved out and allowed to recover in 17.5$^{o}/_{oo}$ salinity water for 2 days. The total wet weight for each tank was then determined after blotting the worms dry.

To distinguish the Avon and R1 worms, the animals were subjected to a 48 hour copper toxicity test (0.5 $mg.l^{-1}$). The exposure was sufficiently long to stress the non-tolerant animals without causing death or tissue damage. Once the contents of each dish had been identified the tolerant and non-tolerant components were reweighed separately.

The total weight for each dish decreased after the exposure to copper, principally due to the evacuation of gut contents, metabolic processes and presumably metal induced osmotic effects. To establish the relative contribution of each genotype to the total weight lost, a second toxicity test was run. 10 acclimated worms from both sites were weighed and exposed to 0.5 $mg.l^{-1}$ copper for 48 hours. The worms were reweighed and the proportion of the total weight loss contributed by the two components was used as a correction factor for the weight lost in the competition experiment.

RESULTS

i) PARAGNATH VARIATION

a) Interpopulation variation

Site means for all paragnath groups are shown in Table 3.1. Standard deviations and ranges are given in Table 3.2. The means and ranges are broadly similar to values previously reported (Barnes and Head, 1977; Muus, 1967a; Smith, 1958).

When calculated over all nine sites, analysis of variance (Table 3.3) showed significant differences between sites for all six paragnath groups ($P<0.001$).

Interpopulation variation within an estuary was similarly determined (Table 3.3). In Restronguet Creek, the four sites studied were significantly different in groups IV, VI, VII-VIII ($P<0.05$) and III ($P<0.001$). No differences were detected in groups I and II. The high significance in group III was primarily due to low paragnath numbers at site R6 and high numbers at R1. In general, the highest numbers of paragnaths were found at the least saline site (R1) but otherwise there were no obvious patterns between numbers and position on the estuary. In the Humber, the three sites studied were sigificantly different in groups VII-VIII ($P<0.01$), II, III and IV ($P<0.001$). No differences were found in groups I and VI. The high significance in groups II, III and IV were mainly due to very low paragnath numbers in the Grimsby population. Numbers at Brough and Whitton were remarkably similar apart from group VII-VIII.

b) Inter-family variation

Offspring from ten specific matings were tested for differentiation for each paragnath group by analysis of variance (Tables 3.4-3.9). The ten families were found to be significantly different in

groups I ($P<0.01$), II, III, IV and VII-VIII ($P<0.001$). Only group VI was found not to be significantly different (Table 3.8).

This considerable differentiation between families grown under identical environmental conditions implies that there must be a significant heritable component amongst the factors determining paragnath patterns.

c) Heritability of paragnath pattern

Heritability is a way of quantifying the degree of resemblance between relatives under given environmental conditions. The heritability of paragnath numbers was estimated through regression of offspring numbers on mid-parental numbers. A regression coefficient significantly greater than zero indicates genetic variation and is a direct estimate of the heritability (Falconer, 1960). The mean values of the offspring were weighted according to the number of offspring in each family (Kempthorne and Tandon, 1953).

Regression plots for each paragnath group are given in Figures 3.1-3.6 and the regression analyses are summarised in Table 3.10.

The plots clearly show that there is considerable intra-family variation for all groups. Groups II, III, IV and VII-VIII all had highly significant regression coefficients (with standard errors) of 0.507 ($\pm$0.089), 0.323 ($\pm$0.090), 0.768 ($\pm$0.128) and 0.476 ($\pm$0.099) respectively ($P<0.001$). This indicates a significant genetic component in the determination of paragnath numbers in these groups. The regression coefficients for groups I and VI were not significantly different from zero. However, numbers of paragnaths in these groups are rather few so there is a particularly limited spread of numbers along the x-axis (mid-parental number) compared with the other groups (see below).

GROUP	RANGE OF MID-PARENTAL NUMBER	X-AXIS SPREAD
I	1-4	3
VI	3-5.5	2.5
II	8-16	8
III	22-35	13
IV	11-20	9
VII-VIII	33-44	11

The limited spread of mid-parental values for these two groups, coupled with the large intra-family variation may well have masked any heritable component.

d) The effect of sediment type on paragnath patterns

The influence of sediment type on paragnath numbers was examined in a small pilot study. Table 3.11 demonstrates that 20 worms from cross H_4G_{35} grown in silt did not differ significantly from 20 sibs grown in sand with respect to paragnath numbers. In the control experiment, sibs from cross A_7A_{26} grown in three tanks of sand under identical conditions also did not differ significantly in groups I, III, IV, VI and VII-VIII. Surprisingly, the three tanks were significantly different for group II ($P<0.001$). This may be an effect of small sample size and large intra-family variation. Only 9 worms could be examined from one dish and 5 from each of the other two dishes.

ii) ALLELE FREQUENCY VARIATION

a) Screening experiments

The results of the screening experiments are summarised in Table 3.12.

Three polymorphic systems were detected; PEP, LDH and EST. The other enzyme systems appeared to be either monomorphic, unreadable (due to blurred or smeared bands) or demonstrated no activity.

On the basis of these results it was decided to concentrate on LDH and EST.

b) Banding characteristics of LDH and EST

The relative mobilities of the principal bands generated on LDH and EST gels are shown in Figure 3.7. In each case the most frequently observed and reliably staining band was nominally assigned the relative mobility (m_r) of 100.

LDH: LDH bands m_r = 100, 94 and 88 were the product of one locus designated LDH_1. The product of the fast (F) allele (subunit A) migrated at m_r = 100 and the slow allele (subunit B) at m_r = 88. The heterozygote (FS) produced a three-banded pattern (Plate 3.1). This is unusual as LDH is a tetrameric protein and the heterozygote usually produces a five-banded pattern with the following subunit compositions; AAAA; AAAB; AABB; ABBB and BBBB (Ferguson, 1980). As the three bands are evenly spaced the two missing bands must correspond to the asymmetric heteropolymers AAAB and ABBB. Similar three-banded patterns have been generated by LDH zymograms from the alewife, Alosa pseudoharengus (Shaklee, 1975), and the leopard frog, Rana pipiens (Goldberg and Wuntch, 1967). In the former case, the absence of the asymmetric heteropolymers was explained through differential instability under certain pH conditions. In the latter case, non-random association of subunits was considered to be responsible. Conceivably, either process may have been occurring in N. diversicolor tissue extracts.

Bands m_r = 61,67,73,78 and 83 were the product of locus LDH_2. The heterozygote produced the expected five-banded pattern (Plate 3.2). Homozygous 'slow' (SS) individuals produced a two-banded pattern corresponding to bands m_r = 61 and 67 (Plate 3.2). Homozygous 'fast' (FF)

individuals were also characterised by a two-banded pattern but surprisingly they corresponded to bands m_r = 73 and 78, and not the faster band m_r = 83 (Plate 3.2). The disappearance of band m_r = 83 and the presence of twin bands for the homozygotes was probably a result of post-translational modification. A variety of processes can cause such changes including denaturation, deamination, phosphorylation, sulphation, oxidation, reduction, aggregation and polypeptide cleavage (Ferguson, 1980). Residual ammonium persulphate in acrylamide gels has been found to split bands (Brewer, 1967) and cause their disappearance (Fantes and Furminger, 1967). Ammonium persulphate was used in this study as an acrylamide polmerisation catalyst and may have been responsible for the unexpected banding patterns obtained. Ideally, riboflavin and light should have been used for gel polymerisation (Brewer, 1967).

Regardless of the gel artefacts mentioned, the observed LDH banding patterns differed markedly from those reported by Jones (1970) and Mustaquim (1988a). The former found one major band and two minor bands, the latter found only a single band. The part of the body sampled and the reproductive state of the animal was probably not critical in the reported variability (c.f. Mustaquim, 1988a). Homogenates of anterior, posterior and mid-body segments from individuals were found to produce identical banding patterns. Similarly, oocytes generated identical bands to the somatic tissue from which they were removed (Personal observations). The use of different buffer systems was the most likely cause for the observed differences. Jones and Mustaquim used a Tris/glycine buffer (pH 8.3-8.5) whereas Tris/citric acid buffer (pH 6.9) was used in this study. Neither worker examined many animals so the low frequency LDH polymorphisms were probably missed.

The genetic basis for the banding configurations were not derived through progeny studies. Without direct evidence from specific matings these bases must remain hypothetical. The genetic interpretations

advanced, however, are supported by the close agreements between the observed proportions of phenotypes and those expected on the basis of the Hardy-Weinberg equilibrium (Selander et al., 1970).

Bands m_r = 104 and 108 stained unreliably. Some samples produced a three-banded pattern (m_r = 100, 104 and 108) which led to speculation that m_r = 108 was the product of a third allele (F*) in locus LDH_1 and that the pattern observed corresponded to the heterozygote FF*. However, despite a reasonably high frequency of the 'F* allele' in many populations, no F*F* homozygotes or F*S heterozygotes were found. In addition, the same samples often produced a strong band at m_r = 104 and no band at m_r = 108 when run on a different gel (Plate 3.2). The third allele hypothesis was rejected when an SS individual was found with a band at m_r = 104. Further research would probably identify bands m_r = 104 and 108 as the products of a third locus or as gel artefacts. For the purpose of this study they were discounted.

EST: EST gels stained inconsistently. Stain intensity varied considerably from individual to individual (Plate 3.3) and also between samples from the same individual. Often the gels displayed zero activity for all samples.

Bands m_r = 100,125 and 130 stained most intensely with α-naphthyl acetate as the substrate, bands m_r = 80 and 94 with β-naphthyl acetate as the substrate. This probably indicates that at least two loci are involved. Although there appeared to be genetic variation in the 'β-naphthyl acetate' locus, the unsatisfactory staining and resolution of the gels made scoring impossible.

The EST zymograms generated in this study bore little resemblance to those reported by Mustaquim (1988a). He ran horse-heart myoglobin (Type III, Sigma) and horse-spleen ferritin (Type I, Sigma) as internal standards (Mustaquim, 1988b) and gave the myoglobin band the

nominal mobility of *100*. His EST enzyme mobilities were measured with reference to this, the fastest band migrating at a mobility of 138. When the same standard was run on gels in this study the bands migrated at mobilities of between *170* (m_r = 80) and *370* (m_r = 135). No significant bands were detected in the mobility range *110-140*. Presumably Mustaquim failed to detect these faster bands because they migrated off the gels on the longer runs he employed (over 3 hours compared with 45 minutes) (Mustaquim, 1988b).

c) Interpopulation variation

The allele frequencies for the two LDH loci at the sites investigated are displayed in Table 3.13.

LDH_1 was fixed for the fast allele at six of the ten sites. The slow allele was found at very low frequencies at Grimsby, Hessle and R13 but was more common at F10. LDH_2 was fixed for the fast allele at four of the sites. The slow allele was present at low frequencies at all Restronguet Creek sites and at very low frequencies at Hessle.

All populations showed no significant departure from Hardy-Weinberg equilibrium (G-test; $P>0.05$).

Interpopulation variation in allele frequencies within an estuary was examined for both the Humber and Restronguet Creek through G-tests for heterogeneity (Table 3.14).

The Humber was essentially homogeneous for both loci. This is not surprising as there was little genetic variation at the three sites and the rarer alleles were at extremely low frequencies.

Restronguet Creek was homogeneous for LDH_2. All five sites had the slow allele at a frequency of approximately 0.1-0.2 (Table 3.13). Significant heterogeneity ($P<0.001$) was found for LDH_1. Sites R1, R4, R6 and R22 were fixed for the fast allele but the slow allele was present at R13 at very low levels (Table 3.13).

These results would indicate that there is some differentiation within Restronguet Creek.

Tests for variation in allele frequencies between estuaries are summarised in Table 3.15. Data from the Humber sites were pooled as the populations were not significantly different at both loci. As the Restronguet Creek sites were heterogeneous for LDH_1, similar pooling could not be justified. R13 is therefore considered separately in the analyses for this locus.

LDH_1: F10 was significantly different from all other estuaries ($P<0.001$). The considerable differentiation was due to the relatively high frequency of the slow allele (Table 3.13). Site R13 differed significantly from the Humber ($P<0.05$), Avon ($P<0.01$) and the pooled Restronguet Creek sites ($P<0.001$). The Avon, Humber and Restronguet Creek were not significantly different.

LDH_2: Restronguet Creek was significantly different ($P<0.001$) from the other three estuaries due to the relatively high frequency of the slow allele. There was no apparent difference between the Humber, Avon and F10.

These results demonstrate considerable differentiation between estuaries. Studying LDH allele frequencies would not enable a distinction to be made between the Humber and Avon, but F10 and Restronguet Creek are highly differentiated with respect to each other and the other two estuaries. There is also some evidence for limited differentiation within Restronguet Creek.

iii) COMPETITION EXPERIMENTS

19 worms were removed from the dishes during the experiment because they sustained damage. The number removed may have been density related (see below).

Density (m^{-2})	Number of animals removed (and percentage of total)
650	0 (0%)
1300	3 (15%)
1950	3 (10%)
2600	5 (12.5%)
3250	8 (16%)

The majority of the animals removed had suffered damage to the proboscis and were unable to burrow. This sort of damage is consistent with that inflicted by intra-specific aggression (Personal observations). One worm from the 2600 m^{-2} dish and two from the 3250 m^{-2} dish were unrecovered on completion of the experiment and were probably eaten. A total of 8 Avon and 14 R1 animals were either removed or unrecovered.

Weight loss during the exposure to copper was the same for all five treatments at approximately 22% ($\pm$2%) of pre-exposure wet weight. One could not assume that the weight loss was equally shared between the tolerant and non-tolerant animals as the Avon animals were more stressed. A control experiment demonstrated that Avon worms lost 17.9% of their initial wet weight and R1 worms lost 10.3% during identical exposures. This enabled a correction factor (CR) to be calculated for estimating the contribution of each genotype to the total weight loss:

For example;

$$CR_{Avon} = \frac{17.9}{17.9 + 10.3} = 0.634$$

0.634 x total weight lost = weight lost from Avon animals during copper exposure

Similarly;

$$CR_{R1} = \frac{10.3}{17.9 + 10.3} = 0.366$$

Using the corrected weights, the mean weight change and the % weight change were calculated for each genotype at all densities (Table 3.16).

The growth rates were highest at the lowest densities. The mean percentage weight change was +34.9% at 650 worms m^{-2} and 33.1% at 1300 worms m^{-2}. As density increased, the mean growth rate decreased from +13.4% at 1950 worms m^{-2} to zero at 2600 worms m^{-2}. This is explained by increasing numbers of worms competing for the same limited food supply. Suprisingly, growth continued at the highest density. This was probably a result of the surviving worms supplementing their intake by consuming the two unrecovered worms.

Non-tolerant animals performed better than tolerant animals at the three lowest densities. There were particularly marked differences in growth rates at 650 worms m^{-2} and 1950 worms m^{-2}. At higher densities (2600 worms m^{-2} and above) this advantage appears to have been lost. Performance was not related to the initial mean size of the animals. At three of the densities (1300, 1950 and 3250 worms m^{-2}) the smaller animals had higher weight increases per unit initial weight.

DISCUSSION

Variation in paragnath numbers in all groups (Table 3.3) appear to indicate differentiated populations. Similar variations have been determined in populations from Danish estuaries (Muus, 1967a, 1967b), British estuaries including the Severn (Barnes and Head, 1977; Barnes, 1978) and a number of sites in the Soviet Union (Khlebovich, Komendantov and Yakovishina, 1984). The differences can be quite marked and have prompted speculation that N. diversicolor exists in isolated races or entities (Muus, 1967b).

Although a genetic component has been found in determining paragnath numbers in groups II, III, IV and VII-VIII, it is still unclear whether population variation in these groups is due to limited gene flow or through varying natural selection at each site. Barnes (1978) considered whether there was a correlation between paragnath numbers and sediment type or approximate habitat salinity. No relationship with sediment was found but there was some indication that paragnath numbers in groups II and VII-VIII were highest at the sites nearest the mouth of the Severn. A similar decrease in numbers with decreasing salinity was noted by Muus (1967b) and Vignocchi (1981). Barnes suggested that this fitted a general pattern in estuaries as, "[a] reduction in the number of the units comprising meristically-varying characters on penetration of dilute media is a well attested phenomenon in the estuarine fauna (Remane and Schlieper, 1971)".

Paragnath variation in this work does not support this statement. In the Humber, the lowest paragnath numbers in each group were generally found at Grimsby, the most saline site (Table 3.1). In Restronguet Creek, the highest numbers of paragnaths were generally found at R1, the least saline site. There is, therefore, no evidence that salinity selects for particular paragnath patterns. Similarly, the laboratory sediment

experiments conducted in this work support Barnes' field experiments indicating sediment type also does not select for paragnath patterns.

Despite these results, it would seem a little premature to state that paragnath numbers are selectively neutral and variation between populations is due to genetic drift (i.e. limited gene flow) (Barnes, 1978). Other obvious environmental factors such as temperature or primary food type have not been considered and may prove to be important selective agents. Primary food type varies considerably from site to site. At R1 and the Avon the principal source of nutrition is probably decaying leaf-litter. At Grimsby, it is probably rich organics from a nearby sewage outlet. N. diversicolor is known to feed by ingesting surface layers of sediment, grasping large particles in its jaws and by filter-feeding (Bryan and Hummerstone, 1973). Paragnaths are probably involved to a different extent in each feeding mechanism. The relative frequency each mechanism is used at a site will depend on the food-source, hence there is potential for selection for different paragnath patterns. The factors determining paragnath variation remain obscure and consequently little emphasis should be placed on them for assessing the extent of gene flow between populations.

Allele frequency analysis has been shown to be a useful tool for estimating gene flow (e.g. Todd, Havenhand and Thorpe, 1988). In the absence of genotype-dependant selection, significant differences in allele frequencies between sites can demonstrate limited genetic interchange. Lactate dehydrogenase (LDH) allele frequency distributions in populations of N. diversicolor clearly demonstrate considerable differentiation between estuaries. There is strong evidence that insignificant genetic interchange occurs between estuaries separated by just 2 kilometers (Restronguet Creek and Mylor Creek). This finding is not particularly surprising for two reasons:

i) N. diversicolor larvae are demersal and therefore subject to bottom water currents rather than surface currents. In a coastal-plain type of estuary there is a net downstream movement of less saline water flowing out along the surface and a non-tidal upstream drift of more dense saline water along the bottom (Pritchard, 1955). The resulting effect is to cause the retention of larvae within the estuary (Scheltema, 1975). At low spring tides Restronguet Creek drains to a fresh-water channel so there is a possibility of larval escape during these periods.

ii) Any larvae or adults that escaped the estuary would have to negotiate a stretch of marine water. Although adults and larvae are able to endure fully saline water (Bogucki, 1953; Smith, 1964), the marine environment is extremely hostile. Apart from having to cope with the extra osmotic stress, there are also numerous predators (e.g. teleosts) in the water column and aggressive competitors in the benthos (Kristensen, 1988).

LDH allele frequency distribution also indicates little variation between populations within an estuary. Both the Humber and Restronguet Creek were essentially homogeneous, with the exception of site R13 for LDH_1. One cannot, therefore, exclude the possibility that extensive gene flow does occur within much of an estuary. Observations by Davey and George (1986a,b) on larval recruitment of transplanted sediments indicate that N. diversicolor larvae are probably tidally dispersed. As Dankers and Binsbergen (1984) demonstrated that adult worms migrate from one site in an estuary to another, there is a strong probability that extensive genetic interchange between populations does occur, and continues through all life-history stages.

There must be an element of caution when making statements regarding population differentiation from allele frequency data. There is always a risk the observed distributions could be a result of genotype

dependant selection and not genetic drift (Ferguson, 1980). Murdock, Ferguson and Seed (1975) demonstrated how allele frequency variation in a leucine aminopeptidase (LAP) locus in Mytilus edulis was determined by the degree of wave action at a site. Similarly, it was considered that the LDH allele frequences observed in Restronguet Creek populations could be explained through the selective forces of metal contamination. The relatively high frequency of the slow (S) allele in LDH_2 at all five sites led to speculation that it may have been selected for by copper contamination. This was shown not to be the case when all three genotypes (SS, FS and FF) were tested for copper tolerance and there was no significant difference in mortality (tested in 1.0 $mg.l^{-1}$ $CuSO_4$. Oneway ANOVA: $F_{2,20} = 0.22$; $P > 0.05$).

Environmental factors other than metal contamination may be selecting for particular LDH genotyopes. Different alleles at the LDH-B locus in Fundulus heterclitus result in different responses of the genotypes to environmental oxygen levels. LDH-B^aB^a embryos were found to hatch before LDH-B^bB^b while heterozygote (LDH-B^aB^b) hatching was intermediate (DiMichele and Powers, 1982). Obviously different environmental oxygen levels between sites could result in different LDH-B allelic frequencies. As LDH plays a critical role in N. diversicolor anaerobic metabolism (D-lactate is the main end product of glycogen break-down; Schottler, 1978), different aerobic conditions at sites could conceivably cause selection for different genotypes. If this were true, there must be considerable differences in the degree of anaerobiosis between estuaries but not within estuaries to explain the distributions of LDH allele frequencies. Considering the wide range of sediment characteristics, organic contents and exposure times at the different sites within Restronguet Creek or the Humber, 'homogeneity of anaerobiosis' within an estuary seems very unlikely.

The absence of genetic heterogeneity within estuaries must not be interpreted as proof of genetic interchange. Only differences between populations can be detected by electrophoresis, not similarities (Ferguson, 1980). LDH allele frequency distribution therefore only fails to exclude the possibility of extensive gene flow. If other enzyme systems had been examined it is possible they would have demonstrated genetic differentiation within the estuaries. Ideally, a large number of enzyme systems should be examined for this sort of study. Not only would it increase the chances of detecting population variation, but it would also reduce the risks of genotype dependant selection and enable genetic distances (Nei, 1972) and direct estimates of gene flow (Slatkin, 1985) to be calculated. Unfortunately only 3 enzymes out of 15 systems screened were found to be polymorphic, and only one of these could be scored. This made such calculations impossible as both estimates of mean genetic distance (D) and average number of migrants exchanged (Nm) require that a large number of loci are sampled (Nei and Roychoudhury, 1974; Slatkin, 1985). The nine worms used for the screening experiments were all from the Humber (but from three different sites). With the benefit of hindsight it may have been better to screen worms from other estuaries (e.g. Restronguet Creek) as they may have been more heterogeneous.

A review of relevant literature has shown that estimates of gene flow between populations of polychaetes are very scarce. Levin (1983) used surface drift tubes to establish that genetic interchange between populations of Pseudopolydora paucibranchiata in isolated bays hundreds of kilometers apart was possible through larval exchange. Rice and Simon (1980) studied gene flow between populations of Polydora ligni in a large bay area through allele frequency analysis. They established the existence of a genetically distinct population in close proximity to other bay populations (approximately 8-10 km apart). Finally, Crisp and

Ekaratne (1984) studied interlocality variations in allele frequencies for populations of Pomatoceros lamarckii and Pomatoceros triqueter in North West Europe. Populations were found to be differentiated with no readily interpretable geographic or environmental trends in allele frequencies. Only the former two cases considered gene flow in a relatively enclosed area of water (i.e. large bays) but direct comparisons cannot be made with gene flow in N. diversicolor because of the very different modes of larval development. Both P. paucibranchiata and P. ligni have larvae with pelagic phases, whereas N. diversicolor has demersal larvae. A further source of gene flow exists for N. diversicolor, as adults are not sessile and can migrate within an estuary (Dankers and Binsbergen,1984). P. paucibranchiata and P. ligni adults are, however, relatively sedentary.

The results of the competition experiment suggest non-tolerant animals have a selective advantage in uncontaminated conditions when resources are relatively plentiful. This advantage appears to be lost when resources are more limited. It is not readily apparent whether the superior performance of non-tolerant animals (as determined by increase in wet weight) was a result of higher dietary intake, through competitive ability, or through superior physiological mechanisms for converting food to tissue. An examination of worms removed during the course of the experiment (due to death or being moribund) showed a high incidence of damage consistent with that obtained by competitive interactions. The epidermis and epithelium of the proboscis was usually split and often one jaw was missing. These observations combined with the biased ratio of damaged animals removed from the experiment (8 non-tolerant: 14 tolerant) suggests there is an element of superior competitive ability in the relative success of non-tolerant worms. Intra-specific aggressions between individual N. diversicolor have been studied (Evans, 1973) but

not between particular genotypes. Little proboscis eversion, wounding and no killing was reported but the experiments were short duration and the animals had not undergone significant periods of starvation (Evans, 1973). Long-term inter-specific aggression experiments between N. diversicolor, Nereis virens and Nereis succinea have been studied by Kristensen (1988) but not long-term intra-specific experiments.

It should be emphasised that the competition experiment needs repeating for these results to be confirmed. Ideally, control experiments should be run simultaneously in the form of tanks containing just tolerant or non-tolerant worms at the same experimental densities. These control experiments would finally enable a distinction to be made between increased aggression and better assimilative capacity in determining the superior performance of non-tolerant worms.

The competition experiment indicates that there may be a cost associated with tolerance. Heavy metal tolerances in plants are also associated with reduced competitive ability in uncontaminated conditions. Zinc/lead tolerant specimens of Agrostis tenius, Anthoxanthum odoratum and Plantago lanceolata produced lower dry weights and fewer tillers or leaves than non-tolerant speciments when grown in competition (Cook, Lefebvre and McNeilly, 1972). Similar results were obtained for copper tolerant ecotypes of Agrostis tenuis (McNeilly, 1968).

Klerks and Levinton (1987) speculated whether there was a cost associated with cadmium/nickel tolerance in the freshwater oligochaete Limnodrilus hoffmeisteri. No competition experiments were performed but tolerant F_2 worms were slightly less resistant than field-collected tolerant worms (the difference was not significant). They considered that the possible reduction in tolerance may have been due to relaxation of selection pressure when culturing worms for two generations in clean sediment. This reduction in fitness may imply a cost associated with increased resistance. There is, however, no strong evidence for this as

the reduction could also be explained through an environmental component in the resistance.

When the results of the allele frequency distribution study and competition experiment are considered together, a conceptual model can be developed to explain how tolerance clines in Restronguet Creek may be maintained.

Although gene flow between estuaries is negligible, there is little evidence to contradict the theory generated by studies of larval and adult movement (Davey and George, 1986a,b; Dankers and Binsbergen, 1984) that considerable gene flow exists between populations of N. diversicolor within an estuary. As the copper and zinc tolerance clines have been shown to be stable over the short-term (2-3 years. Personal observations) they must be maintained despite the 'swamping' effects of this gene flow. Endler (1973) demonstrated how extensive gene flow may be unimportant in the differentation of populations along an environmental gradient. He established clines in the frequency of an eye mutation in artificial populations of Drosophila melanogaster and subjected them to artificial selection and gene flow. The clines were stable after 35 generations despite gene flow being as high as 40%.

The balance between the opposing forces of natural selection and gene flow have been shown to be responsible for maintaining tolerance clines in higher plants (Jain and Bradshaw, 1966; McNeilly, 1968). Strong selection on contaminated soils selected for tolerant plants and weak selection on normal soils selected for non-tolerant plants through superior performance in competition. The clines were fuelled by considerable gene flow in the form of wind-driven pollen. A similar mechanism could explain the maintenance of the cline in copper tolerance in Restronguet Creek. Considerable genetic interchange occurs through adult migration and tidal dispersal of larvae but highly metalliferous

sediments strongly select for tolerant genotypes and relatively uncontaminated sediments may weakly select for the more competitive non-tolerant genotypes. The same mechanism could also explain the maintenance of the disjunct cline in zinc tolerance as "[i]t is not necessary for a sharp spatial environmental change to be present for distinct differentiation to occur". (Endler, 1973). However, there would also have to be a cost associated with zinc tolerances.

To sum up:

i) Allele frequency analysis demonstrated restricted gene flow between populations of N. diversicolor in different estuaries. Similar analyses could not rule out the possibility of extensive gene flow within estuaries.

ii) There is an indication that copper tolerant animals are competitively inferior in uncontaminated conditions (i.e. there may be a cost associated with tolerance). This would satisfy Luoma point (iii).

iii) Tolerance clines in Restronguet Creek may therefore be maintained through a balance between the opposing forces of natural selection and gene flow.

Table 3.1

Site means of paragnath numbers. Groups II, IV and VI are mean values of left and right sides.

SITE	NO. WORMS EXAMINED	MEAN NUMBERS IN EACH GROUP					
		I	II	III	IV	VI	VII-VIII
HUMBER	SITES						
GRIMSBY	60	1.33	9.31	21.22	12.46	4.30	34.62
HESSLE	59	1.32	11.33	26.56	17.10	4.53	34.19
BROUGH	45	1.53	11.10	26.49	17.20	4.58	31.27
RESTRONGUET CREEK SITES							
R1	60	1.77	13.25	30.98	19.03	5.12	41.07
R6	24	1.63	14.04	20.25	16.69	4.15	36.63
R13	59	1.71	14.40	23.68	19.01	4.59	37.46
R22	17	1.53	11.82	25.82	17.35	4.24	42.00
OTHER SOUTH WEST SITES							
AVON	64	2.06	13.77	29.66	22.07	4.21	33.53
F10	53	2.55	17.13	28.64	25.11	5.38	30.91

Table 3.2

Standard deviation and range (beneath) of paragnath numbers in each group for each site.

SITE	NO. WORMS EXAMINED	STANDARD DEVIATION & RANGE FOR EACH GROUP					
		I	II	III	IV	VI	VII-VIII
HUMBER	SITES						
GRIMSBY	60	0.629 0-3	2.124 4-16	5.152 11-33	3.024 7-24	0.884 1-8	5.952 26-57
HESSLE	59	0.655 0-3	2.771 4-19	4.534 12-37	3.632 6-25	1.226 2-8	5.764 21-53
BROUGH	45	0.661 1-3	2.428 6-18	4.832 15-37	3.754 9-27	1.133 1-7	4.131 22-39
RESTRONGUET CREEK SITES							
R1	60	0.998 1-5	2.478 8-22	7.089 15-46	3.861 9-30	1.424 1-9	8.366 22-60
R6	24	0.824 1-4	2.698 9-21	4.533 12-32	3.082 11-25	1.058 2-7	6.513 22-47
R13	59	0.892 1-4	3.079 7-24	5.655 14-35	3.899 10-31	1.254 1-8	7.509 17-53
R22	17	0.875 1-4	2.772 6-18	5.514 17-37	3.762 11-26	1.147 1-8	7.450 28-56
OTHER SOUTH WEST SITES							
AVON	64	0.906 1-5	2.204 1-21	4.906 22-41	3.742 13-37	0.925 0-7	5.061 20-43
F10	53	1.084 1-5	2.739 10-31	4.860 15-43	3.365 16-43	1.282 1-10	6.365 17-47

Table 3.3

Analysis of variance of paragnath numbers for Restronguet Creek sites, Humber sites and all sites.

NS = not significant
* = P<0.05
** = P<0.01
*** = P<0.001

	RESTRONGUET CK		
GROUP	F-RATIO	DF	SIG.
I	0.3545	3,156	NS
II	2.1745	3,156	NS
III	23.3904	3,156	***
IV	2.9292	3,156	*
VI	3.5655	3,156	*
VII-VIII	3.8194	3,156	*

	HUMBER		
GROUP	F-RATIO	DF	SIG.
I	1.6527	2,161	NS
II	10.2946	2,161	***
III	22.8326	2,161	***
IV	32.6160	2,161	***
VI	1.2791	2,161	NS
VII-VIII	5.5170	2,161	**

	ALL SITES		
GROUP	F-RATIO	DF	SIG.
I	11.1273	8,432	***
II	34.4104	8,432	***
III	22.6772	8,432	***
IV	44.9960	8,432	***
VI	4.8466	8,432	***
VII-VIII	15.6094	8,432	***

Table 3.4

Analysis of variance between families for numbers of paragnaths in Group I.

CROSS	NO. EXAMINED	MEAN	SD
G_1PD_3	22	2.3636	1.0486
G_2G_{11}	18	1.8333	1.0981
PP_3A_{15}	6	1.5000	0.5477
H_3A_{15}	24	1.2500	0.4423
PP_4C_4	6	1.3333	0.5164
GPD_1P_{32}	10	1.6000	0.6992
P_5BR_2	6	1.1667	0.4082
A_7A_{26}	19	1.6316	0.5973
H_4G_{35}	20	1.8500	1.1821
H_4G_{45}	15	1.6667	0.7237

SOURCE OF VARIATION	DF	SS	MS	F-RATIO
CROSS	9	18.278	2.031	2.86 ** P<0.01
ERROR	136	96.462	0.709	
TOTAL	145	114.74		

Table 3.5

Analysis of variance between families for numbers of paragnaths in Group II.

CROSS	NO. EXAMINED	MEAN	SD
G_1PD_3	21	12.048	2.012
G_2G_{11}	19	8.684	3.698
PP_3A_{15}	6	10.833	2.137
H_3A_{15}	24	10.083	1.501
PP_4C_4	6	10.167	0.983
GPD_1P_{32}	10	11.200	1.751
P_5BR_2	6	9.167	3.125
A_7A_{26}	19	15.526	3.221
H_4G_{35}	20	11.350	2.059
H_4G_{45}	15	11.200	2.597

SOURCE OF VARIATION	DF	SS	MS	F-RATIO
CROSS	9	552.95	61.44	9.89***P<0.001
ERROR	136	844.68	6.21	
TOTAL	145	1397.62		

Table 3.6

Analysis of variance between families for numbers of paragnaths in Group III.

CROSS	NO. EXAMINED	MEAN	SD
G_1PD_3	22	21.818	3.660
G_2G_{11}	18	23.167	3.485
PP_3A_{15}	6	20.667	3.724
H_3A_{15}	24	18.125	3.097
PP_4C_4	6	17.333	3.933
GPD_1P_{32}	10	21.600	2.633
P_5BR_2	6	21.833	0.753
A_7A_{26}	19	26.474	4.611
H_4G_{35}	20	19.700	4.497
H_4G_{45}	15	23.000	1.890

SOURCE OF VARIATION	DF	SS	MS	F-RATIO
CROSS	9	1002.6	111.4	8.72 *** P<0.001
ERROR	136	1737.2	12.8	
TOTAL	145	2739.8		

Table 3.7

Analysis of variance between families for numbers of paragnaths in Group IV.

CROSS	NO. EXAMINED	MEAN	SD
G_1PD_3	22	16.591	4.159
G_2G_{11}	18	9.444	2.617
PP_3A_{15}	6	17.333	3.077
H_3A_{15}	24	15.917	3.078
PP_4C_4	6	13.833	2.714
GPD_1P_{32}	10	14.900	2.514
P_5BR_2	6	17.667	3.327
A_7A_{26}	19	22.000	4.619
H_4G_{35}	20	13.650	3.200
H_4G_{45}	15	18.267	2.463

SOURCE OF VARIATION	DF	SS	MS	F-RATIO
CROSS	9	1719.5	191.1	16.68 *** P<0.001
ERROR	136	1557.5	11.5	
TOTAL	145	3277.0		

Table 3.8

Analysis of variance between families for numbers of paragnaths in Group VI.

CROSS	NO. EXAMINED	MEAN	SD
G_1PD_3	21	4.762	1.446
G_2G_{11}	19	4.947	1.649
PP_3A_{15}	5	4.600	1.140
H_3A_{15}	24	4.667	1.007
PP_4C_4	6	4.667	0.516
GPD_1P_{32}	10	4.300	1.337
P_5BR_2	6	5.333	0.816
A_7A_{26}	19	5.211	0.976
H_4G_{35}	20	5.750	1.517
H_4G_{45}	15	5.200	1.146

SOURCE OF VARIATION	DF	SS	MS	F-RATIO
CROSS	9	23.63	2.63	1.62 NS P>0.05
ERROR	135	219.36	1.62	
TOTAL	144	242.99		

Table 3.9

Analysis of variance between families for numbers of paragnaths in Group VII-VIII.

CROSS	NO. EXAMINED	MEAN	SD
G_1PD_3	22	32.727	4.672
G_2G_{11}	19	32.263	4.840
PP_3A_{15}	4	29.500	2.380
H_3A_{15}	25	32.840	3.197
PP_4C_4	6	32.167	1.941
GPD_1P_{32}	10	32.400	2.366
P_5BR_2	6	30.500	2.429
A_7A_{26}	19	37.789	3.881
H_4G_{35}	20	36.300	3.230
H_4G_{45}	15	35.267	2.865

SOURCE OF VARIATION	DF	SS	MS	F-RATIO
CROSS	9	727.1	80.8	6.02 *** P<0.001
ERROR	136	1825.4	13.4	
TOTAL	145	2552.5		

Table 3.10

Summary of offspring on mid-parent regressions for each paragnath group.

NS = not significant
*** = P<0.001
M = mean parental value
F_1 = mean offspring value

GROUP	REGRESSION EQUATION	SE	t-RATIO	DF	SIG.
I	F_1=1.85-0.104.M	0.087	-1.19	151	NS
II	F_1=5.47+0.507.M	0.089	5.71	151	***
III	F_1=13.0+0.323.M	0.090	3.60	151	***
IV	F_1=3.27+0.768.M	0.128	5.99	151	***
VI	F_1=5.02-0.018.M	0.136	-0.13	148	NS
VII-VIII	F_1=15.9+0.476.M	0.099	4.80	151	***

Table 3.11

Analysis of variance of paragnath numbers for worms grown in different sediments.

NS = not significant
*** = P<0.001.

	SAND/SILT			SAND/SAND/SAND		
GROUP	F-RATIO	DF	SIG.	F-RATIO	DF	SIG.
I	1.30	1,38	NS	2.70	2,16	NS
II	1.31	1,38	NS	13.31	2,16	***
III	1.20	1,38	NS	3.47	2,16	NS
IV	2.13	1,38	NS	3.49	2,16	NS
VII	0.02	1,38	NS	1.53	2,16	NS
VII-VIII	4.04	1,38	NS	1.95	2,16	NS

Table 3.12

Summary of screening experiments for enzyme activity.

ENZYME SYSTEM	ACTIVITY
APK	NO ACTIVITY
CK	UNREADABLE
EST	POLYMORPHIC
EST-D	NO ACTIVITY
HK	MONOMORPHIC
IDH	MONOMORPHIC
LDH	POLYMORPHIC
LAP	NO ACTIVITY
MDH	MONOMORPHIC
ME	UNREADABLE
PEP	POLYMORPHIC
PGM	MONOMORPHIC
6PGDH	NO ACTIVITY
PGI	MONOMORPHIC
SOD	MONOMORPHIC

Table 3.13

Allele frequencies for the two LDH loci at each of the 10 sites.

n = number of alleles.

	LDH_1			LDH_2		
SITE	FAST	SLOW	n	FAST	SLOW	n
HUMBER SITES						
GRIMSBY	0.991	0.009	234	1.000	0.000	234
HESSLE	0.995	0.005	192	0.995	0.005	192
BROUGH	1.000	0.000	200	1.000	0.000	200
RESTRONGUET CREEK						
R1	1.000	0.000	196	0.862	0.138	196
R4	1.000	0.000	138	0.797	0.203	138
R6	1.000	0.000	200	0.885	0.115	200
R13	0.971	0.029	204	0.887	0.113	204
R22	1.000	0.000	160	0.825	0.175	160
OTHER SOUTH WEST SITES						
AVON	1.000	0.000	202	1.000	0.000	202
F10	0.865	0.135	200	1.000	0.000	200

Table 3.14

G-tests of heterogeneity of LDH allele frequencies between sampling sites within the Humber and Restronguet Creek.

NS = not significant
*** = P<0.001

	LOCUS					
	LDH_1			LDH_2		
ESTUARY	G-VALUE	DF	SIG.	G-VALUE	DF	SIG.
HUMBER	2.489	2	NS	2.367	2	NS
RESTRONGUET CREEK	17.923	4	***	7.937	4	NS

Table 3.15

Matrices of G-tests of heterogeneity between estuaries. R13 is considered separately from the other Restronguet Creek sites for LDH_1.

NS = not significant
* = P<0.05
** = P<0.01
*** = P<0.001
H = Humber
R = Restronguet Creek
R^+ = Restronguet Creek - R13
A = Avon
F = F10

LDH_1

H				
R+	NS			
R13	*	***		
F	***	***	***	
A	NS	NS	**	***
	H	R+	R13	F

LDH_2

H			
R	***		
F	NS	***	
A	NS	***	NS
	H	R	F

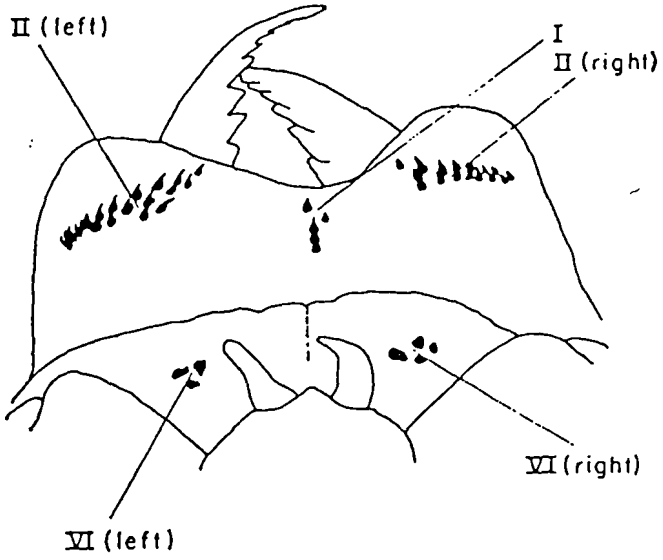
Table 3.16

Relative performances of Avon and R1 worms in competition experiments as determined by changes in wet weight.

		DENSITY (m^{-2})				
	SITE OF ORIGIN	650	1300	1950	2600	3250
INITIAL MEAN WEIGHT (g)	AVON	0.212	0.116	0.135	0.197	0.181
	R1	0.174	0.126	0.166	0.215	0.174
CORRECTED FINAL MEAN WEIGHT (g)	AVON	0.304	0.156	0.163	0.197	0.193
	R1	0.220	0.166	0.176	0.215	0.194
MEAN WEIGHT CHANGE (g)	AVON	+0.092	0.040	0.028	0.000	0.012
	R1	+0.046	0.040	0.010	0.000	0.020
WEIGHT CHANGE AS % INITIAL MEAN WEIGHT	AVON	+43.4%	+34.5%	+20.7%	0%	+6.6%
	R1	+26.4%	+31.7%	+6.0%	0%	+11.5%

Figure 3.A

Diagram of N. diversicolor everted proboscis showing positions of paragnath groups on the dorsal surface (top) and ventral surface (bottom). (Adapted from Barnes and Head, 1977).


II (left)
I
II (right)
VI (right)
VI (left)

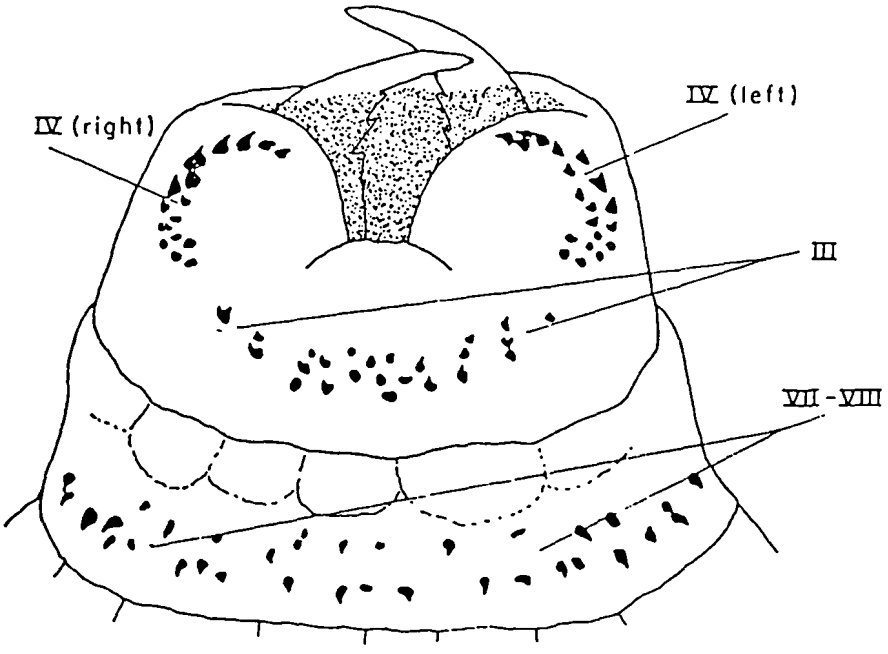

IV (left)
IV (right)
III
VII - VIII

Figure 3.1

Scatter diagram of offspring values against mean parental values for group I paragnath numbers.

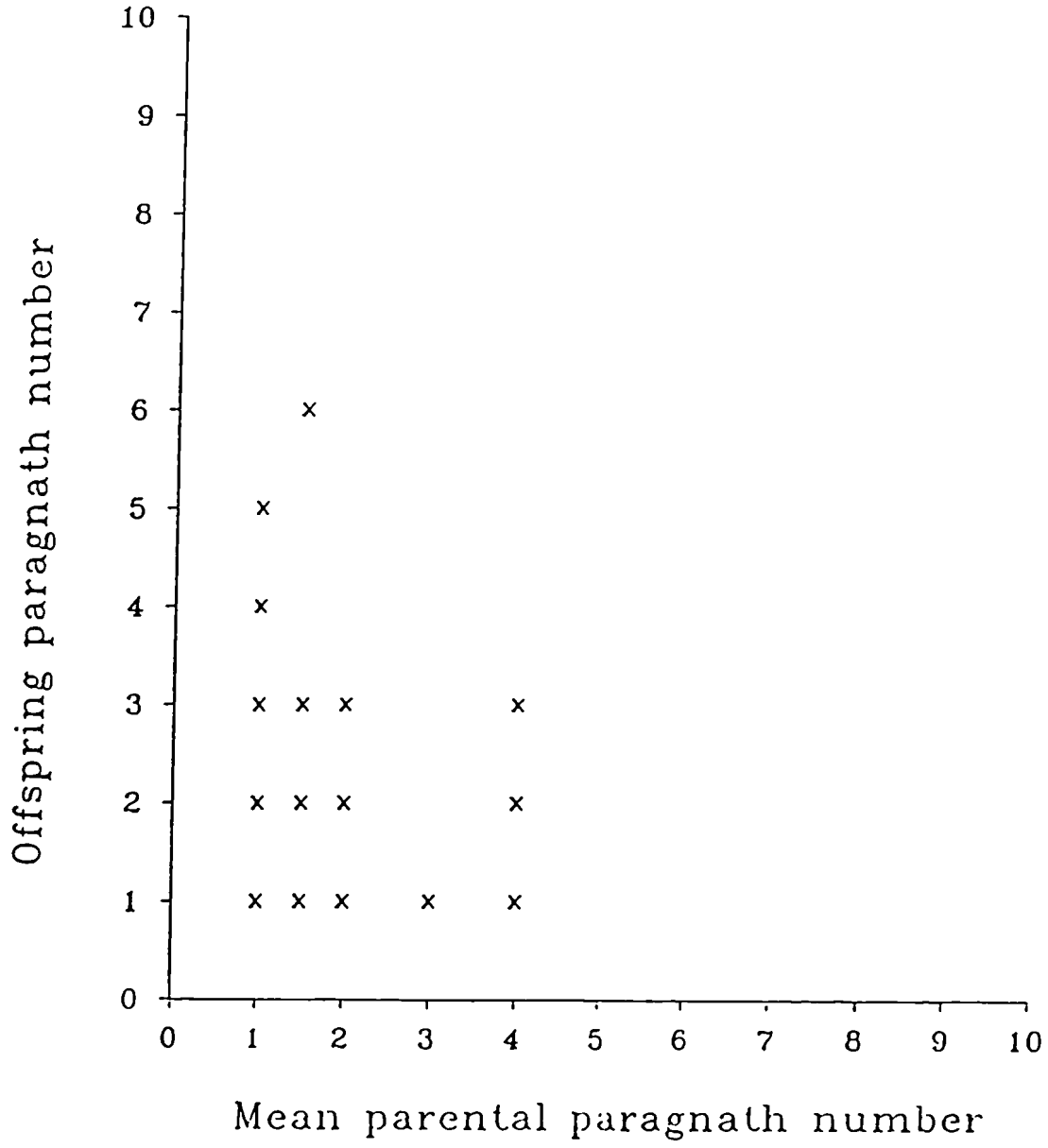

Offspring paragnath number
Mean parental paragnath number
0
1
2
3
4
5
6
7
8
9
10

Figure 3.2

Scatter diagram and regression of offspring values on mean parental values for group II paragnath numbers.

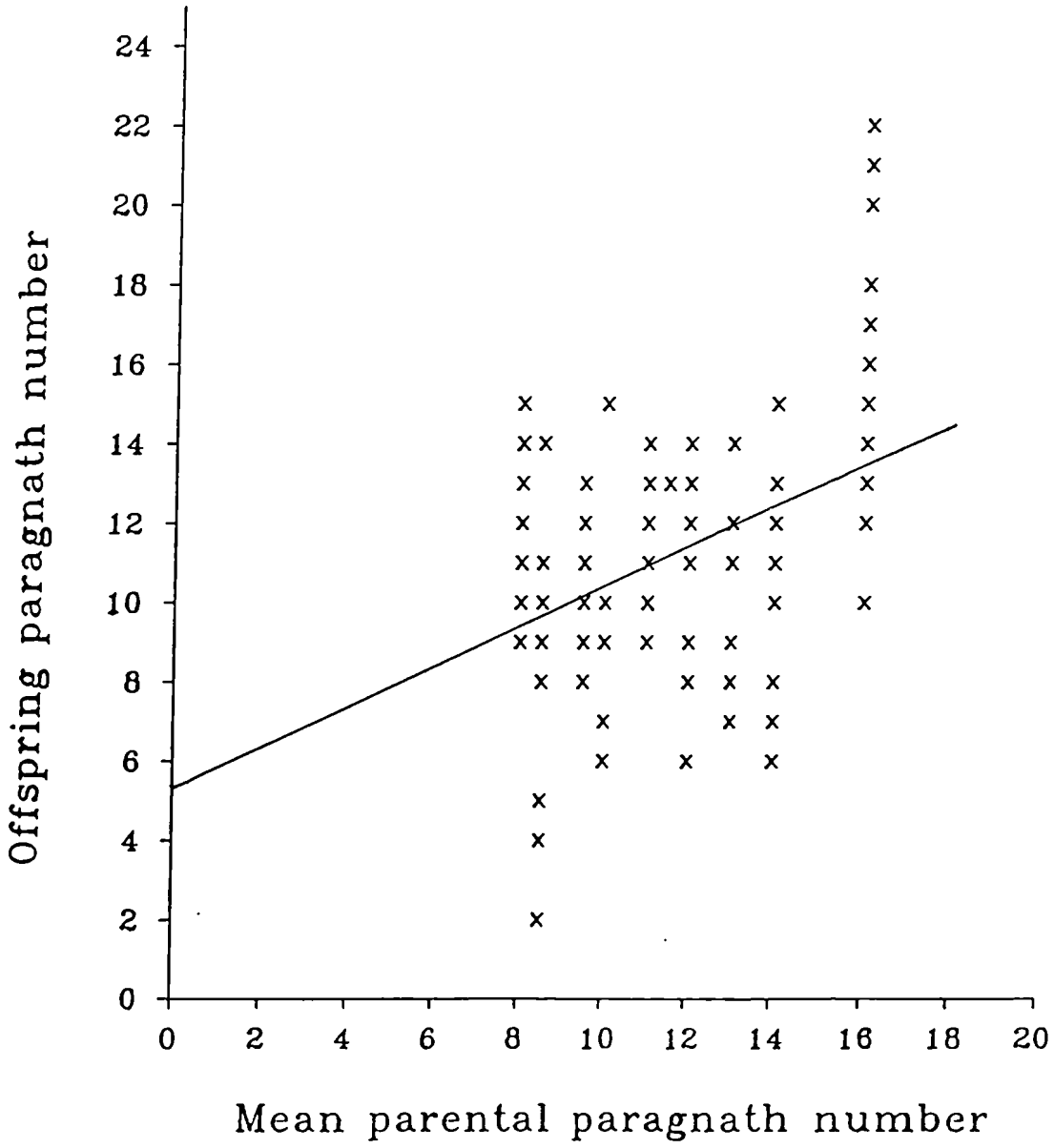

Offspring paragnath number
24
22
20
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
18
20
Mean parental paragnath number

Figure 3.3

Scatter diagram and regression of offspring values on mean parental values for group III paragnath numbers.

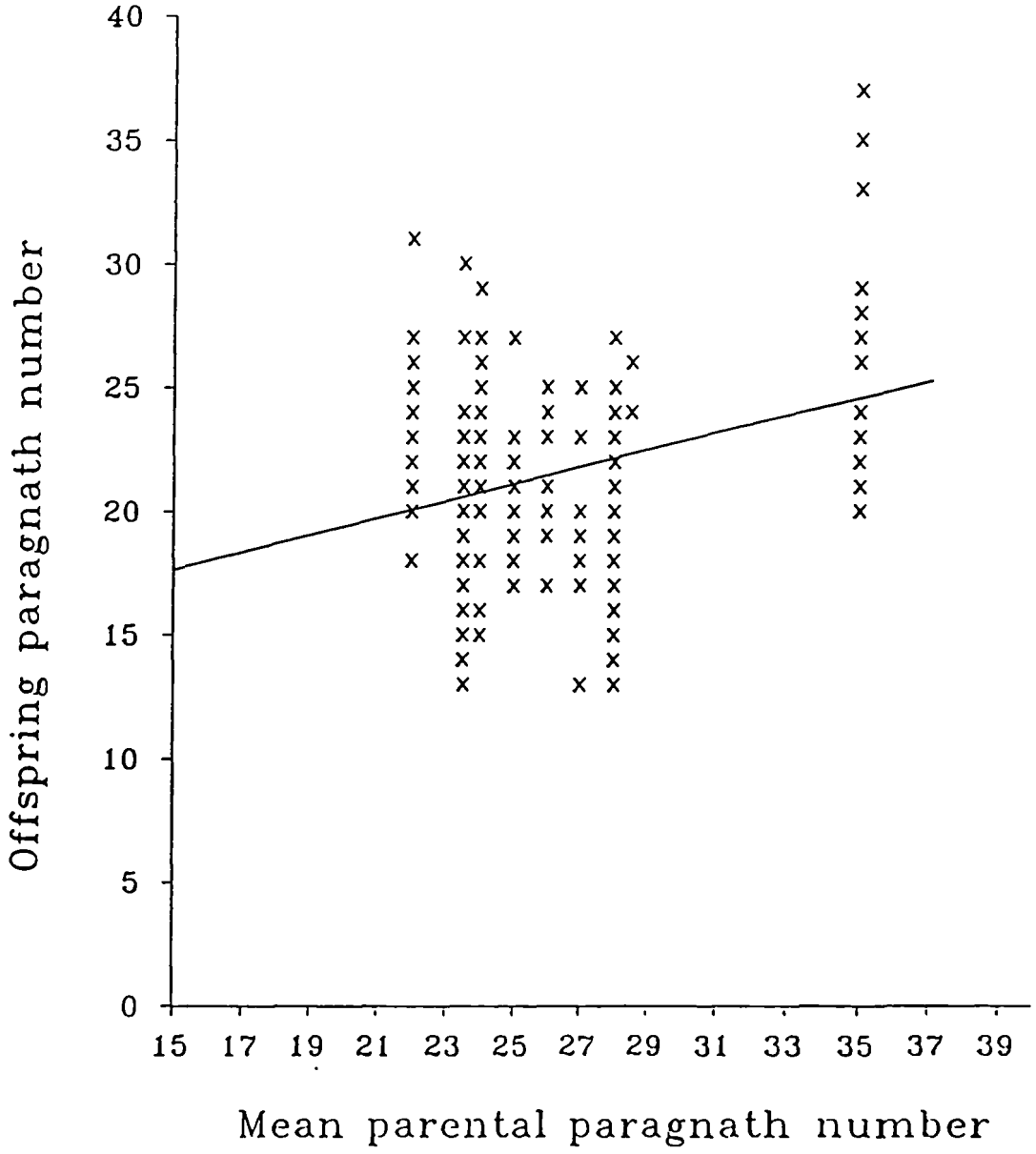

Offspring paragnath number
40
35
30
25
20
15
10
5
0
15 17 19 21 23 25 27 29 31 33 35 37 39
Mean parental paragnath number

Figure 3.4

Scatter diagram and regression of offspring values on mean parental values for group IV paragnath numbers.

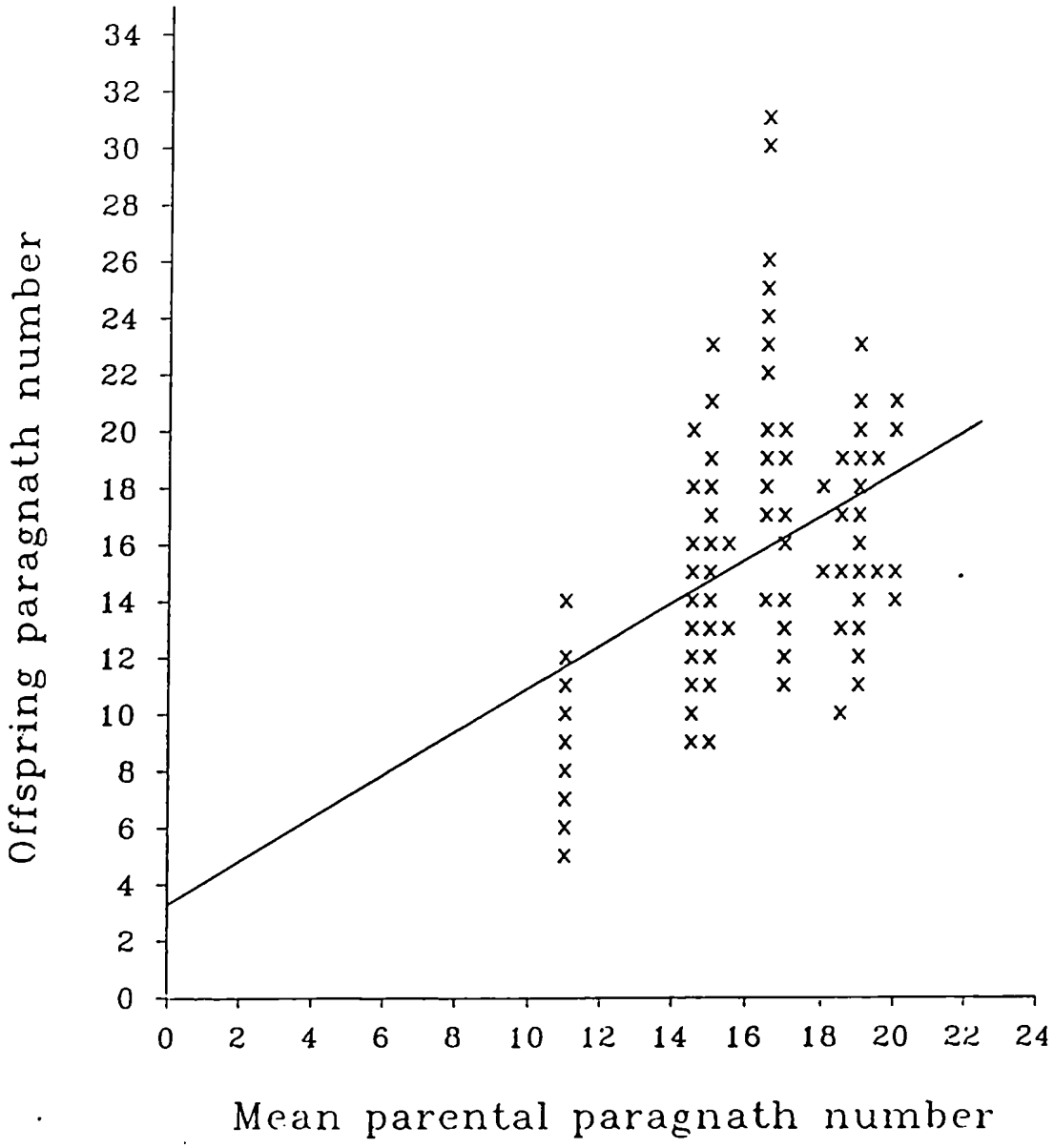

Offspring paragnath number
Mean parental paragnath number
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34

Figure 3.5

Scatter diagram of offspring values on mean parental values for group VI paragnath numbers.

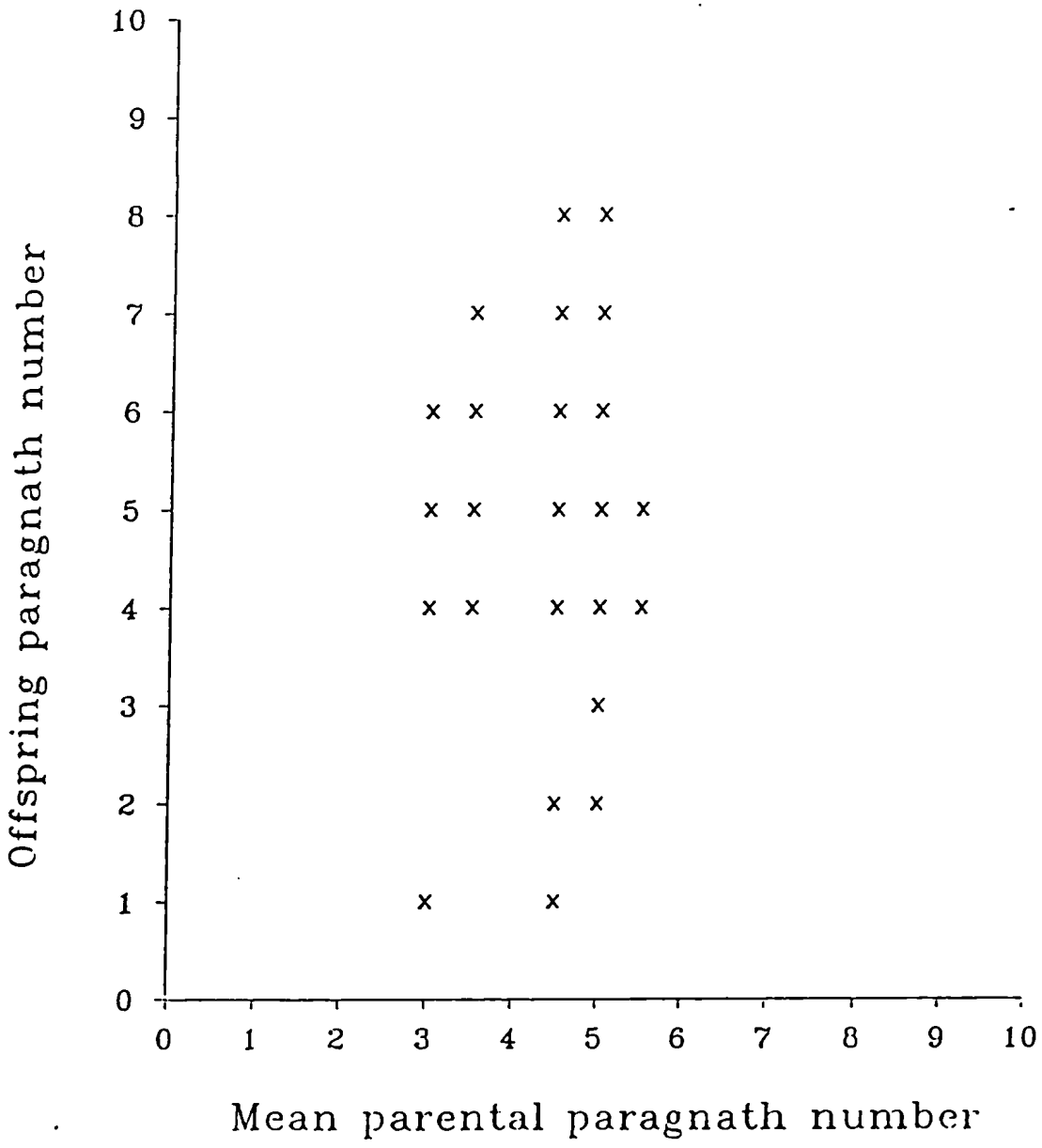

Offspring paragnath number
Mean parental paragnath number
0
1
2
3
4
5
6
7
8
9
10

Figure 3.6

Scatter diagram and regression of offspring values on mean parental values for group VII-VIII paragnath numbers.

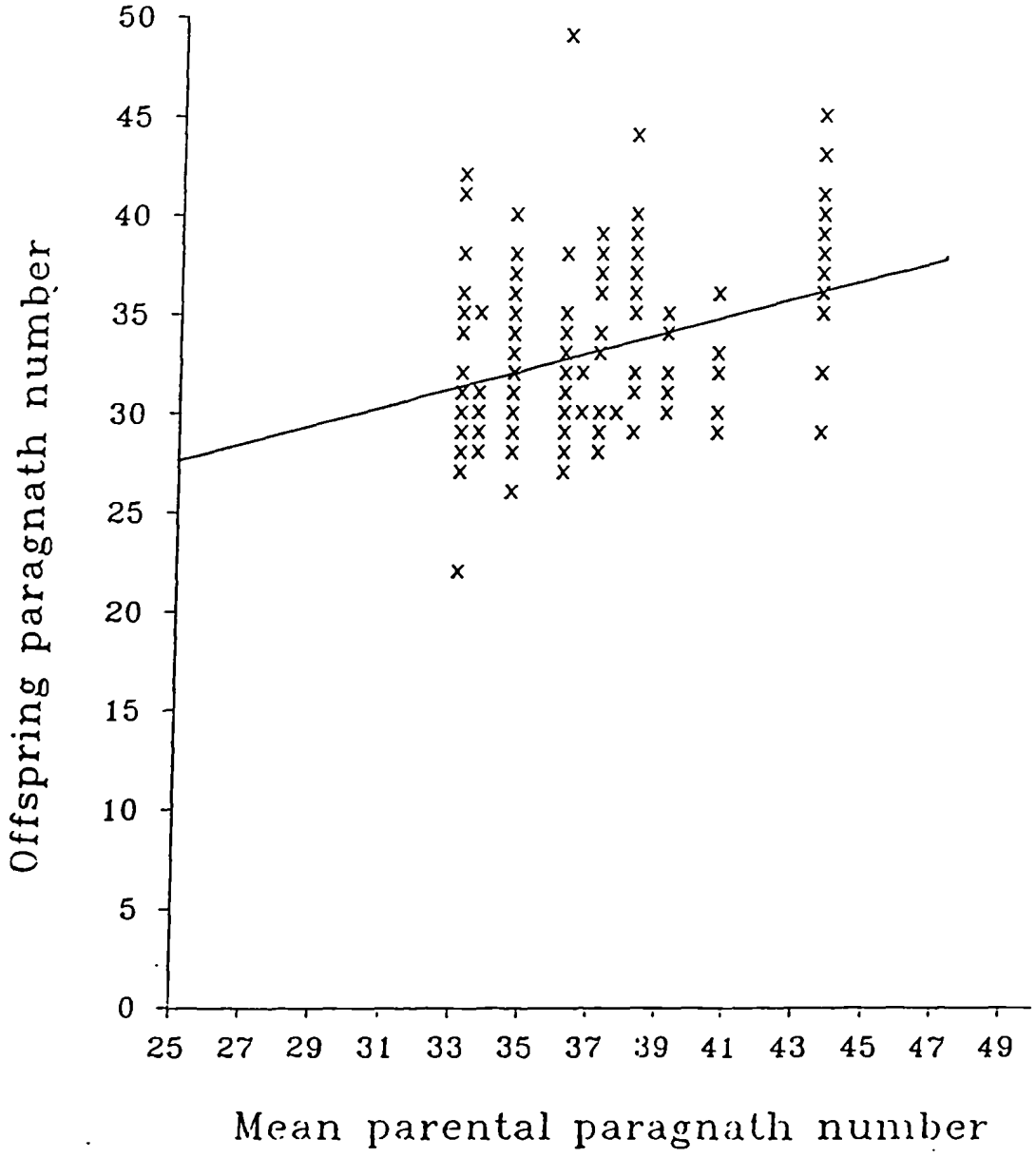
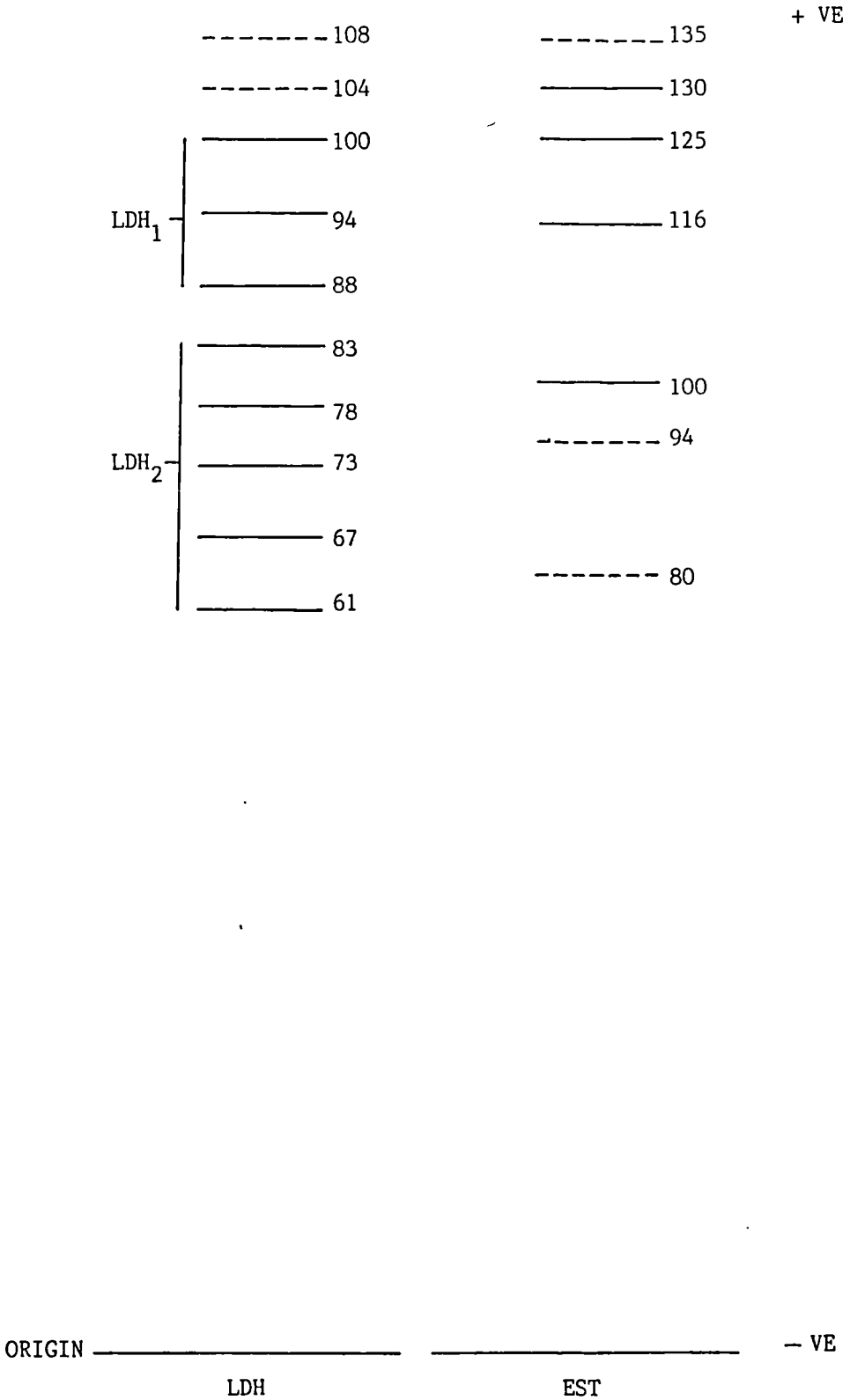

Offspring paragnath number
50
45
40
35
30
25
20
15
10
5
0
25 27 29 31 33 35 37 39 41 43 45 47 49
Mean parental paragnath number

Figure 3.7

Relative mobilities (m_r) of LDH and EST bands. Bands corresponding to hypothesised loci LDH_1 and LDH_2 are shown. Dashed lines indicate bands with inconsistent activity.


+ VE
108
104
100
94
88
LDH1
83
78
73
67
61
LDH2
135
130
125
116
100
94
80
ORIGIN
— VE
LDH
EST

For all three plates, origins are at the bottom of the frames and the bands are migrating up the page.

Plate 3.1

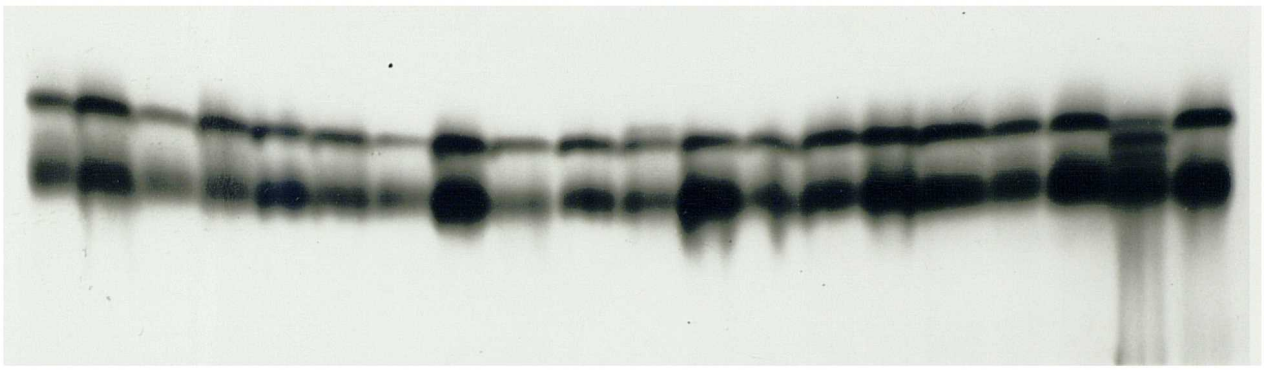
Polyacrylamide gel stained for LDH activity. All individuals are homozygous for the fast allele for LDH_2. Most individuals are homozygous for the fast allele for LDH_1 but note the three-banded heterozygote, two lanes from the right. Band m_r = 104 is visible eleven lanes from the left.

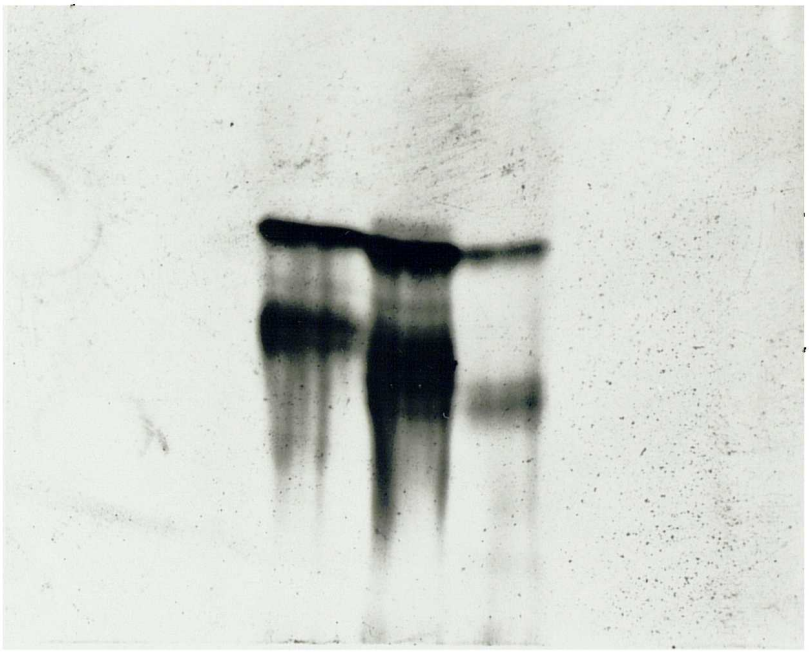
Plate 3.2

Polyacrylamide gel stained for LDH activity. The three LDH_2 genotypes are shown (from left to right); homozygous fast (FF), five-banded heterozygote (FS) and homozygous slow (SS). Note the double bands produced by the homozygotes and band m_r = 104 in the centre lane.

Plate 3.3

Polyacrylamide gel stained for EST activity.





CHAPTER 4

GAMETOGENESIS AND POPULATION DYNAMICS

INTRODUCTION

This chapter discusses the value of studying reproduction in N. diversicolor as a means of monitoring for the effects of estuarine pollution. The reproductive biology of this species has been well studied and the salient points are summarised below.

The animals are monotelic (breed once in a lifetime), do not have an epitokous stage, are non-hermaphroditic and non-viviparous (Dales, 1950; Smith 1964; Bogucki, 1953). The species shows a biased sex ratio of between 2.5 and 10 females for every male (Dales, 1950; Bogucki, 1953).

Endocrinological studies show that gametogenesis is probably mediated by a single hormone originating from the supra-oesophageal ganglion (Golding, 1983; Clark and Ruston, 1963). The duration of gametogenesis has been estimated as being 9-18 months for oogenesis and 6 months for spermatogenesis in the Blyth estuary (Olive and Garwood, 1981). Mettam (1979) also found oocyte growth and development extended over a full year in Severn estuary populations.

Age at onset of spawning appears to vary from site to site. It has previously been estimated as being 12-18 months in Denmark (Kristensen, 1984), 18-24 months in Scotland (Chambers and Milne, 1975), 24-36 months in Sweden (Moller, 1985), and 33-42 months in N.E. England (Olive and Garwood, 1981). Similarly, there is great variation in the timing and length of the breeding season. Most workers report spawning to occur annually in a single discrete period (e.g. Olive and Garwood, 1981; Mettam, 1979), but in the Ythan estuary, Scotland, two discrete spawning periods have been reported (Chambers and Milne, 1975) and Herpin (1925) reported a population that bred throughout the year. Finally, fertilisation and development is known to successfully occur in the salinity range $5^{o}/_{oo}$ - $35^{o}/_{oo}$ (Smith, 1964; Bogucki, 1953).

The larvae are demersal and for the first 3-4 days after fertilisation they have no powers of movement. At the monotrochal and

telotrochal stages (4-8 days), very limited swimming is possible through cilial activity. Chaetae appear at approximately 10 days and subsequent movement through muscular wriggling. When about 2-3 weeks old the larvae have reached a length of approximately 330 μm, have three chaetigerous segments, simple jaws, a complex gut and are capable of feeding. The adult mode of life is assumed by the time the animal is 2.5-3.0 mm long (approximately 20 segments), the young worms being competent swimmers and living in excavated burrows (Dales, 1950;).

Reproduction in marine organisms is particularly vulnerable to the effects of heavy metals. Studies on molluscs (Gibbs et al, 1987), crustacea (Moraitou-Apostolopoulou et al, 1983; Verriopoulos and Hardouvelis, 1988) and teleosts (Kumar and Pant, 1984) have demonstrated that even very low metal concentrations are sufficient to suppress or eliminate reproduction. The toxic effects of metals on annelid reproduction are particularly well documented. Studies on Ctenodrilus serratus, Ophryotrocha diadema and Neanthes arenaceodentata have shown their reproductive capacity to be suppressed by metal levels well below their lethal concentrations (Reish and Carr, 1978; Oshida et al, 1976; Jenkins and Mason, 1988).

There is little information on the impact of heavy metals on reproduction in N. diversicolor. This is particularly surprising considering the importance of the animal in European estuaries. Ozoh (1986) has studied the effect of copper on fertilisation and embryogenesis. He determined that the early life-history stages and cleavage in particular were most vulnerable to the toxic effects of the metal. Just 0.005 $mg.l^{-1}$ copper was sufficient to inhibit cleavage almost completely. Only one brief study has been conducted on the impact of metals on the dynamics of the species. Gillet (1987) monitored the

density and biomass of N. diversicolor in the Bou Regreg (Morocco) over the course of a year. He also measured tissue levels of copper and zinc at regular intervals and tentatively explained poor recruitment in the autumn through seasonally high metal burdens. Copper levels were reasonably constant throughout the year (8.5-54.7 $\mu g.g^{-1}$) but zinc concentrations peaked in autumn at 297.5-337.5 $\mu g.g^{-1}$ compared with the yearly mean of 188 $\mu g.g^{-1}$.

For biomonitoring purposes, the most useful approach for studying the impact of metals on reproduction is believed to be through the study of population structure. One advisory body considered this approach "highly recommended for immediate use in monitoring programmes" (G.E.S.A.M.P, 1980). The same body also considered the study of gametogenic cycles to be potentially useful.

In this chapter both approaches have been field tested. The gametogenic cycle and population dynamics of worms from an uncontaminated estuary were compared with animals from the moderately contaminated Humber and the heavily contaminated Restronguet Creek. Sig nificant perturbations in either element of the worm's reproductive biology would be indicative of ecological impact by metals.

As mentioned earlier, assessing the impact of heavy metals on reproduction is a form of biological effects monitoring and consequently suffers from lack of specificity. However, judging by the extremely low levels of metal in solution required to disrupt reproduction (e.g. Gibbs et al, 1987; Jenkins and Mason, 1988) it has the potential for being a particularly sensitive biomonitoring tool.

MATERIALS AND METHODS

a) Adult worms

The five cores (see General Methods) were sieved through 1000 µm and 500 µm meshes and the worms were allowed to recover in 17.5°/$_{oo}$ salinity water prior to preservation in a 1:1 mixture of formalin and ethanol. The jaws from each worm were removed and their total length measured using a microscope graticule (x24 or x60 magnification). A sample of coelomic fluid from each worm was smeared on a microscope slide and the diameters of 10-20 oocytes measured. Means were calculated for each individual (Olive and Garwood, 1981).

b) Juveniles and larvae

The surface sediment cores were sieved through 500 µm and 120 µm meshes. Juveniles were retained by the larger mesh and the numbers in each core were recorded. The samples of surface sediment preserved in formalin/ethanol (see General Methods) were also sieved through 500 µm and 120 µm meshes. The material trapped in the finer mesh was examined under a dissecting microscope at x10-x50 magnification for the presence of any larval stages. Unfortunately, copious amounts of material were retained by the mesh and it was extremely difficult detecting early life-history stages. Samples where larvae or small worms were found are mentioned in the text.

RESULTS

i) GAMETOGENIC CYCLE

Figures 4.1-4.6 illustrate the female reproductive cycle at the six sites studied in 1987 and 1988.

a) South West sites

At the Avon site (Figure 4.1), oocytes developed during the spring of 1987 and maturation was complete by June/July. Spawning took place during August. Some worms containing immature oocytes in September overwintered and became the following seasons breeding animals. Gametogenesis appeared to take approximately twelve months. 1988 followed the same pattern as for 1987.

Oogenesis at the highly contaminated R1 site (Figure 4.2) took place rapidly during spring (1987) and was complete by July. Somatic growth continued during this period. Spawning again occurred in August. In September the population was dominated by small unsexable worms (jaw-length 0.5-1.0 mm) with very few large animals containing immature oocytes. Oogenesis appeared to begin in March 1988 and was complete for many animals by August. Spawning again occurred in August but in this year many worms did not complete oogenesis and overwintered with immature oocytes. The pathways to maturation can therefore vary from year to year at R1 with oogenesis taking from five to twelve months.

Oogenesis at the less contaminated R13 site (Figure 4.3) followed the same basic pattern as for the Avon in 1987. Some females with mature oocytes were found in November 1987, March 1988 and May 1988 outside the main August breeding season. Ripe males were also found here and at R6 at low frequency throughout the year, suggesting there may be a continuous low-level incidence of breeding in the middle of Restronguet Creek.

These results indicate that the main spawning period at the South West sites is in August. However, different reproductive strategies

appear to exist within this framework. At R1, oogenesis can be completed within five months compared with twelve months at the Avon, and at R13 some spawning appears to occur all year round.

b) Humber sites

At Grimsby (Figure 4.4), the oocytes were full-sized in February 1987 and the majority of the ripe females spawned in February/March. Ripe females continued to be found until May but these eggs tended to be degenerate and there were no mature males present to fertilise them. This is not an uncommon phenomenon as Olive and Garwood (1981) and Moller (1985) have reported similar occurrences. Worms with immature oocytes (approximately 50 μm) were present 12 months prior to spawning. These oocytes increased in size during summer and autumn to be mature by January 1988. The gametogenic cycle for 1988 mirrored that for 1987.

Upstream at Hessle (Figure 4.5), oocyte growth continued during March 1987 until maturity was reached in April/May. Spawning occurred throughout May, approximately 2-3 months after the maximum spawning period in Grimsby. Degenerate eggs were found in June. Immature oocytes developed during autumn and winter and reached maximum size by April 1988. Oogenesis at Hessle therefore, also took approximately 12 months. As at Grimsby, the gametogenic cycle did not differ over the two years studied.

At Whitton (Figure 4.6), the oocytes were fully mature by May 1987 and spawning occurred in June, at least one month after Hessle. Oogenesis during autumn and winter followed a similar pattern to Hessle but the worms did not spawn until after July in 1988.

The Humber sites appeared to behave very differently from the South West sites. The timing of spawning varied considerably with location on the estuary. For both years, populations bred later the further one moved up the Humber. The earliest incidences of spawning occurred in February

at Grimsby, and the latest in July at Whitton. Length of oogenesis was consistent at all three sites at approximately 12 months. There was no evidence for rapid oogenesis as at R1 nor for the continuous breeding found at R13.

ii) POPULATION DYNAMICS

Figures 4.7-4.11 summarise the size frequency data for five of the six populations examined throughout 1987 and 1988. The nature of the shore at Hessle did not allow cores to be taken, so size frequency data could not be obtained.

a) South West sites

There were two distinct cohorts at the Avon site from April to July 1987 (Figure 4.7). In August the ripe females spawned, consequently there were no animals bearing large oocytes found in September. Some large animals bearing immature oocytes remained to overwinter. In November a new cohort appeared containing recruited juveniles (jaw-length approximately 0.5 mm). By May 1988, worms with mature oocytes reappeared and these spawned in August. A large number of juveniles were again recruited in October. Fine sieving of surface sediment confirmed that recruitment occurred in September and October. However, juvenile peaks were also found in April/May for both years. As no animals were found to breed at this site in spring, these animals must have been recruited from another site.

At R1 (Figure 4.8), the population in April 1987 consisted of a single cohort with few animals bearing oocytes. By July, virtually every animal was fully mature and few worms failed to spawn in August. A large number of juveniles were recruited in September. This cohort grew rapidly and approximately half the worms spawned the following August, the remaining half overwintered. Surface sediment cores confirmed that substantial juvenile recruitment took place prior to September/October in

1987 and 1988. In addition, tiny worms (12 segments) were found in the August surface mud for both years. This indicates that the population is not maintained by inward migration of large worms.

The size frequency data of worms from R13 (Figure 4.9) did not appear to follow a simple pattern. Mature females were found virtually throughout 1987 and 1988 suggesting a continuous level of breeding was taking place. Analysis of surface sediment seems to confirm this as juveniles were found in almost all samples. Peak numbers of juveniles appeared from September to November consistent with a main breeding season in August. The presence of tiny worms (14 segments) in July suggests that reproduction is successful here.

At all three sites, mature worms had a jaw-length of 1.6-2.0 mm. The age at spawning varied from site to site and also from year to year. In 1987 and 1988 most breeding worms at the Avon were probably two years old. At R1 in 1987, virtually all spawning worms were one year old. In 1988 however, only half the population spawned when one year old and the remainder overwintered to breed the following year.

b) Humber sites

The Grimsby population in February 1987 (Figure 4.10) was a mixture of large mature worms, intermediate sized worms with small oocytes and smaller worms with no oocytes. As spawning took place in February/March the larger worms disappeared. Maximum juvenile recruitment occurred in July/August, 4-5 months after the main spawning period. This suggests that either the growth rate of the early life-history stages was very slow or the juveniles were recruited from another site. 1988 followed the same pattern but there were fewer worms present than at the corresponding times the previous year. This was probably due to the discontinuation of the sewage outlet adjacent to the sampling site in November 1986. Three-setigerous larvae were found in April surface sediment which indicates

that the juveniles were probably recruited locally and not from another site.

At Whitton, there were two distinct cohorts present from March to May 1987 (Figure 4.11). Juveniles were found in the surface sediment in considerable numbers during this period. The worms spawned in June but there must have been recruitment failure as no juveniles were found for the remainder of 1987 or the whole of 1988. The mean salinity at Whitton is approximately $6^{o}/_{oo}$ which is very near the lower limit for successful breeding (Smith, 1964; Bogucki, 1953). The presence of juveniles in early 1987 however, would tend to negate low salinity as being the limiting factor. It is more probable that a summer storm stripped the surface sediment away, taking the young worms with it. Following the July spawning in 1988, very few worms were left at the site. Numbers had not recovered by June 1989 (Personal observation).

Unlike the South West sites, the breeding worm size varied in the Humber. For both years, Whitton animals spawned at a size corresponding to jaw-length 1.4-1.5 mm. At Grimsby, the breeding worms were much larger. In 1987 the jaw-lengths were approximately 2.0-3.0 mm and in 1988 they were at least 2.0 mm. The age at spawning appeared to be 2 years at Whitton and 2-3 years at Grimsby.

DISCUSSION

Studies on gametogenesis in N. diversicolor almost invariably concentrate on oocyte maturation. Oocyte diameters give a more finely graded index of maturity than do stages of spermatogenesis. Oogenesis takes a considerably longer time than spermatogenesis (Olive and Garwood, 1981) and females are available in larger numbers than males, due to the biased sex-ratio. Considerably more information on the gametogenic cycle of a population can thus be readily obtained this way (Grant, Hateley and Jones, In Press).

The length of oogenesis showed considerable heterogeneity both between populations and between seasons. Oocyte maturation was completed in approximately 12 months at all the Humber sites and the Avon site for both 1987 and 1988. This corresponded reasonably well with the 9-18 months reported by Olive and Garwood (1981) in the Blyth estuary (N.E. England) and "over a year" recorded by Mettam (1979) for a number of populations in the Severn estuary. At R1, however, oogenesis (including both oocyte proliferation and maturation) was completed within 5 months in 1987, considerably faster than found in any previous studies.

The large variations in oocyte maturation time would seem to indicate inter-population differences in the detailed control of reproduction (Grant, Hateley and Jones, In Press). The final stages of oocyte maturation are dependant on a decrease in cerebral endocrine activity, as the hormone produced inhibits oocyte maturation at high concentrations (Golding, 1983). Golding considered that the first decline in hormone secretion for the population studied by Olive and Garwood (1981) would begin in the last 5 months of oogenesis when the mean oocyte diameter was approximately 180 μm. Obviously the decline in hormone secretion at R1 must be considerably faster as the first immature oocytes were only just appearing 5 months prior to spawning. As the timing of

gametogenesis has been found to be unaffected by photoperiod and temperatures in the range of 5-15°C (Olive and Garwood, 1983) there would therefore appear to be a genetic component in the factors determining the rate of oocyte growth to maturation.

There was also variation in the length of oogenesis between seasons. Although all R1 worms completed oogenesis in 5 months in 1987, the following year a large proportion of the animals overwintered with immature oocytes to spawn the next season. Oogenesis in 1988 therefore continued for up to 12 months (from oocytes first appearing to maturity). This seasonal variation indicates that the length of oogenesis is also partially determined by environmental factors (Clark and Ruston, 1963).

There was considerable heterogeneity in the timing of spawning both between and within estuaries. In the South West, the main spawning period for all sites studied was August. There does, however, appear to be a continuous low-level incidence of spawning at R13 and bimodal recruitment of juveniles at the Avon site may indicate a spring spawning season elsewhere in this estuary. Spawning started earlier in the Humber (February/March at Grimsby) but occurred later in the year as one moved up the estuary. Hessle worms spawned in May, approximately 2-3 months after Grimsby, and Whitton worms spawned 1 month after Hessle. This asynchronous breeding does not seem to be caused by differences in tidal levels between sites (as noted by Chambers and Milne (1975) in the Ythan estuary). Although the Grimsby site was lower down the shore than Hessle and Whitton, specimens collected from the upper shore at Grimsby were also mature in February/March.

Regional variability in spawning time has been noted by a number of workers. Herpin (1925) found continuous breeding in a population at Cherbourg; breeding throughout spring, summer and autumn (with a peak in spring) at Tourlaville, and breeding which did not pass July at Nacqueville. N. diversicolor spawns in March/April in the Blyth (Olive

and Garwood, 1981), May on the west coast of Sweden (Moller, 1985), May to December (with a peak in August) in a brackish water pond in Belgium (Heip and Herman, 1979) and early spring/summer in Norsminde Fjord, Denmark (Kristensen, 1984). Only Mettam (1979) studied reproduction at a number of sites within one estuary (Severn). Five widely spaced populations living under different environmental regimes were examined, but all spawned synchronously in the summer.

The 'proximate' factors determining spawning timing are unknown but Smith (1976) suggested it may be linked to an increase in environmental temperature. For this to be true the critical temperatures would have to be specific to individual populations, otherwise the warmer waters in the South West would be expected to induce earlier spawning than the cooler Humber waters. As the South West sites spawn at the hottest period in the year, increase in environmental temperature is clearly not the stimulus for spawning in this region. The 'ultimate' factors that favour spawning at a particular time of the year are also unknown. It is possible that availability of food for larvae and juveniles is an important determinant of spawning timing. The early spawning populations (e.g. Grimsby) may rely on the spring phytoplankton bloom whereas the larvae of summer breeders may feed on surface diatoms or leaf-litter (particularly in the South West) (Grant, Hateley and Jones, In Press). This explanation is probably a little too simplistic as nutritional input at Grimsby through phytoplankton is almost certainly negligible compared with that from the adjacent domestic sewage outfall.

The large variations in length of oogenesis and the timing of spawning are not related in any simple way to the level of metal contamination. Although oogenesis is remarkably rapid at the most contaminated site (R1), gametogenesis is not abnormal in any other respect. Gametes from both R1 males and females have been successfully used in artificial fertilisations in the laboratory. Similarly, the

presence of ripe females at all times of the year at the less contaminated R13 site cannot be directly attributed to metal induced asynchrony of breeding. Fertile males were also found during most of the year and the gametes were again demonstrated to be fully viable in the laboratory. As a population at Cherbourg has been found to breed throughout the year (Herpin, 1925) this reproductive behaviour is not necessarily unusual.

Studies of population structure also demonstrated considerable variation between sites, particularly in the age and size of worms at maturity. In 1987, the R1 population reproduced almost entirely at the age of 12 months. The other populations studied all appeared to reproduce when aged at least 24 months. More definite estimates of breeding age are prevented by the difficulty of interpreting data in the form of size/frequency histograms to produce age classes without knowing juvenile growth rates (Grant, Morgan and Olive, 1987; Grant, 1989). A review of the literature also shows great geographical variability in the age of worms at maturation. It has been previously estimated as being 12 months in the Thames (Dales, 1951), 12-18 months in Denmark (Kristensen, 1984), 18-24 months in Scotland (Chambers and Milne, 1975), 24-36 months in Sweden (Moller, 1985) and 33-42 months in North East England (Olive and Garwood, 1981).

In 1988 the worms spawning at R1 were, like the previous year, aged 12 months. Not all of the cohort spawned, however, and a large proportion overwintered with immature oocytes to spawn in 1989 at the age of 24 months (Figure 4.8). The age at maturation can therefore vary from season to season. This guarantees that generations at R1 are overlapping and there is no genetic isolation between consecutive year classes (Olive and Garwood, 1981). It also demonstrates that the 'decision' to reproduce in

a particular year is not a simple function of age (c.f. Olive, Grant and Cowin, 1986).

The size of mature worms varied considerably from site to site. The minimum jaw-length at maturity can be estimated from the plots of oocyte diameter against jaw-length; Grimsby = 2.0 mm; Hessle = 1.6 mm, Whitton = 1.4 mm, R1 = 1.6 mm, R13 = 1.6 mm and Avon = 1.6 mm. The largest breeding worms were therefore found at Grimsby, with size at maturity declining as one moved up the Humber estuary. In the South West, size at maturity was remarkably constant. Although minimum breeding size varied considerably by site, it was temporally stable within a site (i.e. 1987 and 1988 values did not differ substantially). This suggests worm size plays an important role in the 'decision' to reproduce (Grant, Hateley and Jones, In Press; c.f. Olive, Grant and Cowin, 1986).

Age at maturity would therefore primarily be determined by growth rate. Worms at R1 are able to breed in 12 months because tolerance to copper has enabled them to exploit an environment rich in organic matter (Bryan and Gibbs, 1983), with no inter-specific competition and few predators. These conditions are highly conducive to fast growth. The failure of many worms to breed in 12 months in 1988 suggests their growth rate had been slower and they had not attained the minimum size required when the 'decision' to reproduce was made.

The presence of very small worms at R1 in August/September surface mud demonstrates that the population is being maintained through the annual recruitment of juveniles, and not by the inward migration of adults. This contrasts with polluted environments on the West coast of Sweden and South West Finland where specimens of N. diversicolor found in the most heavily contaminated areas were considered to be adult invaders (Leppakoski, 1975; Tulkki, 1960; Laakso, 1965). At R13, peak juvenile recruitment also occurred in September but there was a level of continuous recruitment throughout the year. This would support the

contention that successful breeding at the mid-estuary sites does occur all year round with the main breeding season in August. There is, therefore, no evidence from studying population dynamics to indicate that reproduction in Restronguet Creek is significantly perturbed by heavy metals. Both populations spawned and recruited successfully, although the reproductive strategies involved were very different.

This work indicates that studies of the reproductive biology of N. diversicolor specifically for the detection of metal impact in an estuary are unlikely to yield useful information. In a long-term contaminated estuary like Restronguet Creek, tolerance has enabled populations to reproduce even at the most contaminated sites. In short-term contaminated estuaries where tolerance would not have had time to develop, the natural variability in the gametogenic cycle (e.g. length of oogenesis and timing of spawning) could well obscure any detectable metal induced perturbations in gametogenesis. Even studies of population dynamics in such estuaries are limited and vulnerable to misinterpretation. The apparent failure of juvenile recruitment in the heavily contaminated waters around Helsinki and Airisto Sound could simply have been a function of low salinity (under $6^{o}/_{oo}$) rather than pollution (Leppakoski, 1975; Tulkki, 1960; Laakso, 1965). Similarly, it would seem rather optimistic of Gillet (1987) to attribute bad autumn recruitment in the Bou-Regreg (Morocco) to fluctuating levels of copper and zinc. Sediment concentrations were just 19-30 $\mu g.g^{-1}$ copper and 78-172 $\mu g.g^{-1}$ zinc, similar values to those obtained in the uncontaminated Kingsbridge Avon! Poor recruitment was most probably a function of unpredictable rainfall and extremely high salinities ($40-57^{o}/_{oo}$).

A considerable portion of the literature devoted to reproductive biology and population dynamics is based on single populations. As mentioned earlier, only Mettam (1979) examined a number of populations

within a single estuary. The work in this thesis has demonstrated the considerable variability in the reproductive patterns within an estuary and this highlights the dangers of making generalised statements about an estuary from evidence provided by a single population. For life-history studies to truly reflect reproduction in an estuary, a number of populations must be examined.

In summary;

i) Studies on population dynamics and the gametogenic cycle in N. diversicolor are unsuitable for detecting ecological impact by metals.

Figure 4.1

Time-series plots of mean oocyte diameter against jaw-length for individuals sampled from the Avon. Animals lacking oocytes are plotted as having an oocyte diameter of zero. Sampling dates as shown.

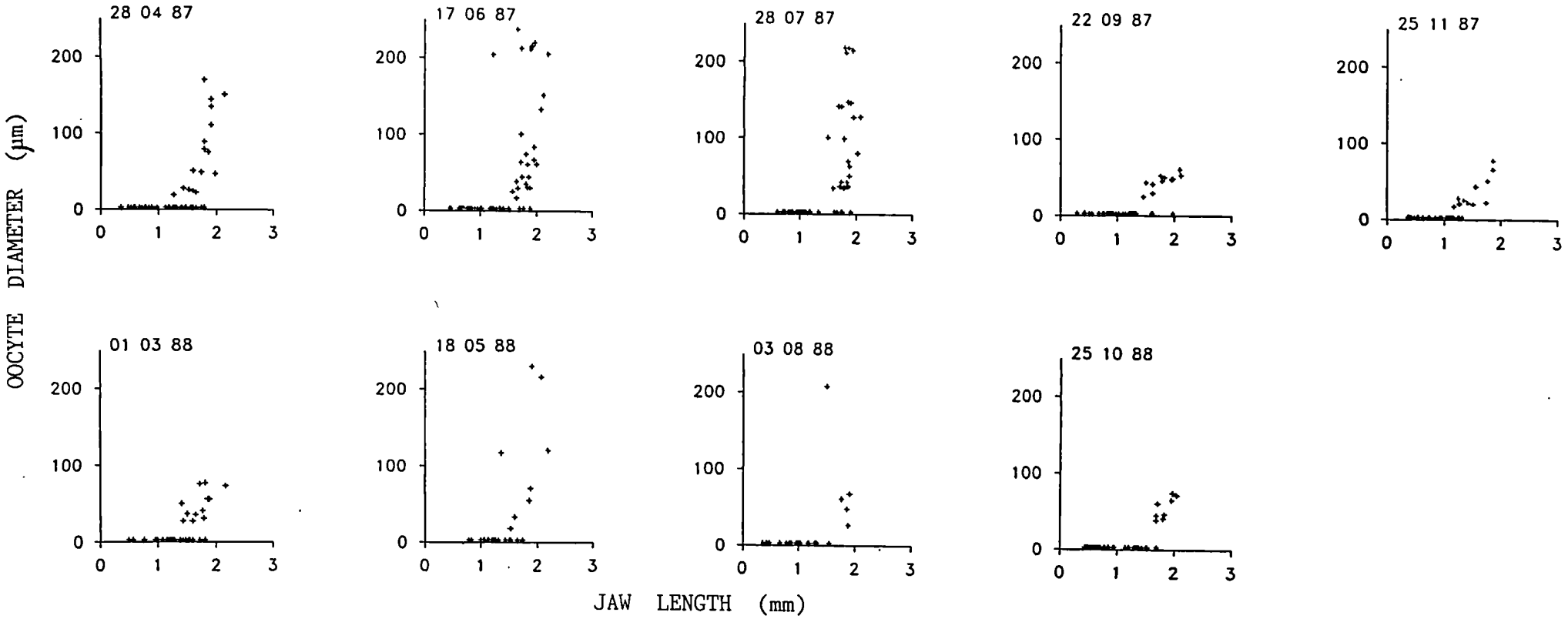

28 04 87
17 06 87
28 07 87
22 09 87
25 11 87
01 03 88
18 05 88
03 08 88
25 10 88
OOCYTE DIAMETER (µm)
JAW LENGTH (mm)
0
100
200
0
1
2
3

Figure 4.2

Time-series plots of mean oocyte diameter against jaw-length for individuals sampled from R1. Animals lacking oocytes are plotted as having an oocyte diameter of zero. Sampling dates as shown.

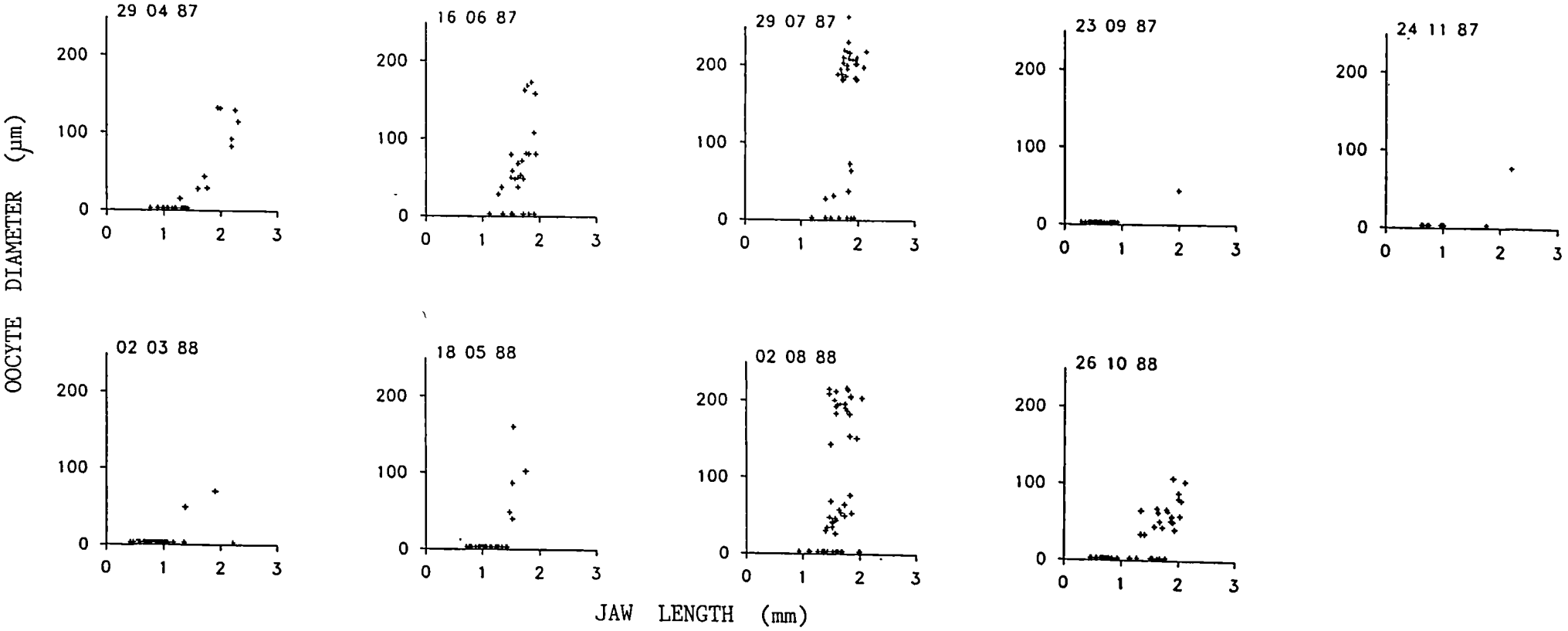

29 04 87
16 06 87
29 07 87
23 09 87
24 11 87
02 03 88
18 05 88
02 08 88
26 10 88
OOCYTE DIAMETER (µm)
JAW LENGTH (mm)
0
100
200
0
1
2
3

Figure 4.3

Time-series plots of mean oocyte diameter against jaw-length for individuals sampled from R13. Animals lacking oocytes are plotted as having an oocyte diameter of zero. Sampling dates as shown.

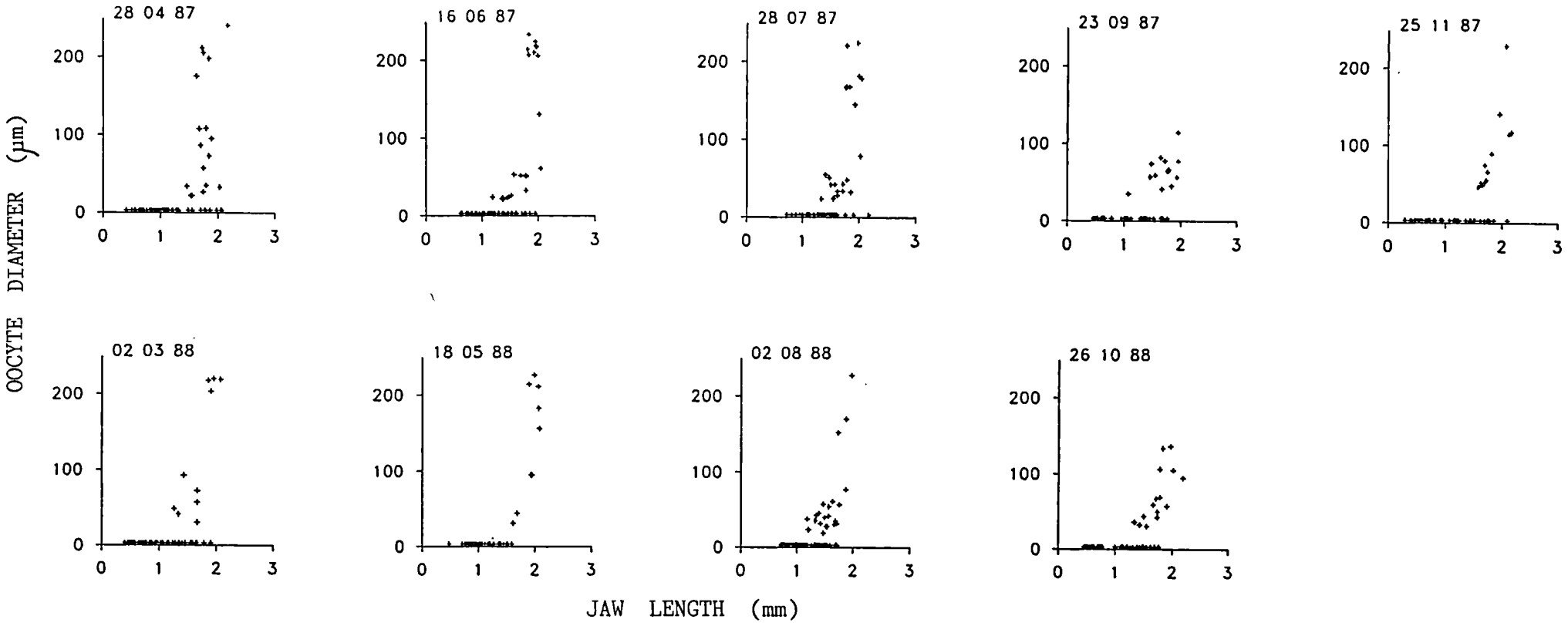

28 04 87
16 06 87
28 07 87
23 09 87
25 11 87
02 03 88
18 05 88
02 08 88
26 10 88
OOCYTE DIAMETER (µm)
JAW LENGTH (mm)
0
100
200
0
1
2
3

Figure 4.4

Time-series plots of mean oocyte diameter against jaw-length for individuals sampled from Grimsby. Animals lacking oocytes are plotted as having an oocyte diameter of zero. Sampling dates as shown.

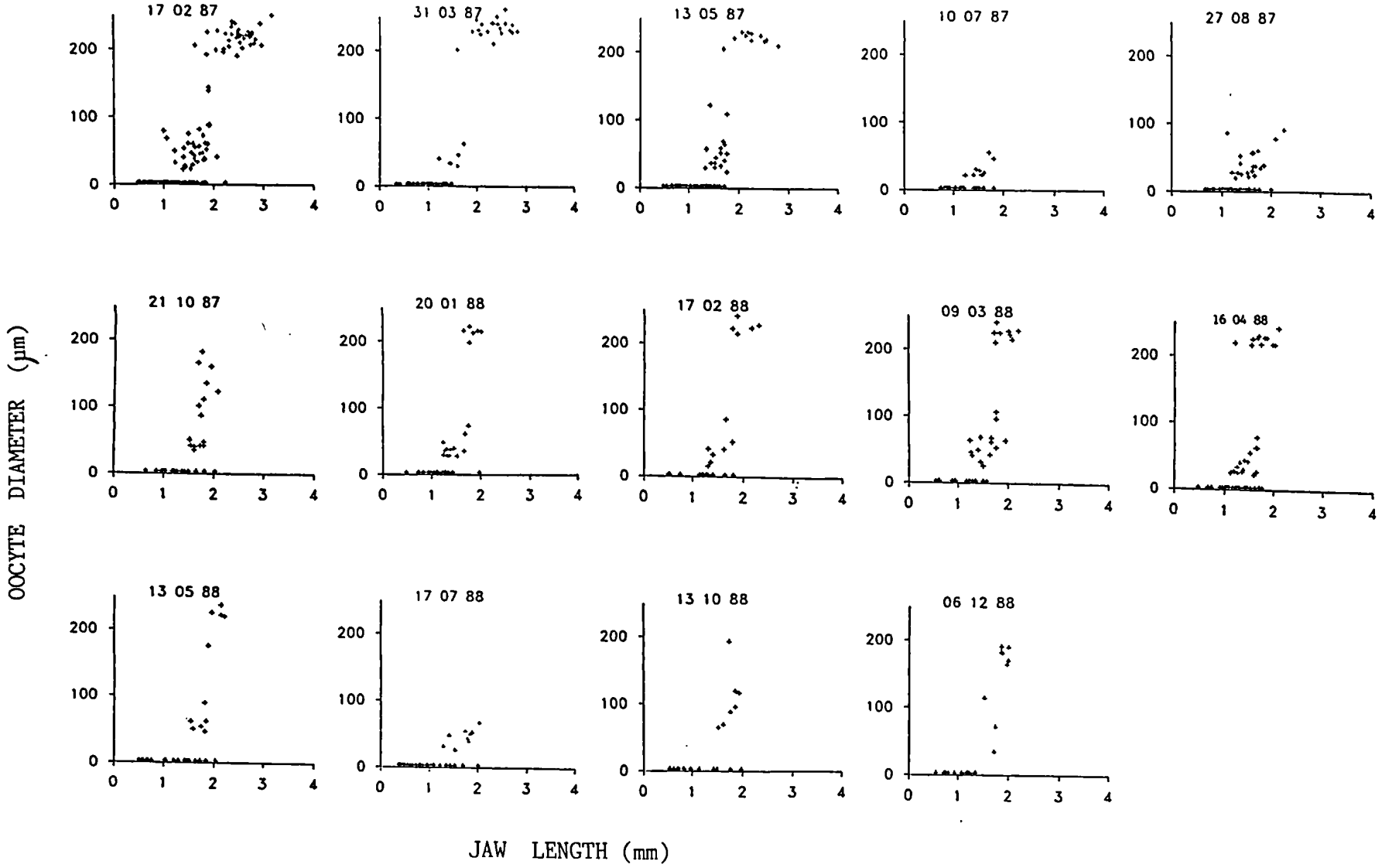

17 02 87
31 03 87
13 05 87
10 07 87
27 08 87
21 10 87
20 01 88
17 02 88
09 03 88
16 04 88
13 05 88
17 07 88
13 10 88
06 12 88
OOCYTE DIAMETER (µm)
JAW LENGTH (mm)

Figure 4.5

Time-series plots of mean oocyte diameter against jaw-length for individuals sampled from Hessle. Animals lacking oocytes are plotted as having an oocyte diameter of zero. Sampling dates as shown.

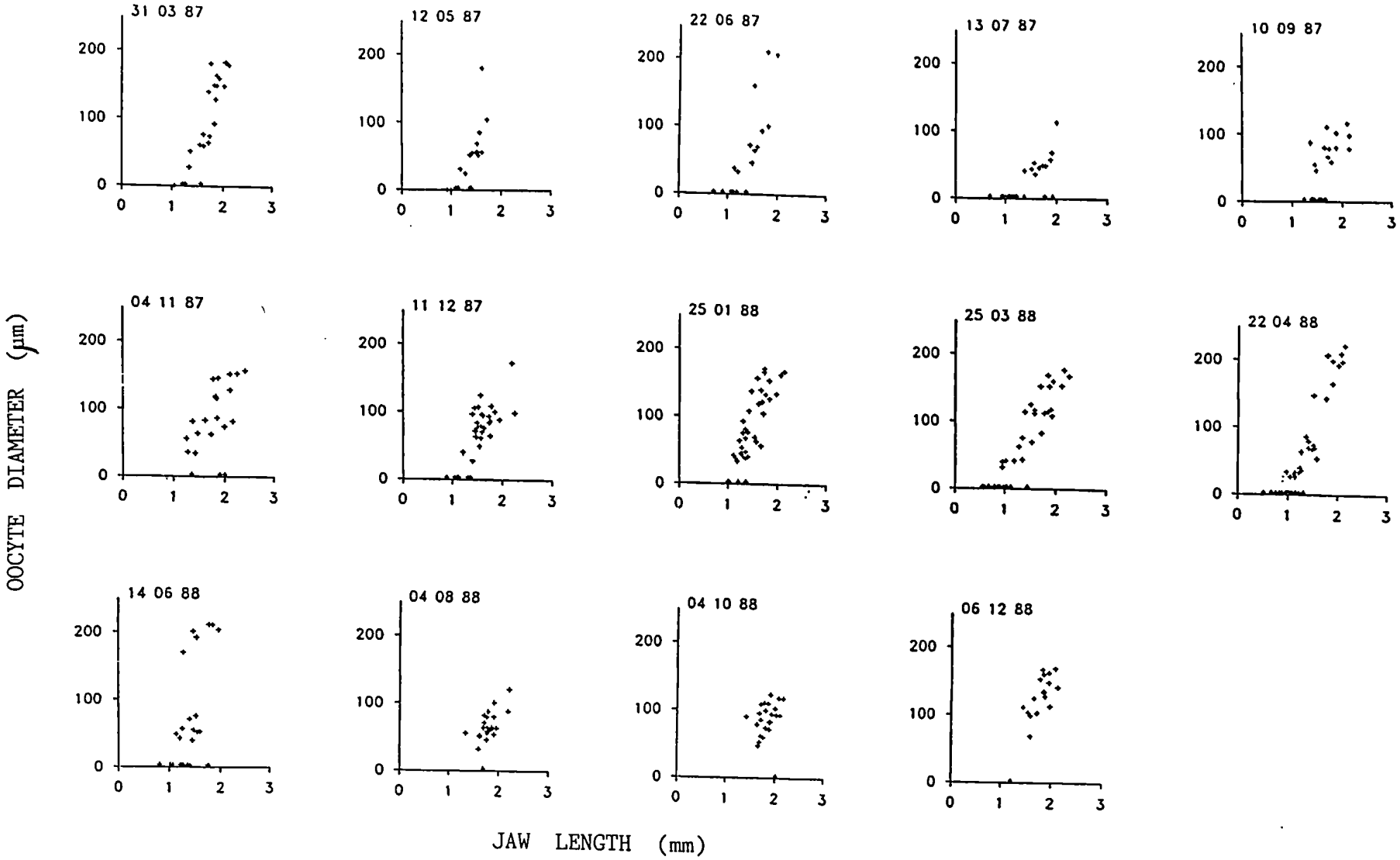

31 03 87
12 05 87
22 06 87
13 07 87
10 09 87
04 11 87
11 12 87
25 01 88
25 03 88
22 04 88
14 06 88
04 08 88
04 10 88
06 12 88
OOCYTE DIAMETER (µm)
JAW LENGTH (mm)

Figure 4.6

Time-series plots of mean oocyte diameter against jaw-length for individuals sampled from Whitton. Animals lacking oocytes are plotted as having an oocyte diameter of zero. Sampling dates as shown.

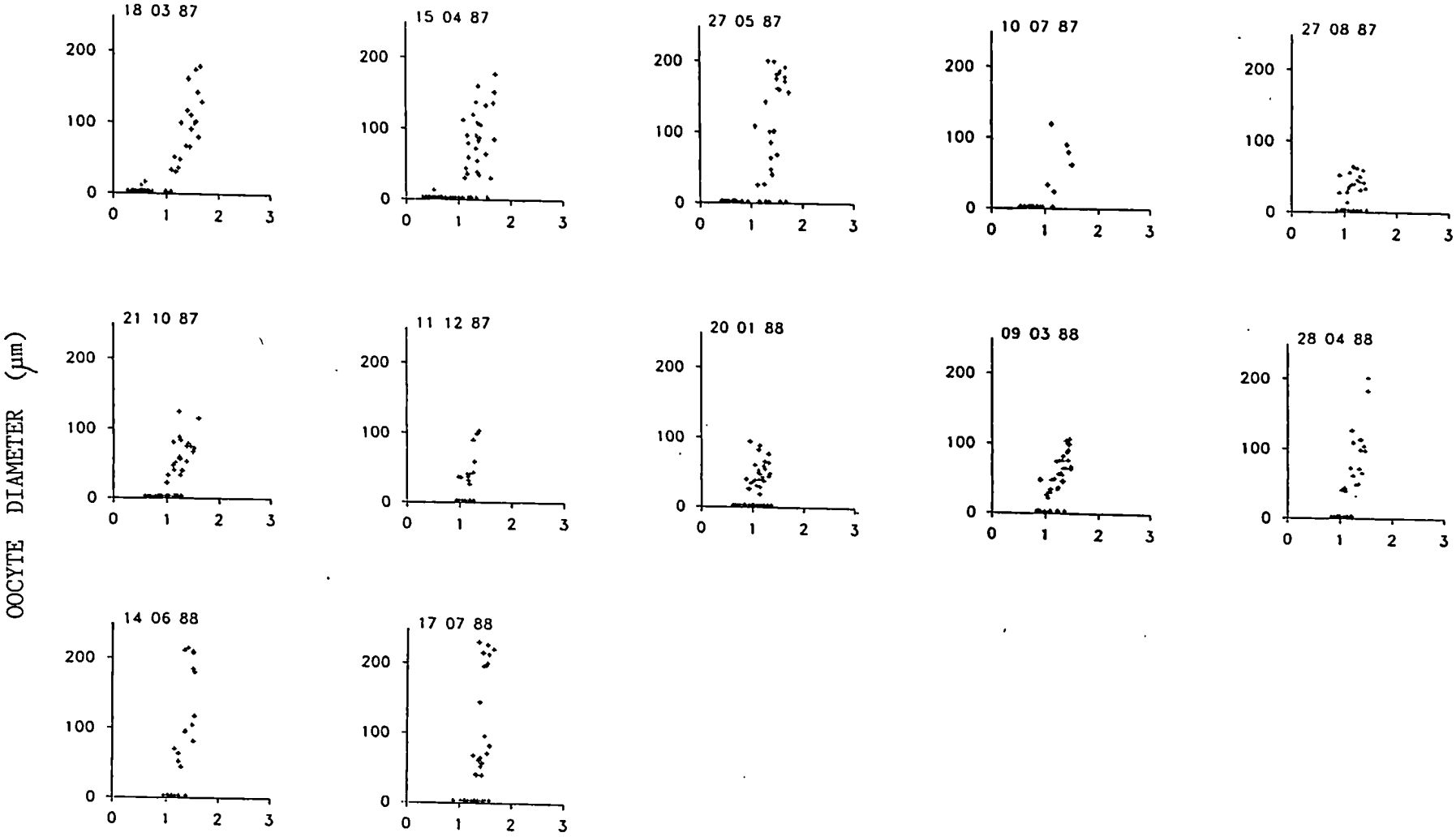

18 03 87
15 04 87
27 05 87
10 07 87
27 08 87
21 10 87
11 12 87
20 01 88
09 03 88
28 04 88
14 06 88
17 07 88
OOCYTE DIAMETER (µm)
JAW LENGTH (mm)

Figure 4.7

Time-series plots of size frequency histograms for Avon animals.

Dark shading = animals with mean oocyte diameter > 80 μm.

Light shading = animals with mean oocyte diameter < 80 μm.

JUV = number of juveniles (worms < 15 mm) in surface core of 0.01 m^2.

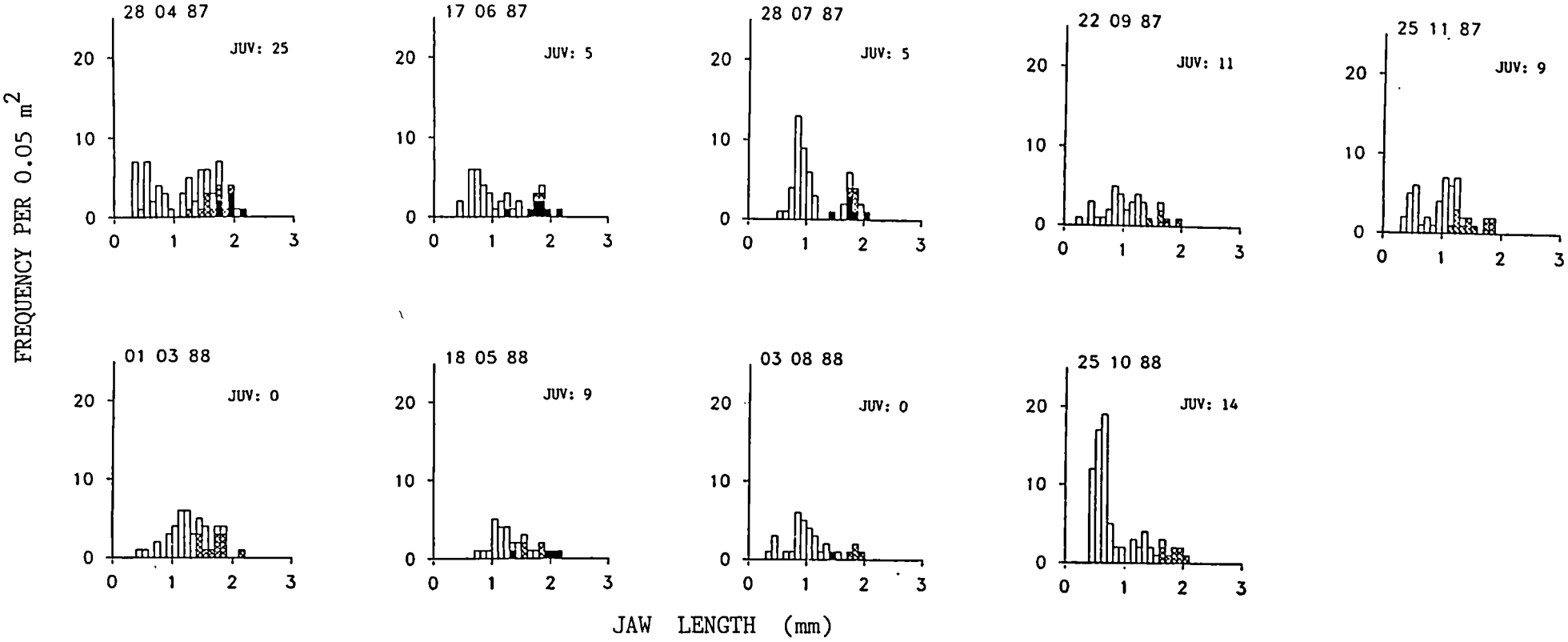

28 04 87
JUV: 25
17 06 87
JUV: 5
28 07 87
JUV: 5
22 09 87
JUV: 11
25 11 87
JUV: 9
01 03 88
JUV: 0
18 05 88
JUV: 9
03 08 88
JUV: 0
25 10 88
JUV: 14
FREQUENCY PER 0.05 m2
JAW LENGTH (mm)

Figure 4.8

Time-series plots of size frequency histograms for R1 animals.

Dark shading = animals with mean oocyte diameter > 80 μm.

Light shading = animals with mean oocyte diameter < 80 μm.

JUV = number of juveniles (worms < 15 mm) in surface core of 0.01 m^2.

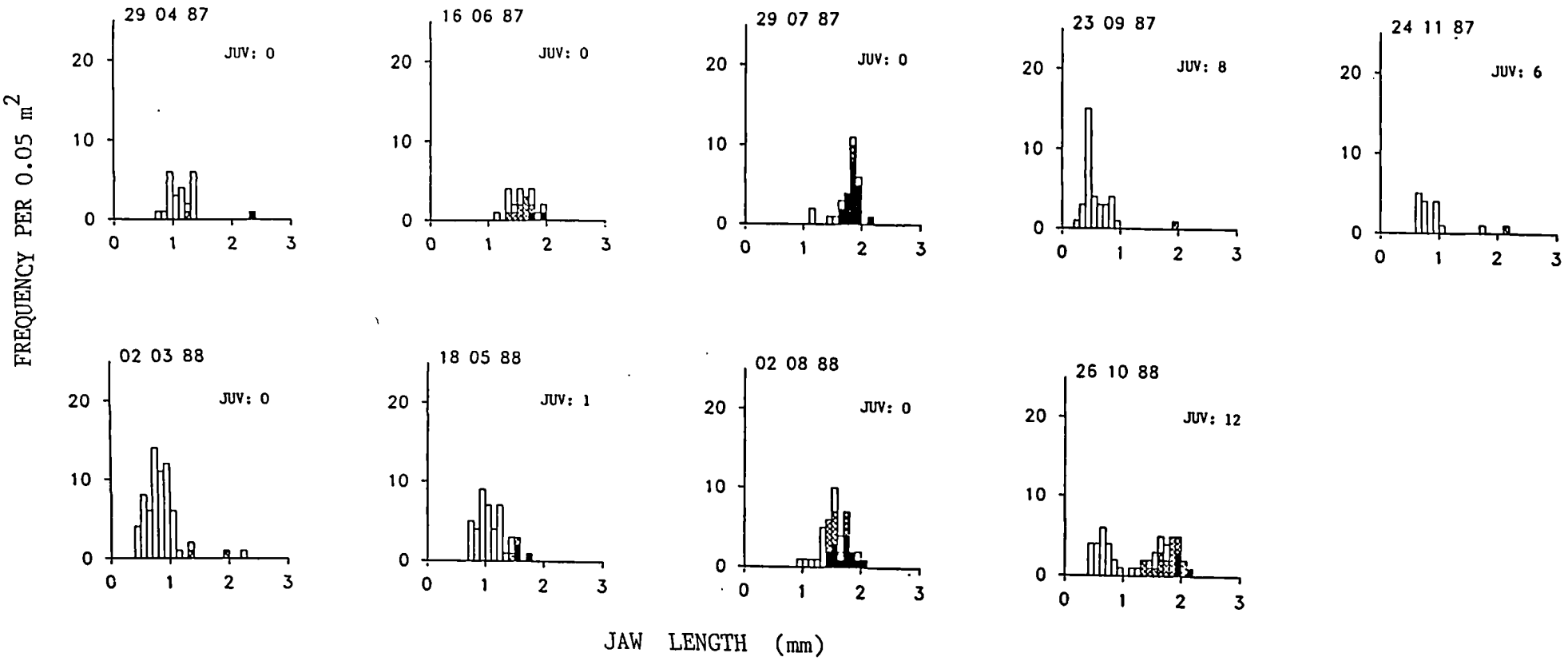

29 04 87
JUV: 0
16 06 87
JUV: 0
29 07 87
JUV: 0
23 09 87
JUV: 8
24 11 87
JUV: 6
02 03 88
JUV: 0
18 05 88
JUV: 1
02 08 88
JUV: 0
26 10 88
JUV: 12
FREQUENCY PER 0.05 m2
JAW LENGTH (mm)

Figure 4.9

Time-series plots of size frequency histograms for R13 animals.

Dark shading = animals with mean oocyte diameter > 80 μm.

Light shading = animals with mean oocyte diameter < 80 μm.

JUV = number of juveniles (worms < 15 mm) in surface core of 0.01 m^2.

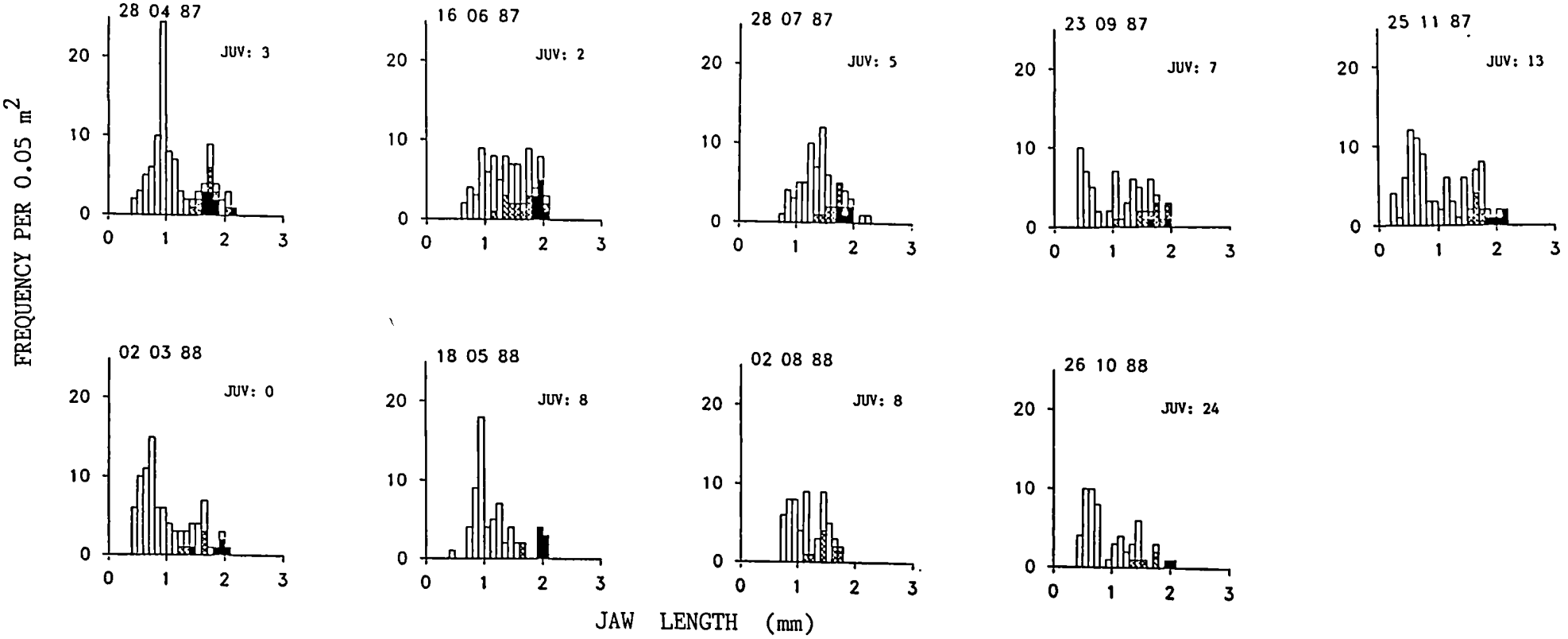

28 04 87
JUV: 3
16 06 87
JUV: 2
28 07 87
JUV: 5
23 09 87
JUV: 7
25 11 87
JUV: 13
02 03 88
JUV: 0
18 05 88
JUV: 8
02 08 88
JUV: 8
26 10 88
JUV: 24
FREQUENCY PER 0.05 m2
JAW LENGTH (mm)

Figure 4.10

Time-series plots of size frequency histograms for Grimsby animals.

Dark shading = animals with mean oocyte diameter > 80 μm.

Light shading = animals with mean oocyte diameter < 80 μm.

JUV = number of juveniles (worms < 15 mm) in surface core of 0.01 m^2.

* = core spoilt.

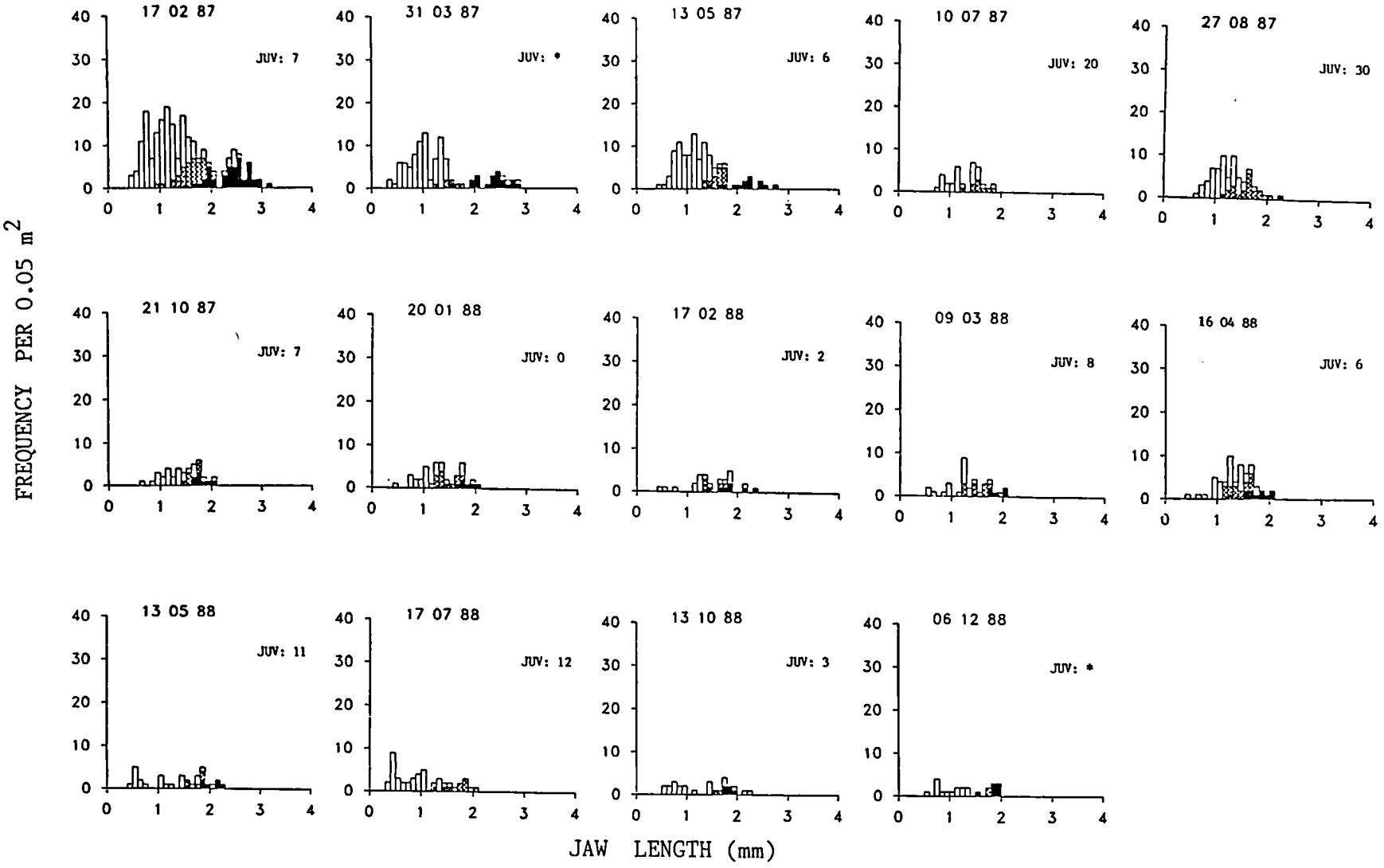

17 02 87
JUV: 7
31 03 87
JUV: *
13 05 87
JUV: 6
10 07 87
JUV: 20
27 08 87
JUV: 30
21 10 87
JUV: 7
20 01 88
JUV: 0
17 02 88
JUV: 2
09 03 88
JUV: 8
16 04 88
JUV: 6
13 05 88
JUV: 11
17 07 88
JUV: 12
13 10 88
JUV: 3
06 12 88
JUV: *
FREQUENCY PER 0.05 m2
JAW LENGTH (mm)

Figure 4.11

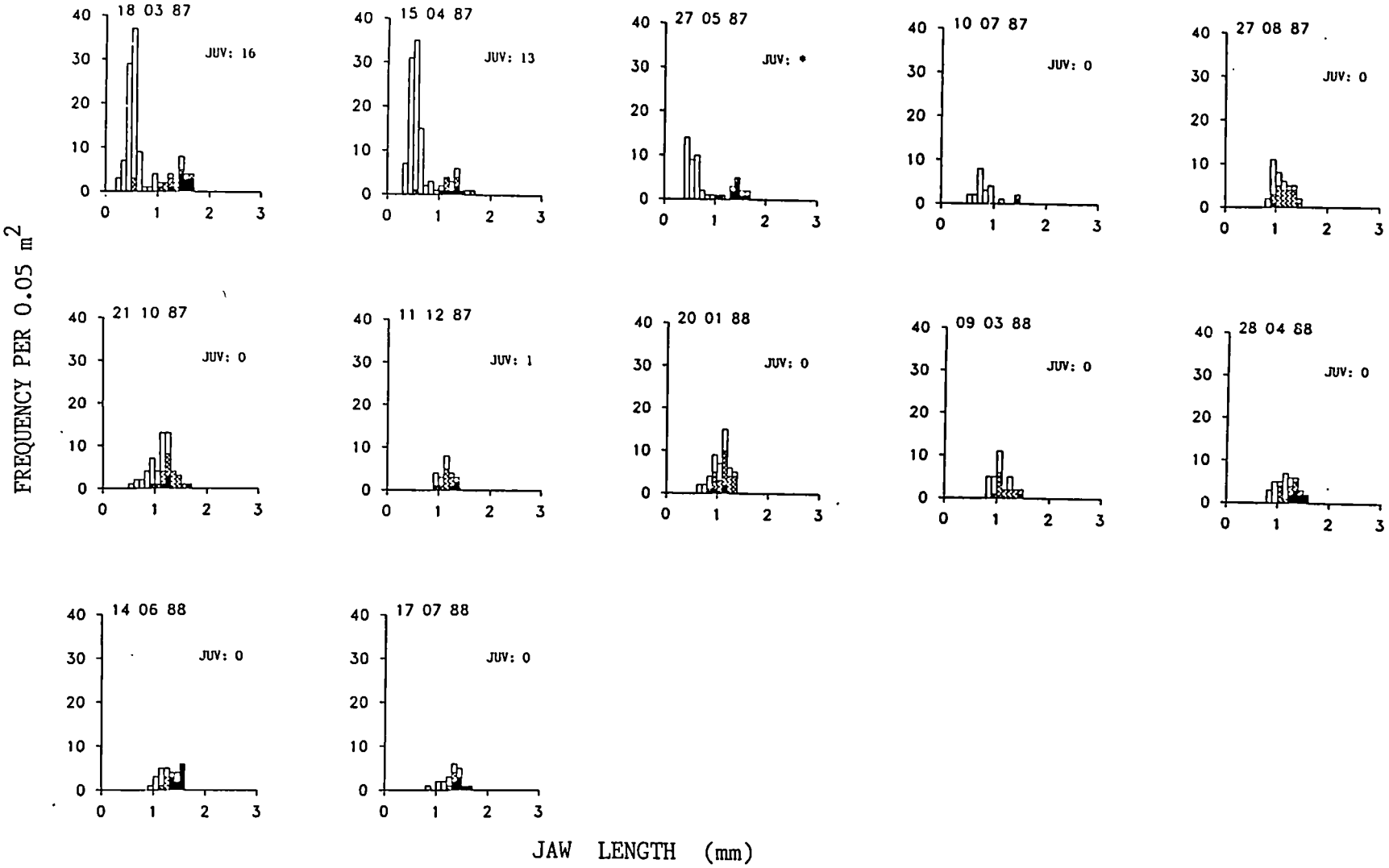
Time-series plots of size frequency histograms for Whitton animals.

Dark shading = animals with mean oocyte diameter > 80 μm.

Light shading = animals with mean oocyte diameter < 80 μm.

JUV = number of juveniles (worms < 15 mm) in surface core of 0.01 m^2.

* = spoilt core.


18 03 87
JUV: 16
15 04 87
JUV: 13
27 05 87
JUV: *
10 07 87
JUV: 0
27 08 87
JUV: 0
21 10 87
JUV: 0
11 12 87
JUV: 1
20 01 88
JUV: 0
09 03 88
JUV: 0
28 04 88
JUV: 0
14 06 88
JUV: 0
17 07 88
JUV: 0
FREQUENCY PER 0.05 m2
JAW LENGTH (mm)

GENERAL DISCUSSION

GENERAL DISCUSSION

The main aims of this thesis were to determine the suitability of heavy metal tolerance and the reproductive biology of N. diversicolor as variables for inclusion in marine pollution monitoring schemes. The former approach was advocated by Luoma (1977) who stated, "[f]urther study of toxicant resistance in natural systems would increase its usefulness as a tool in environmental studies." The latter approach was considered by G.E.S.A.M.P. (1980) to be potentially useful for biomonitoring purposes.

Copper and zinc tolerance in N. diversicolor have been compared with the four characteristics of resistance determined by Luoma (1977) from pesticide resistance in insects and metal tolerance in plants. It was considered that any significant departure from these characteristics would limit their use for biomonitoring purposes. The extent to which copper and zinc tolerances conformed to each of Luoma's four points are summarised below:

Luoma point (i). "Resistance results from Darwinian selection for resistant genotypes; it cannot be induced over the life-time of individuals". In Chapter 2 both copper and zinc tolerance were determined to have significant heritable components. In addition, copper tolerance could not be induced in N. diversicolor through long-term exposure to sub-lethal levels.

Luoma point (ii). "Physiological mechanisms specific for a single toxicant or group of toxicants are usually involved in resistance, rather than selection for vigorous strains." The significantly different distributions of copper and zinc tolerance demonstrated in Chapter 1 combined with uptake and body compartmentalisation studies (Bryan and Hummerstone, 1971, 1973; Pirie, Liu Fayi and George, 1985) unequivocally

show different physiological mechanisms to be responsible for these tolerances.

Luoma Point (iii). "Resistance reduces the overall fitness of the population; when the selective pressure of the toxicant is removed the populations revert, in a few generations, to dominance by intolerant genotypes." Competition experiments in Chapter 3 provided weak evidence that there was a cost associated with copper tolerance. No such experiments were conducted for zinc tolerance. However, as there is evidence for extensive larval and adult movement in estuaries (Davey and George, 1986a,b; Dankers and Binsbergen, 1984) with the resultant genetic interchange between populations (a possibility not excluded by allele frequency analysis), the disjunct cline in zinc tolerance is unlikely to be explained through genetic isolation. It is more likely a result of natural selection which implies a cost associated with zinc tolerance. However, considerably more work is required in this area.

Luoma point (iv). "The degree of tolerance is related to the level of exposure to the toxicant." Chapter 1 demonstrated that tolerance was roughly related to environmental levels. However, salinity and bioavailability can affect the toxicity of a given environmental concentration resulting in difficulties establishing precise threshold concentrations where detrimental ecological effects begin.

Although there is still some uncertainty over the effect of tolerance on fitness (especially zinc), it would appear that copper and zinc tolerances in N. diversicolor conform reasonably well to Luoma's four characteristics of resistance. The distribution of tolerance would therefore appear to have the basic qualities suitable for biomonitoring purposes. A large number of other factors must be considered however. Its usefulness in pollution monitoring is also dependant on performance in a number of criteria established by G.E.S.A.M.P. (1980). These criteria

were based on the findings of previous workshops (e.g. I.C.E.S., 1978; F.A.O., 1977) and are loosely packaged into three categories;

A: Fundamental scientific aspects of assessing the biological impact of an environmental change.

B: Efficiency of the biological measurement and practical value as an indicator of impact.

and C: Administration.

G.E.S.A.M.P. evaluated 37 biological variables using these criteria (which will be mentioned in detail later) and categorised them according to their suitability for immediate use in monitoring programmes. 20 variables were considered "highly recommended". These included lysosomal stability, feeding rate, 'scope for growth', ratio of oxygen consumed to nitrogen excreted, fin erosion, ulcers and vertebral damage (biochemical, physiological and morphological affects). Highly recommended studies based on ecological effects were community biomass, abundance, diversity, alterations in distribution, species density, growth rate and population structure.

The distribution of inherited tolerance as a biological variable was not considered by G.E.S.A.M.P. This is not entirely surprising as the report was published in 1980 and two of the three examples of inherited metal tolerance in aquatic metazoans were not published until later (Nevo, Ben-Shlomo and Lavie, 1984; Klerks and Levinton, 1987). As tolerance distribution would appear to have enormous potential as an indicator of marine pollution, it requires evaluation using the same criteria recommended by G.E.S.A.M.P. (1980). Heavy metal tolerance in N. diversicolor will be considered as an example.

Category A: 1. Ecological significance: Can tolerance be shown to be related to an adverse effect on the growth, reproduction or survival of the individual or the population, and ultimately on the well-being of the community/ecosystem? The presence of inherited metal tolerance is

direct evidence that metals are having an adverse effect on a particular population, as the contamination must have selected against non-tolerant genotypes.

2. Relevance to other effects: Can tolerance be related to other effects at higher or lower levels of organisation? Although tolerance is determined at the individual and population level, the distribution of tolerance enables the extent of metal impact within an ecosystem to be determined.

3. Specificity: How specific is tolerance in relation to the causative agent? As different physiological mechanisms are involved in copper and zinc tolerance the response to the causative agent is completely specific with respect to these metals. There is however an indication that tolerance to silver depends on the presence of tolerance to copper and perhaps lead (Bryan, 1976). It would therefore be important for silver tolerance to be correlated with high environmental levels of silver, and low levels of copper and lead before confidently attributing an ecological impact to silver.

4. Reversibility: To what degree can tolerance return to its original level when the causative agent is removed? As copper and zinc tolerances are genetically determined, it is irreversible within an individual. Within a population, tolerance is reversible but the rate of change will depend on the cost associated with the tolerance. If selection against tolerant genotypes in uncontaminated conditions is weak (as suggested by the competition experiment), it will take many generations before tolerance in a population is removed. The rate of change will be more rapid if the cost (and hence selection) is greater. In the special case of estuarine metal contamination the question is probably purely academic. Even after removal of the causative agent (e.g. an industrial discharge) environmental levels remain high because

estuarine sediments are efficient traps of metal contamination (Turekian, 1977; Bryan et al, 1987).

5. Range of taxa: Is tolerance specific to particular taxa? Inherited metal tolerance has been identified in bacteria (Nelson and Colwell, 1975; Timoney and Port, 1982), algae (Russell and Morris, 1970) annelids (Klerks and Levinton, 1987), molluscs and crustacea (Nevo, Ben-Shlomo and Lavie, 1984). There is therefore nothing to indicate that tolerance is restricted to particular taxa.

Category B. 1. Quantitive aspects: Does tolerance bear a quantitative or predictable relationship to the cause (i.e. pollution)? The magnitude of copper and zinc tolerances in N. diversicolor accurately reflect the extent of environmental impact at a site. The cost associated with copper tolerance ensures that the impact is not over-estimated. 'Hard' selection against non-tolerant genotypes ensures it is not under-estimated. It is important to emphasise that the influences of salinity and bioavailability result in tolerance magnitude being related to the level of impact and not just environmental levels.

2. Sensitivity: What intensity of stressor is required to elicit tolerance? It is not possible to precisely state the environmental levels of copper and zinc that elicit tolerance for reasons stated above. The presence of copper tolerant worms in sediment levels of approximately 700 $\mu g.g^{-1}$ copper (Hayle estuary; Bryan, 1976) suggests levels above approximately 500-600 $\mu g.g^{-1}$ are required for tolerance. Similarly, sediment levels of approximately 4000 $\mu g.g^{-1}$ zinc appear to be required before there is sufficient bioavailable zinc to elicit tolerance. Evidence that a toxicant has exerted selective pressure on an ecological 'opportunist' like N. diversicolor makes it likely that it is having adverse effects on other more specialised species within a community (Luoma, 1977). It is probable that these more sensitive species will be seriously affected by environmental metal levels below those required to

elicit tolerance in N. diversicolor (e.g. Cerastoderma edule in Restronguet Creek; Bryan et al, 1987). Thus, although heavy metal tolerance in N. diversicolor is a sensitive method for detecting ecological impact on populations of N. diversicolor, it is probably an insensitive determinant of impact on other species.

3. Scope: Over what range of intensity of stressor is tolerance observable? Tolerant animals were found in sediment concentrations in excess of 3500 $\mu g.g^{-1}$ copper and 5000 $\mu g.g^{-1}$ zinc. These were the highest levels recorded in Restronguet Creek and they occurred at a site of low mean salinity (R1; approximately $9^{o}/_{oo}$). Copper and zinc are more toxic at low salinities (McLusky, Bryant and Campbell, 1986) indicating that higher environmental concentrations could probably be tolerated at more favourable salinities. Higher sediment metal concentrations have been found in Sorfjord (Norway) (Skei, Price and Calvert, 1972) but the occurrence or distribution of N. diversicolor within the fjord have not been studied.

4. Response rate: How quickly is there an observable effect? It is difficult to state precisely how quickly measurable levels of tolerance will appear after significant impact by metal contamination. The rate of response will depend on the heritability of tolerance (Falconer, 1960) and also on the rate of contamination (i.e. the rate of selection). Even if selection was fast (e.g. sudden heavy contamination), it would take a few generations for the tolerance to become measurable and therefore the response rate must be measured in years. Heavy metal tolerance in N. diversicolor in Restronguet Creek has evolved over 200 years, but as tolerance in the oligochaete Limnodrilus hoffmeisteri appeared within 30 years (Klerks and Levinton, 1987) the true response time was probably an order of magnitude less. Allele frequency analysis and studies of larval and adult movement indicate that once tolerance has developed in one part of an estuary, recolonisation of severely impacted sites would be rapid.

5. Signal/noise ratio: Can the signal (tolerance) be easily detected above the noise (natural variability)? The LT_{50} for copper tolerant worms was approximately five times greater than that for non-tolerant worms. For zinc tolerance, the difference was a factor of two. As there is no overlap between the 95% confidence limits for tolerant and non-tolerant animals for both metals (Figures 1.1 and 1.2), the natural variability does not obscure the signal. Fully tolerant or partially tolerant populations can easily be detected provided sufficient animals are tested, control animals are included, and suitable statistical analyses are applied.

6. Precision: Can tolerance be measured accurately and precisely? The measurement of tolerance within an individual is very precise as it is the time taken until mortality. The tolerance of a population is also accurately determinable through relative performance in toxicity tests when compared with control animals.

Category C: 1. Cost: How expensive is the measurement of tolerance in terms of capital equipment, running costs, training costs and manpower? Static toxicity tests are quick (96 hours or less if LC_{50} data used), simple and very inexpensive. They also require minimal supervision.

2. Application: To what extent has tolerance been used in a field monitoring programme and shown to be related to pollution? This is the first study in which heavy metal tolerance in N. diversicolor has been used to assess the ecological impact of metals within an estuary. The results of the trial demonstrate that the distribution of tolerance is a direct measure of pollutant effect. As other estuaries are known to harbour populations of tolerant N. diversicolor (e.g. Hayle estuary, Cornwall; Bryan, 1976), further trials are possible and recommended.

In summary, the only major concerns of using N. diversicolor tolerance distribution as a pollution monitoring variable are its

insensitivity and slow response rate. Its performance in the other criteria established by G.E.S.A.M.P. (1980) were very favourable and the cheapness and simplicity of operation would probably give it a high general evaluation. Bearing these points in mind the next stage would be to determine where in an estuarine pollution monitoring scheme metal tolerance would be most suitable. G.E.S.A.M.P. (1980) recognised three phases in a monitoring programme;

Phase I: Identifcation

Phase II: Quantification

Phase III: Causation

'Identification' is concerned with detecting 'hot-spots' of contamination to enable effort in the later phases to be concentrated where pollutant effects are most likely. 'Quantification' is the period where the degree or extent of the pollutant effect is determined. 'Causation' is establishing the specific contaminant or contaminants responsible for the pollution.

The variables suitable for study in the 'identification' phase must be sensitive, precise and have a rapid response rate. G.E.S.A.M.P. (1980) and I.C.E.S. (1978) recommended study of contaminant levels in the sediment and biota (biological indicators) combined with observations of morphological abnormalities in fish and bioassays of water samples on oyster larvae. The relatively low sensitivity and slow response rate of heavy metal tolerance in N. diversicolor makes it wholly unsuitable for implementation at this stage.

At present, the best monitoring variables available for determination of 'quantification' and 'causation' can be broadly divided into two groups; those measuring biochemical, physiological and morphological effects (nominally called biological effects) and those measuring population/species distribution (ecological effects).

The principal advantages of biological effects measurements are that they are sensitive, respond rapidly and can be quantitative to the effects of pollution. Bayne et al (1979) discussed the benefits of three such variables that were rated highly by G.E.S.A.M.P. (1980). Scope for growth is the energy available to an organism from the food consumed minus loss through respiration, excretion and egestion. A similar physiological index is the ratio of oxygen consumed to nitrogen excreted (O:R ratio). Both have been evaluated in field monitoring programmes using Mytilus edulis in the contaminated Narragansett Bay. There was a clear relationship between environmental condition and physiological condition using these indices demonstrating their value in the 'quantification' phase of pollution monitoring. A cytochemical index of stress was also discussed (lysosomal stability). The lysosomal membrane is often a target for injury by contaminants and damage results in destabilisation of the membrane. The stability of the membrane can be measured and is related to the degree of stress (Bayne et al, 1970; Moore et al, 1986).

Although biochemical and physiological variables are very sensitive and responsive measures of stress at the cellular and individual level, it is very difficult extrapolating such effects to the population and ecosystem level. They should therefore only be used in monitoring programmes in conjunction with other effects measurements at higher levels of organisation in order to distinguish a physiological effect from an ecological effect.

The biggest drawback of biological effects measurements are their lack of specificity. Scope for growth, O:N ratio and lysosomal stability are all measures of a general syndrome of stress but they do not allow the identification of specific stressors. Efforts have been made to fine-tune biochemical measurements to detect the effects of individual contaminants. Few have been developed successfully, the notable

exceptions with respect to metal contamination being specific inhibition of a blood enzyme and metallothionein induction. In the former case, as little as 0.01 $mg.l^{-1}$ lead was required to depress erythrocyte D-amino lavulinic acid dehydratase activity in the rainbow trout Salmo gairdneri. This effect was lead specific as copper, cadmium, zinc and mercury had no inhibiting effect (Hodson et al, 1977). Metallothionein induction in salmon may be metal specific as high environmental levels of copper induced synthesis of copper-thionein but not zinc-thionein (McCarter and Roch, 1984). Similarly, metals in mussels appear to be associated with different metallothioneins (Pavicic et al, 1987). Not only are biochemical measurements which are useful for 'causation' rare, but they are also prohibitively expensive for extensive use (G.E.S.A.M.P., 1980).

The ultimate interest in pollution-induced disturbance is at the population/community level of organisation. As there are difficulties in extrapolating biological effects at the biochemical, cellular or individual level to the community as a whole, direct measurements of ecological variables are required. Ecological effects measurements are, however, less sensitive, responsive and quantitative than biological effects (G.E.S.A.M.P., 1980). They are also harder to interpret because one rarely has the luxury of historic data on which to construct a base-line for comparison. A number of methods have been proposed for identifying significant changes in aquatic communities:

The presence or absence of key indicator species may demonstrate a polluted environment. Reish (1979) discussed the usefulness of Capitella capitata as an indicator of marine or estuarine pollution. Since Capitella has a short life-history and can reproduce all year round, it is capable of rapidly colonising an area following the demise of the resident population through pollution (Grassle and Grassle, 1974). However, Capitella is also found in large numbers in unpolluted estuaries, emphasising the importance of historic data (Reish, 1979).

Rafaelli and Mason (1981) noted that the ratio of nematodes to copepods was extremely high in samples taken from polluted beaches and proposed this as an ecological effects monitoring variable. Lambshead (1984) has since shown that the ratio varies with a variety of environmental parameters and recommended the ratio be abandoned as a practical pollution indicator.

Gray and Mirza (1979) suggested that a departure from the log-normal distribution of individuals/species at á site was accompanied by significant pollution. There is, however, some doubt as to whether undisturbed benthic communities actually conform to the log-normal model (Lambshead and Platt, 1985; Hughes, 1985). Similarly, the 'ABC' method ('abundance/biomass comparison') proposed by Warwick (1986), where the distribution of numbers of individuals among species behaves differently from the distribution of biomass among species under pollution induced stress, has been shown to be unsuitable for use in tidal flat communities (Beukema, 1988). Consequently, there are considerable difficulties in quantifying pollutant impact through studies on ecological variables such as community biomass, abundance, alterations in species distribution etc. These problems are particularly exacerbated in estuaries with their inherent low species diversity. Ecological effects are also non-specific to pollutants and therefore cannot be implemented in the 'causation' phase.

The greatest void in pollution monitoring programmes would therefore appear to be direct evidence for the causative agent(s). As ecological variables and the vast majority of biochemical/physiological variables are non-specific, the causative agent must be determined through circumstantial evidence from chemical analyses of the sediment/biota and through bioassays (G.E.S.A.M.P., 1980). Clearly the specificity of metal tolerance is an invaluable device for determining

causation, particularly when the contamination is a complex mixture of metals.

The distribution of inherited metal tolerance is also an ideal mechanism for quantifying the ecological impact at a site (Luoma, 1977). It has considerable advantages over both biological effects measurements (through ecological relevance) and ecological effects measurements (through ease of interpretation and sensitivity). Klerks and Levinton (1987) also determined the latter case when studying metal pollution of Foundry Cove, Hudson River. They concluded, "Our survey for differences in resistance to pollutants has proved more sensitive in detecting effects of pollution than a taxonomic survey".

In conclusion, the distribution of inherited metal tolerance as a general approach to pollution monitoring would appear to be vindicated through the evaluation of tolerance in N. diversicolor. Other examples will have to be considered on their own merits but in principle it would appear to be a very powerful biomonitoring tool.

The occurrence of inherited metal tolerance also has relevance to the establishment of environmental quality standards (EQSs). At present, the lack of information on the sort of levels in the field that produce an ecological effect have resulted in EQSs being set in the laboratory. In the U.K., the lowest chronic toxicity value obtained from toxicity tests is arbitrarily divided by an application factor (10 in the case of copper; Gardiner and Mance, 1984). As there are difficulties in extrapolating results from the laboratory to the field (Burton, 1979; Lee, 1973) there is uncertainty how realistic these figures may be. Could the distribution of inherited metal tolerance be used as a basis for calculating EQSs? It might be argued that the spatial information on ecological impact enables effort to be concentrated on determining which aspects of the sediment and water chemistry are correlated with the

ecological effect. Consequently, EQSs directly relevant to field conditions could then be determined. Unfortunately, this approach is limited by the number of species that have the genetic diversity to develop tolerance (Nevo et al, 1986). Specialised (narrow-niche) species may be more sensitive to the effects of contamination but lack the genetic plasticity to develop tolerance. Conceivably, they will be eliminated by levels of contamination that would be too low to elicit tolerance in the more robust, broad-niche species. There is therefore a danger that EQS values established this way will be set too high.

In contrast to tolerance distribution, studies on the gametogenic cycle and population dynamics of N. diversicolor demonstrated them to be wholly unsuitable variables for inclusion in pollution monitoring schemes. Tolerance evolved in a long-term contaminated estuary enabled populations at even the most contaminated sites to apparently reproduce normally. In short-term contaminated estuaries (where tolerance would not have had time to evolve), it is probable that the natural variation in length of gametogenesis, breeding age, breeding size and timing of spawning would obscure any pollution related effect. Although G.E.S.A.M.P. (1980) highly recommended the monitoring of population structure and considered gametogenesis appropriate for inclusion in monitoring programmes, the unsuitability of studies on N. diversicolor reproduction only serves to illustrate how each species must be evaluated independantly.

SUMMARY

SUMMARY

Studies of reproductive biology and metal tolerance in Nereis diversicolor have been assessed for their potential value as biomonitoring tools. These variables were studied in populations from the heavily contaminated Restronguet Creek, the moderately contaminated Humber and the uncontaminated Avon estuary.

Populations at even the most contaminated sites completed gametogenesis and apparently spawned and recruited successfully. There are substantial variations in gametogenesis and life-history between sites which are not related to levels of contamination. Studies of population dynamics and gametogenesis in this species thus appear to be of limited value for pollution monitoring purposes.

By contrast, metal tolerance would appear to have considerable potential as an approach to biomonitoring. Copper and zinc tolerances have substantial heritable components, copper tolerance is not induced by exposure to sub-lethal metal concentrations and appears to confer a competitive disadvantage in uncontaminated environments. Copper and zinc tolerance are independent phenomena.

In view of this, the presence of metal tolerant animals at a site is strong evidence of ecological impact. Such impact is restricted to the most contaminated parts of Restronguet Creek (total sediment concentration; 3000 $\mu g.g^{-1}$ copper and 4000 $\mu g.g^{-1}$ zinc) although there is not a perfect correlation between total metal levels and ecological impact.

Information is also presented on the degree of genetic differentiation within and between estuaries.

BIBLIOGRAPHY

BIBLIOGRAPHY

Banat, K., U. Forstner and G. Muller, 1972. Schwermetalle in sedimenten von Donau, Rhein, Ems, Weser und Elbe im bereich der Bundesrepublik Deutschland.- Naturwissenschaften **59**: 525-528.

Barnes, R.S.K., 1978. Variation in paragnath number of Nereis diversicolor in relation to sediment type and salinity regime.- Est. Coast. Mar. Sci. **6**: 275-283.

Barnes, R.S.K. and S.M. Head, 1977. Variation in paragnath number in some British populations of the estuarine polychaete Nereis diversicolor.- Est. Coast. Mar. Sci. **5**: 771-781.

Barnett, B.E. and C.R. Ashcroft, 1989. Heavy metals on the South Bank of the Humber estuary.- Mar. Poll. Bull. **20**: 17-21.

Bayne, B.L., P.A. Gabbott and J. Widdows, 1975. Some effects of stress in the adult on the eggs and larvae of Mytilus edulis.- J. Mar. Biol. Ass. U.K. **55**: 675-689.

Bayne, B.L., M.N. Moore, J. Widdows, D.R. Livingstone and P. Salkeld, 1979. Measurement of the responses of individuals to environmental stress and pollution; studies with bivalve molluscs.- Phil. Trans. R. Soc. Lond. B. **286**: 563-581.

Ben-Shlomo, R. and E. Nevo, 1988. Isozyme polymorphism as monitoring of marine environments: The interactive effect of cadmium and mercury pollution on the shrimp, Palaemons elegans.- Mar. Poll. Bull. **19**: 314-317.

Beukema, J.J., 1988. An evaluation of the ABC-method (abundance/biomass comparison) as applied to macrozoobenthic communities living on tidal flats in the Dutch Wadden Sea.- Mar. Biol. **99**: 425-433.

Bloxam, T.W., S.N. Aurora, L. Leach and T.R. Rees, 1972. Heavy metals in some river and bay sediments near Swansea.- Nature (Phys. Sci.) **239**: 158-159.

Bogucki, M., 1953. The reproduction and the development of Nereis diversicolor (O.F. Muller) in the Baltic.- Polskie Archiwm Hydrobiol. **1**: 251-270.

Bogucki, M., 1962. Rearing N. diversicolor in laboratory conditions.- Przeglad Zool. **VI**: 232-234.

Boyden, C.R., 1977. Effect of size upon metal content of shellfish.- J. Mar. Biol. Ass. U.K. **57**: 675-714.

Bradshaw, A.D., 1970. Pollution and plant evolution.- New Sci. **48**: 497-500.

Brewer, J.M., 1967. Artifact produced in disc electrophoresis by ammonium persulphate.- Science **156**: 256-257.

Brown, A.W.A. and R. Pal, 1971. Insecticide resistance in arthropods.- World Health Organisation, Geneva: 491pp.

Brown, B.E., 1976. Observations on the tolerance of the isopod Asellus meridianus Rac. to copper and lead.- Wat. Res. **10**: 555-559.

Brown, B.E., 1978. Lead detoxification by a copper tolerant isopod.- Nature **276**: 388-390.

Bryan, G.W., 1974. Adaptation of an estuarine polychaete to sediments containing high concentrations of heavy metals.- *In* Vernberg, F.W. and W.B. Vernberg (eds.): Pollution and physiology of marine organisms. Acad. Press, New York: 123-135.

Bryan, G.W., 1976. Some aspects of heavy metal tolerance in aquatic organisms.- *In* A.P.M. Lockwood (ed.): Effects of pollutants on aquatic organisms. Cambridge University Press: 3-35.

Bryan, G.W., 1984. Pollution due to heavy metals and their compounds.- *In* O. Kinne (ed.): Marine Ecology V. Wiley, Chichester: 1289-1431.

Bryan, G.W. and P.E. Gibbs, 1980. Metals in nereid polychaetes: the contribution of metals in the jaws to the total body burden.- J. Mar. Biol. Ass. U.K. **60**: 641-654.

Bryan, G.W. and P.E. Gibbs, 1983. Heavy metals in the Fal Estuary, Cornwall: A study of long-term contamination by mining waste and its effects on estuarine organisms.- Mar. Biol. Ass. Occasional Publication No. 2, Plymouth: 112pp.

Bryan, G.W., P.E. Gibbs, L.G. Hummerstone and G.R. Burt, 1987. Copper, Zinc, and organotin as long-term factors governing the distribution of organisms in the Fal estuary in South West England.- Estuaries **10**: 208-219.

Bryan, G.W. and L.G. Hummerstone, 1971. Adaptation of the polychaete Nereis diversicolor to estuarine sediments containing high concentrations of heavy metals. 1. General observations and adaptations to copper.- J. Mar. Biol. Ass. U.K. **51**: 845-863.

Bryan, G.W. and L.G. Hummerstone, 1973. Adaptation of the polychaete Nereis diversicolor to estuarine sediments containing high concentrations of zinc and cadmium.- J. Mar. Biol. Ass. U.K. **53**: 839-857.

Bryan, G.W. and L.G. Hummerstone, 1977. Indicators of heavy metal contamination in the Looe estuary (Cornwall) with particular regard to silver and lead.- J. Mar. Biol. Ass. U.K. **57**: 75-92.

Bryan, G.W., W.J. Langston and L.G. Hummerstone, 1980. The use of biological indicators of heavy metal contamination in estuaries, with special reference to an assessment of the biological availability of metals in estuarine sediments from South West Britain.- Mar. Biol. Ass. Occasional Publication No. 1, Plymouth: 73pp.

Burton, J.D., 1979. Physico-chemical limitations in experimental investigations.- Phil. Trans. R. Soc. Lond. B. **286**: 443-456.

Calabrese, A. and H.C. Davis, 1966. The pH tolerance of embryos and larvae of Mercenaria mercenaria and Crassostrea virginica.- Biol. Bull. **131**: 427-436.

Chambers, M.R. and H. Milne, 1975. Life cycle and production of Nereis diversicolor O.F. Muller in the Ythan estuary, Scotland.- Est. Coast. Mar. Sci. **3**: 133-144.

Clark, R.B. and R.J.G. Ruston, 1963. The influence of brain extirpation on oogenesis in the polychaete Nereis diversicolor.- Gen. Comp. Endocr. **3**: 529-541.

Connor, P.M. and K.W. Wilson, 1972. A continuous-flow apparatus for assessing the toxicity of substances to marine animals.- J. Exp. Mar. Biol. Ecol. **9**: 209-215.

Cook, S.C.A., C. Lefebvre and T. McNeilly, 1972. Competition beween metal tolerant and normal plant populations on normal soil.- Evolution **26**: 366-372.

Crisp, D.J. and K. Ekaratne, 1984. Polymorphism in Pomatoceros.- J. Linn. Soc. (Zool.) **80**: 157-175.

Dales, R.P., 1950. The reproduction and larval development of Nereis diversicolor O.F. Muller.- J. Mar. Biol. Ass. U.K. **29**: 321-360.

Dales, R.P., 1951. An annual history of a population of Nereis diversicolor O.F. Muller.- Biol. Bull. **101**: 131-137.

Dankers, N. and M.A. Binsbergen, 1984. The polychaete Nereis diversicolor is also migrating.- Levende Nat. **85**: 14-15.

Davey, J.T. and C.L. George, 1986a. Factors in the distribution of intertidal, estuarine polychaetes: A field experiment with Nereis (Hediste) diversicolor and Nephtys hombergi in the Tamar at Plymouth.- Est. Coast. Shelf Sci. **22**: 603-618.

Davey, J.T. and C.L. George, 1986b. Species interactions in soft sediments: Factors in the distribution of Nereis (Hediste) diversicolor in the Tamar estuary.- Ophelia **26**: 151-164.

DiMichele, L. and D.A. Powers, 1982. LDH-B genotype-specific hatching times of Fundulus heteroclitus embryos.- Nature **296**: 563-564.

Edwards, A.M.C., R. Freestone and C. Urquhart, 1987. The water quality of the Humber estuary 1986.- Humber Estuary Committee: 41pp.

Elderfield, H., L. Thornton and J.S. Webb, 1971. Heavy metals and oyster culture in Wales.- Mar. Poll. Bull. **2**: 44-47.

Endler, J.A., 1973. Gene flow and population differentiation.- Science **179**: 243-250.

Evans, S.M., 1973. A study of fighting reactions in some nereid polychaetes.- Anim. Behav. **21**: 138-146.

Falconer, D.S., 1960. Introduction to quantitative genetics. Oliver and Boyd, Edinburgh: 365pp.

Fantes, K.H. and I.G.S. Furminger, 1967. Proteins, persulphate and disc electrophoresis.- Nature **215**: 750-751.

F.A.O., 1977. Manual of methods in aquatic environment research. Part 4. Bases for selecting biological tests to evaluate marine pollution.- F.A.O. Fisheries Technical Paper 164, F.A.O., Rome: 31pp.

Ferguson, A., 1980. Biochemical systematics and evolution. Blackie, Glasgow: 194pp.

Fernandez, T.V., 1983. Some studies on the toxic effects of heavy metals to a polychaete Hediste diversicolor with particular reference to zinc.- Ph.D. Thesis, University of Hull: 160pp.

Forstner, U. and G.T.W. Wittmann, 1979. Metal pollution in the aquatic environment.- Springer-Verlag, Berlin.

Fraser, J., D.T. Parkin and E. Verspoor, 1978. Tolerance to lead in the freshwater isopod Asellus aquaticus.- Wat. Res. **12**: 637-641.

Fraser, J., 1980. Acclimation to lead in the freshwater isopod Asellus aquaticus.- Oecologia **45**: 419-420.

Gameson, A.L.H., 1976. Routine surveys of the tidal waters of the Humber basin. 1. Physical parameters.- Water Research Centre Technical Report TR25: 51pp

Gardiner, J. and G. Mance, 1984. Environmental quality standards for list II substances. Water Research Centre Technical Report TR206: 12pp.

Gartside, D.W. and T. McNeilly, 1974. Genetic studies in heavy metal tolerant plants II. Zinc tolerance in Agrostis tenuis.- Heredity **33**: 303-308.

G.E.S.A.M.P. (IMCO/FAO/UNESCO/WMO/WHO/IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of Marine Pollution), 1980. Monitoring biological variables related to marine pollution.- Reports and Studies **12**: 22pp.

Gibbs, P.E., G.W. Bryan, P.L. Pascoe and G.R. Burt, 1987. The use of the dog-whelk Nucellus lapillus, as an indicator of tributyltin (TBT) contamination.- J. Mar. Biol. Ass. U.K. **67**: 507-523.

Gillet, P., 1987. Bioaccumulation du cuivre et du zinc chez Nereis diversicolor (Annélide Polychète) de l'estuaire du Bou Regreg (Maroc).- Cah. Biol. Mar. **28**: 339-350.

Goldberg, E. and T. Wuntch, 1967. Electrophoretic and kinetic properties of Rana pipiens lactate dehydrogenase isozymes.- J. Exp. Zool. **165**: 101-110.

Golding, D.W., 1983. Endocrine programmed development and reproduction in Nereis.- Gen. Comp. Endocr. **52**: 456-466.

Grant, A., 1989. The use of graphical methods to estimate demographic parameters from size/frequency data.- J. Mar. Biol. Ass. U.K. **69**: 367-371.

Grant, A., J.G. Hateley and N.V. Jones, In Press. Interpopulation variation in life history and metal tolerance in Nereis diversicolor (Polychaeta; Nereidae).- *In* J.C. Aldrich (ed.): Phenotypic responses and individuality in aquatic ectotherms. JAPAGA, Dublin: 40-60.

Grant, A. and D. Middleton, Submitted. An assessment of metal contamination in the Humber estuary.

Grant, A., P.J. Morgan and P.J.W. Olive, 1987. Use made in marine ecology of methods for estimating demographic parameters from size/frequency data.- Mar. Biol. **95**: 201-208.

Grant, B.F., 1976. Endrin toxicity and distribution in freshwater: A review.- Bull. Environ. Contam. Toxicol. **15**: 283-290.

Grassle, J.F. and J.P. Grassle, 1974. Opportunistic life histories and genetic systems in marine benthic polychaetes.- J. Mar. Res. **32**: 253-284.

Gray, J.S. and F.B. Mirza, 1979. A possible method for the detection of pollution-induced disturbance on marine benthic communities.- Mar. Poll. Bull. **10**: 142-146.

Gregory, R.P.G. and A.D. Bradshaw, 1965. Heavy metal tolerance in populations of Agrostis tenuis Sibth. and other grasses.- New Phytol. **64**: 131-143.

Hamilton, E.I., P.G. Watson, J.J. Cleary and R.J. Clifton, 1979. The geochemistry of recent sediments of the Bristol Channel - Severn Estuary system.- Mar. Geol. **31**: 139-182.

Heip, C. and R. Herman, 1979. Production of Nereis diversicolor O.F. Muller (Polychaeta) in a shallow brackish-water pond.- Est. Coast. Mar. Sci. **8**: 297-305.

Herpin, R., 1925. Recherches biologiques sur la reproduction et le développement de quelques annélides polychètes.- Bull. Soc. Sci. Ouest Fra. **4**: 1-250.

Hodson, P.V., B.R. Blunt, D.J. Spry and K. Ansten, 1977. Evaluation of erythrocyte delta-amino levulinic acid dehydratase as a short-term indicator in fish of a harmful exposure to lead.- J. Fish. Res. Bd Can. **34**: 501-508.

Hosking, K.G.F. and R. Obial, 1966. A preliminary study of the distribution of certain metals of economic interest in the sediments and waters of the Carrick Roads (West Cornwall) and of its 'feeder' rivers.- Camborne School of Mines Magazine, **66**: 17-37.

Howard, A.G. and G. Nickless, 1974. Protein binding of cadmium, zinc and copper in environmentally insulted limpets Patella vulgata.- J. Chromat. **104**: 457-459.

Howard, L.S. and B.E. Brown, 1983. Natural variations in tissue concentration of copper, zinc and iron in the polychaete Nereis diversicolor.- Mar. Biol. **78**: 87-97.

Hughes, R.G., 1985. A hypothesis concerning the influence of competition and stress on the structure of marine benthic communities.- *In* P.E. Gibbs (ed.): Proceedings of the 19th European Marine Biology Symposium. Cambridge University Press, Cambridge: 391-400.

I.C.E.S., 1978. On the feasibility of effects monitoring.- I.C.E.S. Co-operative Research Report No. 75. Charlottenlund, Denmark: 41pp.

Imber, B.E., J.A.J. Thompson and S. Ward, 1987. Metal-binding protein in the pacific oyster Crassostrea gigas: Assessment of the protein as a biochemical environmental indicator.- Bull. Environ. Contam. Toxicol. **38**: 707-714.

Jain, S.K. and A.D. Bradshaw, 1966. Evolutionary divergence among adjacent plant populations. I. The evidence and its theoretical analysis.- Heredity **21**: 407-441.

Jaffe, D. and J.K. Walters, 1977. Intertidal trace-metal concentrations in some sediments from the Humber estuary.- Sci. Tot. Env. **7**: 1-15.

Jenkins, K.D. and A.Z. Mason, 1988. Relationships between subcellular distributions of cadmium and perturbations in reproduction in the polychaete Neanthes arenaceodentata.- Aquatic Toxicol. **12**: 229-244.

Jones, L.H., N.V. Jones and A.J. Radlett, 1976. Some effects of salinity on the toxicity of copper to the polychaete Nereis diversicolor.- Est. Coast. Mar. Sci. **4**: 107-111.

Jones, M.L., 1970. The presence of certain dehydrogenases among polychaetous annelids as shown by disc electrophoresis.- Comp. Biochem. Physiol. **36**: 605-611.

Kempthorne, O. and O.B. Tandon, 1953. The estimation of heritability by regression of offspring on parent.- Biometrics **9**: 90-100.

Khlebovich, V.V., A.Y. Komendantov and L.A. Yakovishina, 1984. On variation of paragnath number in Nereis diversicolor, N. japonica and N. limnicola (Polychaeta, Nerediae).- Zool. Zh. **62**: 1110-1113.

Klerks, P.L. and J.S. Levinton, 1987. Effects of heavy metals in a polluted aquatic ecosystem. *In* S.A. Levin (ed.): Ecotoxicology. Springer-Verlag, Berlin: 41-67.

Klerks, P.L. and J.S. Weis, 1987. Genetic adaptation to heavy metals in aquatic organisms: A review.- Env. Poll. **45**: 173-205.

Knutzen, J., 1981. Effects of decreased pH on marine organisms.- Mar. Poll. Bull. **12**: 25-29.

Krauskopf, K.B., 1967. Introduction to geochemistry.- McGraw Hill, New York.

Kristensen, E., 1984. Life cycle, growth and production in estuarine populations of the polychaete Nereis virens and N. diversicolor.- Holarct. Ecol. **7**: 249-256.

Kristensen, E., 1988. Factors influencing the distribution of nereid polychaetes in Danish coastal waters.- Ophelia **29**: 127-140.

Kumar, S. and S.C. Pant, 1984. Comparative effects of the sublethal poisoning of zinc, copper and lead on the gonads of the teleost Puntius conchonius Ham.- Toxicol. Lttrs. **23**: 189-194.

Laakso, M., 1965. The bottom fauna in the surroundings of Helsinki.- Ann. Zool. Fenn. **2**: 19-37.

Lambshead, P.J.D., 1984. The nematode/copepod ratio. Some anomalous results from the Firth of Clyde.- Mar. Poll. Bull. **15**: 256-259.

Lambshead, P.J.D. and H.M. Platt, 1985. Structural patterns of marine benthic assemblages and their relationship with empirical statistical models.- *In* P.E. Gibbs (ed.): Proceedings of the 19th European Marine Biology Symposium. Cambridge University Press, Cambridge: 371-380.

Lavie, B. and E. Nevo, 1986. The interactive effects of cadmium and mercury pollution on allozyme polymorphisms in the marine gastropod Cerithium scabridum. Mar. Poll. Bull. **17**: 21-23.

Lee, G.F., 1973. Review paper: Chemical aspects of bioassay techniques for establishing water quality criteria.- Wat. Res. **7**: 1525-1546.

Leppakoski, E., 1975. Assessment of degree of pollution on the basis of macrozoobenthos in marine and brackish-water environments.- Acta. Acad. Abo. Ser. B. **35**(2): 1-91.

Levin, L.A., 1983. Drift tube studies of bay-ocean water exchange and implications for larval dispersal.- Estuaries **6**: 364-371.

Levinton, J.S., 1980. Genetic divergence in estuaries.- *In* V.S. Kennedy (ed.): Estuarine perspectives. Acad. Press, New York: 509-520.

Lewis, J.R., 1978. The implications of community structure for benthic monitoring studies.- Mar. Poll. Bull. **9**: 64-67.

Lowe, D.M. and M.N. Moore, 1979. The cytochemical distributions of zinc (Zn II) and iron (Fe III) in the common mussel, Mytilus edulis, and their relationship with lysosomes.- J. Mar. Biol. Ass. U.K. **59**: 851-858.

Luoma, S.N., 1977. Detection of trace contaminant effects in aquatic ecosystems.- J. Fish. Res. Bd Can. **34**: 436-439.

Luoma, S.N. and G.W. Bryan, 1982. A statistical study of environmental factors controlling concentrations of heavy metals in the burrowing bivalve Scrobicularia plana and the polychaete Nereis diversicolor.- Est. Coast. Shelf Sci. **15**: 95-108.

Macnair, M.R., 1979. The genetics of copper tolerance in the yellow monkey flower, Mimulus guttatus. I. Crosses to non-tolerants.- Genetics **91**: 553-563.

McCarter, J.A. and M. Roch, 1983. Hepatic metallothionein and resistance to copper in juvenile coho salmon.- Comp. Biochem. Physiol. **74**C: 133-137.

McCarter, J.A. and M. Roch, 1984. Chronic exposure of coho salmon to sublethal concentrations of copper. III. Kinetics of metabolism of metallothionein.- Comp. Biochem. Physiol. **77**C: 83-87.

McIntyre, A.D., 1984. What happened to biological effects monitoring?- Mar. Poll. Bull. **15**: 391-392.

McLusky, D.S., V. Bryant and R. Campbell, 1986. The effects of temperature and salinity on the toxicity of heavy metals to marine and estuarine invertebrates.- Oceanogr. Mar. Biol. Ann. Rev. **24**: 481-520.

McNeilly, T., 1968. Evolution in closely adjacent plant populations III. Agrostis tenuis on a small copper mine.- Heredity **23**: 99-108.

Majori, L., G. Nedoclan, G.B. Modonutti and F. Daris, 1978. Study of the seasonal variations of some trace elements in the tissues of Mytilus galloprovincialis taken in the Gulf of Trieste.- Rev. Int. Oceanogr. Med. **49**: 37-40.

Mettam, C., 1979. Seasonal changes in populations of Nereis diversicolor O.F. Muller from the Severn estuary, U.K.- *In* E. Naylor and R.G. Hartnoll (eds.): Cyclic phenomena in marine plants and animals. Pergamon, Oxford: 123-130.

Middleton, D. and A. Grant, Submitted. Heavy metals in the Humber estuary: Scrobicularia clay as a pre-industrial datum.

Moller, P., 1985. Production and abundance of juvenile Nereis diversicolor and oogenic cycle of adults in shallow waters of Western Sweden.- J. Mar. Biol. Ass. U.K. **65**: 603-616.

Moore, M.N., D.M. Lowe, D.R. Livingstone and D.R. Dixon, 1986. Molecular and cellular indices of pollutant effects and their use in impact assessment.- Wat. Sci. Tech. **18**: 223-232.

Moraitou-Apostolopolou, M., M. Kiortsis, V. Verriopoulos and S. Platanistioti, 1983. Effects of copper sulphate on Tisbe holothuriae Humes (Copepoda) and development of tolerance to copper.- Hydrobiologia **99**: 145-150.

Murdock, E.A., A. Ferguson and R. Seed, 1975. Geographical variation in leucine aminopeptidase in Mytilus edulis L. from the Irish coasts.- J. Exp. Mar. Biol. Ecol. **19**: 33-41.

Mustaquim, J., 1988a. Isozyme variation in three Nereis species from five localities in the U.K.- J. Mar. Biol. Ass. U.K. **68**: 549-553.

Mustaquim, J., 1988b. Electrophoretic variation of isozymes in Polydora ciliata complex (Polychaeta: Spionidae).- Comp. Biochem. Physiol. **91**B: 197-205.

Muus, B.J., 1967a. The fauna of Danish estuaries and lagoons.- Medd. Danm. Fisk. Og Havund. **5**: 1-316.

Muus, B.J., 1967b. Some problems facing the ecologist concerning races and subspecies of brackish water animals.- *In* Lauff, G.H. (ed.): Estuaries. A.A.A.S, Washington: 558-563.

Nei, M., 1972. Genetic distance between populations.- Am. Nat. **106**: 283-292.

Nei, M. and A.K. Roychoudhury, 1974. Sampling variances of heterozygosity and genetic distance.- Genetics **76**: 379-390.

Nelson, J.D. and R.R. Colwell, 1975. The ecology of mercury-resistant bacteria in Chesapeake Bay.- Microbiol. Ecol. **1**: 191-218.

Nevo, E., 1978. Genetic variation in natural populations: Patterns and theory.- Theor. Populat. Biol. **13**: 121-177.

Nevo, E., R. Ben-Shlomo and B. Lavie, 1984. Mercury selection of allozymes in marine organisms: Prediction and verification in nature.- Proc. Natl. Acad. Sci. U.S.A. **81**: 1258-1259.

Nevo, E., R. Roy, B. Lavie, A. Beiles and S. Muchtar, 1986. Genetic diversity and resistance to marine pollution.- Biol. J. Linn. Soc. **29**: 139-144.

Noel-Lambot, F., 1975. Distribution of cadmium, zinc and copper in the mussel Mytilus edulis. Existence of cadmium-binding proteins similar to metallothioneins.- Experimentia **32**: 324-326.

Nyberg, D., 1975. Genetic analysis of copper resistance in Paramecium aurelia syngen 4.- Genetics **80**: 463-473.

O'Brien, R.D., 1967. Insecticides: action and metabolism.- Acad. Press, New York: 332pp.

Olive, P.J.W. and P.R. Garwood, 1981. Gametogenic cycle and population structure of Nereis (Hediste) diversicolor and Nereis pelagica from North-East England.- J. Mar. Biol. Ass. U.K. **61**: 193-213.

Olive, P.J.W. and P.R. Garwood, 1983. The importance of long-term endogenous rhythms in the maintenance of reproductive cycles of marine invertebrates: A reappraisal.- Int. J. Invert. Reprod. **6**: 339-347.

Olive, P.J.W., A. Grant and P.D.B. Cowin, 1986. Decision and choice in polychaete reproductive cycles.- Adv. Invert. Reprod. **4**: 371-378.

Oshida, P.S., A.J. Mearns, D.J. Reish and C.S. Word, 1976. The effects of hexavalent and trivalent chromium on Neanthes arenaceodentata (Polychaeta: Annelida).- Southern California Coastal Water Research Project, El Segundo, California: 58pp.

Ozoh, P.T.E., 1986. Studies on the toxicity of copper to various stages in the life-cycle of Hediste (Nereis) diversicolor (O.F. Muller).- Ph.D. Thesis, University of Hull: 127pp.

Pagenkopf, G.K., R.C. Russo and R.V. Thurston, 1974. Effect of complexation on toxicity of copper to fishes.- J. Fish. Res. Bd Can. **31**: 462-465.

Pavicic, J., M. Skreblin, B. Raspor, M. Branica, M. Tusek-znidaric, I. Kregar and P. Stegnar, 1987. Metal pollution assessment of the marine environment by determination of metal-binding proteins in Mytilus sp.- Mar. Chem. **22**: 235-248.

Pesch, C.E. and G.L. Hoffman, 1982. Adaptation of the polychaete Neanthes arenaceodentata to copper.- Mar. Env. Res. **6**: 307-317.

Phillips, D.J.H., 1977. The use of biological indicator organisms to monitor trace metal pollution in marine and estuarine environments - A review.- Env. Poll. **13**: 281-317.

Phillips, D.J.H., 1980. Quantitative aquatic biological indicators.- Applied Science, London: 488pp.

Pirie, B, Liu Fayi and S. George, 1985. Ultrastructural localisation of copper and zinc in the polychaete, Nereis diversicolor, from a highly contaminated estuary.- Mar. Env. Res. **17**: 197-198.

Pritchard, D.W., 1955. Estuarine circulation patterns.- Proc. Amer. Soc. Civil Eng. **81**: 717-811.

Raffaelli, D.G. and C.F. Mason, 1981. Pollution monitoring with meiofauna, using the ratio of nematodes to copepods.- Mar. Poll. Bull. **12**: 158-163.

Reish, D.J., 1979. Bristle worms (Annelida: Polychaeta).- *In* Hart, C.W. and S.L.H. Fuller (eds.): Pollution ecology of estuarine invertebrates. Acad. Press, New York: 77-125.

Reish, D.J. and R.S. Carr, 1978. The effect of heavy metals on the survival, reproduction, development and life-cycles for two species of polychaetous annelids.- Mar. Poll. Bull. **9**: 24-27.

Remane, A. and C. Schlieper, 1971. Biology of brackish water. (Die Binnengewasser, Vol. 25).- Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.

Rice, S.A. and J.L. Simon, 1980. Intraspecific variation in the pollution indicator polychaete Polydora ligni (Spionidae).- Ophelia **19**: 79-115.

Rocovich, S.E. and D.A. West, 1975. Arsenic tolerance in a population of the grass Andropogon scoparius Michx.- Science **188**: 263-264.

Russell, G. and O.P. Morris, 1970. Copper tolerance in the marine fouling alga Ectocarpus siliculosus.- Nature **228**: 288-289.

Saliba, L.G. and M. Ahsanullah, 1973. Acclimation and tolerance of Artemia salina and Ophryotrocha labronica to copper sulphate.- Mar. Biol. **23**: 297-302.

Scheltema, R.S., 1975. Relationship of larval dispersal, gene-flow and natural selection to geographic variation of benthic invertebrates in estuaries and along coastal regions.- *In* L.E. Cronin (ed.): Estuarine research: Volume 1. Acad. Press, New York: 372-391.

Schottler, U., 1978. Investigations of the anaerobic metabolism of the polychaete worm Nereis diversicolor M.- J. Comp. Physiol. **125**: 185-189.

Selander, R.K., S.Y. Yang, R.C. Lewontin and W.E. Johnson, 1970. Genetic variation in the horseshoe crab (Limulus polyphemus), a phylogenetic "relic".- Evolution **24**: 402-414.

Shaklee, J.B., 1975. The role of subunit interactions in the genesis of non-binomial lactate dehydrogenase isozyme distributions.- *In* C.L. Markert (ed.): Isozymes I: Molecular structure. Academic Press, London: 101-118.

Shaw, C.R. and A.L. Koen, 1968. Starch gel zone electrophoresis of enzymes.- *In* I. Smith (ed.): Chromatographic and electrophoretic techniques. Volume II. Zone electrophoresis. Heinemann: 325-364.

Shaw, C.R. and R. Prasad, 1970. Starch gel electrophoresis of enzymes: A compilation of recipes.- Biochem. Gen. **4**: 297-320.

Skei, J.M., N.B. Price and S.E. Calvert, 1972. The distribution of heavy metals in sediments of Sorfjord, West Norway.- Wat. Air Soil Poll. **1**: 452-461.

Slatkin, M., 1985. Rare alleles as indicators of gene flow.- Evolution **39**: 53-65.

Smith, R.I., 1958. On reproductive pattern as a specific characteristic among Nereid polychaetes.- Syst. Zool. **7**: 60-73.

Smith, R.I., 1964. On the early development of N. diversicolor in different salinities.- J. Morph. **114**: 437-464.

Smith, R.I., 1976. Further observations on the reproduction of Nereis diversicolor (Polychaeta) near Tvarminne, Finland, and Kristineberg, Sweden.- Ann. Zool. Fenn. **13**: 179-184.

Stokes, P.M. and S.I. Dreier, 1981. Copper requirement of a copper tolerant isolate of Scenedesmus and the effect of copper depletion on tolerance.- Can. J. Bot. **59**: 1817-1823.

Stokes, P.M., T.C. Hutchinson and K. Krauter, 1973. Heavy metal tolerance in algae isolated from polluted lakes near the Sudbury, Ontario smelters.- Wat. Poll. Res. Can. **8**: 178-201.

Timoney, J.F. and J.G. Port, 1982. Heavy metal and antibiotic resistance in Bacillus and Vibrio from sediments of New York Bight. *In* G.F. Mayer (ed.): Ecological stress and the New York Bight: Science and management. Columbia, Estuarine Research Federation: 235-248.

Todd, C.D., J.N. Havenhand and J.P. Thorpe, 1988. Genetic differentiation, pelagic larval transport and gene flow between local populations of the intertidal marine mollusc Adalaria proxima (Alder and Hancock).- Func. Ecol. **2**: 441-451.

Tulkki, P., 1960. Studies on the bottom fauna of the Finnish southwestern archipelago. I. Bottom fauna of the Airisto Sound.- Ann. Zool.-Bot. Soc. Fenn. Vanamo **21**(3): 1-26.

Turekian, K.K., 1977. The fate of metals in the oceans.- Geochim. Cosmochim. Acta. **41**: 1139-1144.

Turner, K. and T.A. Lyerla, 1980. Electrophoretic variation in sympatric mud crabs from North Inlet, South Carolina.- Biol. Bull. **159**: 418-427.

Urquhart, C., 1971. Genetics of lead tolernce in Festuca ovina.- Heredity **26**: 19-33.

Vareille-Morel, C. and C. Chaisemartin, 1982. Approche des mécanismes de la tolérance naturelle et de l'acclimation aux métaux lourds (Cr et Pb) chez différentes populations d'Austropotamobius pallipes (Le.).- Acta Oecologica Oecol. Applic. **3**: 105-122.

Verriopoulos, G. and D. Hardouvelis, 1988. Effects of sublethal concentration of zinc on survival and fertility in four successive generations of Tisbe.- Mar. Poll. Bull. **19**: 162-166.

Vignocci, M.G., 1981. Variabilita' in una popolazione di Nereis diversicolor in acque salmastre di Marina di Pisa.- Atti Soc. Nat. Mat. di Modena **112**: 5-8.

Warwick, R.M., 1986. A new method for detecting pollution effects on marine macrobenthic communities.- Mar. Biol. **92**: 557-562.

Watling, H.R. and R.J. Watling, 1976. Trace metals in Choromytilus meridionalis.- Mar. Poll. Bull. **7**: 91-94.

Wilkins, D.A., 1960. The measurement and genetical analysis of lead tolerance in Festuca ovina.- Rep. Scott. Pl. Breeding Stn. 85-98.

Wolynetz, M.S., 1979. Maximum likelihood estimation from confined and censored Normal data.- Appl. Stat. **28**: 185-195.

PUBLICATIONS RESULTING FROM THIS WORK

Grant, A., J.G. Hateley and N.V. Jones, 1989. Mapping the ecological impact of heavy metals on the estuarine polychaete Nereis diversicolor using inherited metal tolerance.- Mar. Poll. Bull. **20**: 235-238.

Grant, A., J.G. Hateley and N.V. Jones, In Press. Interpopulation variation in life history and metal tolerance in Nereis diversicolor (Polychaeta; Nereidae).- *In* J.C. Aldrich (ed.): Phenotypic responses and individuality in aquatic ectotherms. JAPAGA, Dublin: 40-60.

Grant, A. and D. Middleton, Submitted. An assessment of metal contamination in the Humber estuary.

Hateley, J.G., A. Grant and N.V. Jones, 1989. Heavy metal tolerance in estuarine populations of Nereis diversicolor.- *In* J.S. Ryland and P.A. Tyler (eds.): Reproduction, genetics and distributions of marine organisms. Olsen & Olsen, Fredensborg: 379-385.

Middleton, D. and A. Grant, Submitted. Heavy metals in the Humber estuary: Scrobicularia clay as a pre-industrial datum.

APPENDIX; ELECTROPHORESIS BUFFERS AND STAINS

The electrode and gel buffers used in the starch gel screening experiments are summarised in Table A.1. The equivalent buffers used in the polyacrylamide gel scoring experiments are summarised in Table A.2.

i) STAINING PROCEDURES

a) Arginine phosphokinase (APK):

Stain buffer: 30 ml 0.2M Tris/HCl, pH 8.0

Tris	22.1 g
H_2O to	1 litre

pH adjusted with 1.0M HCl.

Stain:

Phospho-L-arginine	10 mg
$MgCl_2.6H_2O$	10 mg
ADP	15 mg
NADP	4 mg
Glucose	30 mg
MTT	7 mg
G6PDH/HK	80/160 units
PMS	Pinch

An agar overlay was used to improve resolution. 0.5 g agar was dissolved in 30 ml H_2O and heated. The warm agar solution was added to the stain and this was immediately poured over the gel. The gel was incubated at 37°C until blue bands appeared.

b) Creatine kinase (CK): Adapted from Shaw and Koen (1968).

Stain buffer: 30 ml 0.2M Tris/HCl, pH 8.0
(As for APK)

Stain:

Phosphocreatine	40 mg
ADP	15 mg
Glucose	30 mg
$MgCl_2.6H_2O$	5 mg
NADP	6 mg
MTT	7 mg
G6PDH/HK	80/160 units
PMS	Pinch

An agar overlay was used (as for APK). The gel was incubated at 37°C until blue bands appeared.

c) Esterase (EST): Adapted from Shaw and Koen (1968).

Stain buffer: 30 ml 0.5M Tris/HCl, pH 7.0

Tris	20 g
H_2O to	1 litre

pH adjusted with 1.0M HCl.

Substrate: Either 0.5% α-naphthyl acetate or 0.5% β-naphthyl acetate.

α or β-naphthyl acetate	0.25 g
Acetone	25 ml
H_2O to	25 ml

Stain:

Fast blue RR	20 mg
α or β-naphthyl acetate solution	2 ml

Incubated at room temperature. Blue/brown bands appeared with α-napthyl acetate as the substrate, pink bands with β-naphthyl acetate as the substrate.

d) Esterase-D (EST-D):

Stain buffer: 30 ml 0.1M Tris/maleic acid, pH 5.3

Tris	12.1 g
Maleic acid	11.6 g
H_2O to	1 litre

pH adjusted with 1.0M NaOH.

Stain:

4-methylumbelliferate	Pinch

Bands would appear when viewed under UV.

e) Hexokinase (HK): Adapted from Shaw and Koen (1968).

Stain buffer: 30 ml 0.5M Tris/HCl, pH 7.0
(As for EST)

Stain:

Glucose	60 mg
$MgCl_2.6H_2O$	10 mg
ATP	15 mg
NADP	4 mg
MTT	7 mg
G6PDH	80 units
PMS	Pinch

Agar overlay used (as for APK). Incubated until dark blue lines appeared.

f) Isocitrate dehydrogenase (IDH): Adapted from Shaw and Koen (1968).

Stain buffer: 30 ml 0.2M Tris/HCl, pH 8.0
(As for APK)

Stain:

NADP	4 mg
MTT	7 mg
Sodium isocitrate	100 mg
$MgCl_2.6H_2O$	10 mg
PMS	Pinch

Incubated at 37°C until blue bands appeared.

g) Lactate dehydrogenase (LDH): Adapted from Shaw and Koen (1968).

Stain buffer: 30 ml 0.2M Tris/HCl, pH 8.0
(As for APK)

Stain:

NAD	15 mg
MTT	7 mg
Sodium-DL-lactate	1 ml
PMS	Pinch

Incubated at 37°C until blue bands appeared.

h) Leucine aminopeptidase (LAP): Adapted from Shaw and Koen (1968).

Stain buffer: 30 ml 0.1M phosphate buffer, pH 7.0

$Na_2HPO_4.7H_2O$	17.2 g
$NaH_2PO_4.2H_2O$	4.94 g
H_2O to	1 litre

pH adjusted with 1.0M NaOH.

Stain:

L-leucyl-β-naphthylamide	25 mg
Fast black potassium salt	25 mg

Incubated at room temperature until black bands appeared.

i) Malate dehydrogenase (MDH): Adapted from Shaw and Koen (1968).
Stain buffer: 30 ml 0.5M Tris/HCl, pH 7.0
(As for EST)

Stain:

L-malic acid	150 mg
MTT	7 mg
NAD	10 mg
PMS	Pinch

Incubated at 37°C until blue bands appeared.

j) Malic enzyme (ME): Adapted from Turner and Lyerla (1980).
Stain buffer: 30 ml 0.2M Tris/HCl, pH 8.0
(As for APK)

Stain:

DL-malic acid	60 mg
NADP	10 mg
$MgCl_2.6H_2O$	10 mg
MTT	7 mg
PMS	Pinch

Incubated at 37°C until blue bands appeared.

k) Peptidase (PEP): Adapted from Shaw and Prasad (1970).
Stain buffer: 30 ml 0.2M Tris/HCl, pH 8.0
(As for APK)

Stain:

L-leucyl leucine	20 mg
Peroxidase	10 mg
L-amino acid oxidase	5 mg
O-dianisidine	5 mg

Agar overlay used (see APK). Incubated at room temperature.

l) Phosphoglucomutase (PGM): Adapted from Shaw and Koen (1968).
Stain buffer: 30 ml 0.2M Tris/HCl, pH 8.0

Stain:

Glucose-1-phosphate	50 mg
$MgCl_2.6H_2O$	70 mg
NADP	4 mg
G6PDH	20 units
MTT	7 mg
PMS	Pinch

Incubated at 37°C until blue bands appeared.

m) 6-phosphogluconate dehydrogenase (6PGDH): Adapted from Shaw and Koen (1968).
Stain buffer: 30 ml 0.2M Tris/HCl, pH 8.0
(As for APK)

Stain:

Sodium-6-phosphogluconate	30 mg
NADP	10 mg
MTT	7 mg
$MgCl_2.6H_2O$	10 mg
PMS	Pinch

Incubated at 37°C until blue bands appeared.

n) Phosphoglucose isomerase (PGI): Adapted from Turner and Lyerla (1980).

Stain buffer: 30 ml 0.2M Tris/HCl, pH 8.0
(As for APK)

Stain:

Fructose-6-phosphate	20 mg
NADP	4 mg
$MgCl_2.6H_2O$	20 mg
MTT	7 mg
G6PDH	20 units
PMS	Pinch

Incubated at 37°C until blue bands appeared.

o) Superoxide dismutase (SOD):

Stain buffer: 30 ml 0.2M Tris/HCl, pH 8.0
(As for APK)

Stain:

MTT	7 mg
$MgCl_2.6H_2O$	20 mg
PMS	Pinch

15 ml of staining solution was added to the gel which was then exposed to UV light for 15 minutes until it turned violet. The remaining 15 ml of stain was added and the gel was incubated at 30°C until white bands appeared.

Table A.1

Buffers used in starch gel screening experiments and the enzyme systems studied.

BUFFER	ELECTRODE BUFFER CONTENTS		GEL BUFFER CONTENTS		ENZYMES SCREENED
Tris/citric acid/EDTA	Tris Citric acid EDTA H_2O pH 7.1	16.35g 8.26g 0.46g 1 litre	Tris Citric acid EDTA H_2O pH 7.1 Dilute 1:15 in gel	16.35g 9.03g 6.90g 1 litre	Hexokinase (HK) Superoxide dismutase (SOD) Malic enzyme (ME) Peptidase (PEP) Isocitrate dehydrogenase (IDH)
Boric Acid/ NaOH	Boric acid NaOH H_2O pH 8.2	18.55g 2.40g 1 litre	Tris Citric Acid H_2O pH 8.2 Dilute 1:15 in gel	9.21g 1.05g 1 litre	6-phosphogluconate dehydrogenase (6PGDH) Malic enzyme (ME) Esterase (ES) Leucine aminopeptidase (LAP) Phosphoglucose isomerase (PGM) Superoxide dismutase (SOD) Malate dehydrogenase (MDH)
Tris/citric acid	Tris Citric acid H_2O pH 6.9	18.20g 10.50g 1 litre	Tris Citric acid H_2O pH 6.9 Dilute 1:14 in gel	18.20g 10.50g 1 litre	Arginine phosphokinase (APK) Creatine Kinase (CK) Esterase-D (EST-D) Leucine aminopeptidase (LAP) Lactate dehydrogenase (LDH)

Table A.2

Buffers used in polyacrylamide gel scoring experiments and the enzyme systems studied.

BUFFER	ELECTRODE BUFFER CONTENTS		ENZYMES STUDIED
Tris/citric acid	Tris	18.20g	Lactate dehydrogenase (LDH)
	Citric acid	10.50g	
	H_2O	1 litre	
	pH 6.9		
Tris/glycine	Tris	6.00g	Esterase (EST)
	Glycine	28.80g	
	H_2O	1 litre	
	pH 8.5		

ACKNOWLEDGMENTS

I wish to extend my sincere gratitude to Dr. Nev Jones and Dr. Alastair Grant for all their help, encouragement and patience during the course of these studies. It couldn't have been easy converting a molecular geneticist into an ecologist!

Thanks also to Dr. Geoff Bryan for stimulating discussions and early help with the project.

To Dick Middleton for technical assistance with the XRF.

Special thanks to Dr. Geoff Oxford at York for advice and discussion on the electrophoresis work. Also to Professor Beardmore and the people in Swansea for all their help and for making me feel welcome.

To Jon Marshall for even more advice on electrophoresis and for letting me make a mess, and to Chris Park for clearing it all up!

To Roland Wheeler-Osman for photography and Sue Taylor for AAS supervision.

A special thank-you to the typist for all her patience and for marrying me.

Finally, I would like to acknowledge my gratitude to Capper Pass Ltd for funding this research.