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Correlative Studies of the Ecophysiology and Community Structure of Benthic Macrofauna

by

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ABSTRACT

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CORRELATIVE STUDIES OF THE ECOPHYSIOLOGY AND COMMUNITY STRUCTURE OF BENTHIC MACROFAUNA

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The use of biochemical measurements in ecological studies are described that quantify the effects on individual organisms of environmental disturbance and to predict subsequent changes at the population or community level. Validation of the methodologies was performed using animals from two sites in the Baie de Somme estuary, France. The population dynamics and biochemistry of two representative species, Cerastoderma edule (Linnaeus) and Nereis diversicolor (O.F. Müller), were studied between September 1990 and September 1992, in addition to the macrofaunal community structure at both sites. The biochemical variables measured were adenosine tri-, di- and monophosphate, arginine or creatine phosphate, succinate, lactate, pyruvate, glucose, glycogen and total carbohydrates, proteins and lipids.

Correlations between the biochemistry and environmental factors revealed detectable differences in the biochemical cycles of both species between the two sites attributable to their differing levels of environmental disturbance. This approach involved the novel application of multivariate analyses to describe the metabolic patterns. The metabolite glycogen was isolated as a representative index which responds to long term environmental disturbance. As such, it could be applied to animals collected from the deep-sea, where the stresses of capture and removal to the surface cause extreme physiological changes and alter levels of short term response metabolites.

The glycogen assay was modified so that it could be applied to formaldehyde-preserved material. Experiments revealed no effects on tissue glycogen levels caused by the preservation procedure or long term storage. The application of this new method to an archive of formaldehyde-preserved specimens showed a potential ability to predict subsequent population changes. Preliminary studies on chemically preserved deep-sea organisms revealed that ecological information can be gained with this index.

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CHAPTER 1

General Introduction

1.1 Background

The term "ecophysiology" requires some definition since it has been used in the literature to describe a multitude of experimental approaches. In the present study the term ecophysiology is used to describe the quantification of the responses of individual organisms to their environment. These responses help to elucidate ecological studies at the population or community level and can assist in the formulation of predictive models of the ecological consequences of environmental change.

Ecological changes are generally the result of environmental change, either natural variability or a more sudden, drastic occurrence which may itself be natural or man-induced. When such a change occurs individual organisms either resist it or adapt to it to a degree within their ability. When this ability is over-stretched, either in magnitude or duration, the organism becomes "stressed" and less able to cope with its environment (the organism's fitness is reduced). Its energetic resources are being used to adapt to or resist the changes and so fewer are available to support metabolism, growth and reproduction. Disease susceptibility will increase (Cheng, 1989; Cheng and Combes, 1990) and ensuing mortalities, coupled with a reduction in reproductive output, will lead to a change in community structure. Different organisms within the community will respond to the same perturbation in different ways, some even benefitting from it. The resulting shifts in community patterns are therefore a consequence of the environmental disturbance. Such a premise has been assumed for many years in ecological studies, where structuring processes have been derived from species abundance data.

The term "disturbance" also requires definition as it has been given different ecological meanings in the literature. Huston (1979) defined disturbance as "environmental change which affects growth rates. It is an unpredictable check reducing, but not necessarily eliminating populations." Petraitis *et al.* (1989) used

a similar more comprehensive definition, stating that disturbance is "any relatively discrete event in time that disrupts ecosystem, community or population structure and changes resources, substrate availability or the physical environment." This definition was considered the most pertinent to the present study but as pointed out by Sousa (1984), any change varies from negligible to extreme depending on the intensity of the force and the susceptibility of the organism. The point at which this force constitutes a disturbance is difficult to decide objectively.

The above definition of Petraitis *et al.* (1989) also includes factors such as the presence of dead organisms or faecal material which may not generally be thought of as a disturbance, but which in deep-sea communities can significantly affect community structure (Smith, 1986).

Changes in a species' environmental fitness, whilst ultimately evident in changes in abundance, are also measurable at the individual, metabolic level. Indeed, this will provide a more accurate indication of the organism's response since abundance changes can be caused by a multitude of factors such as larval recruitment, predation, and mortality on spawning, which are not always quantifiable. When attempting to correlate changes in the levels of biochemical components to environmental disturbances it is essential to consider an animal's natural cycles, both seasonally, as patterns of reproduction and growth will have an effect, and over shorter time-scales. For example, immersion and exposure during a tidal cycle will alter the rate and type of respiration in many bivalves and so alter the balance of metabolites (Hawkins and Hutchinson, 1990). The basal levels of metabolites at the particular stage of the organism's cycles must be known before the influence of disturbance can be inferred. A great deal of work has been carried out on the establishment of suitable physiological indices to detect "stress effects" and these will be reviewed later in this chapter.

There are certain environments, such as the deep sea, where it is extremely difficult to obtain information, other than species abundance data, because of the physical difficulties of sampling. The depths from which the samples are taken mean that the organisms are either dead or dying when brought aboard ship, and in any case, the extreme changes in pressure (and also temperature and light) which occur mean that it is virtually impossible to measure normal *in vivo* metabolic levels, post-capture. *In situ* experiments are also (to date) impractical

and costly.

In the past the deep sea has been thought of as an unchanging, aseasonal environment where metabolic rates are slow and disturbance negligible. This concept has now been disproved; seasonality has been observed in deep sea benthos as a result of pulses of surface phytodetrital material derived from phytoplankton blooms (Billet *et al.*, 1983) and many species of deep-sea echinoderms have seasonal reproductive patterns (Gage and Tyler, 1991). Atmospheric storms may cause periodicity in deep ocean currents (Dickson *et al.*, 1982) and can transport huge loads of fine sediment that dramatically modify the sea floor (Hollister *et al.*, 1984). Intermittent high near-bottom current velocities were recorded at the HEBBLE site, off Newfoundland (High Energy Benthic Boundary Layer Experiment), coincident with abnormally high macrofaunal numbers. It was suggested by Thistle *et al.* (1985) that these currents were bearing enhanced concentrations of food that supported the high faunal densities. Grassle and Morse-Porteous (1987) detected disturbance effects in a deep-sea community resulting from current fluctuations and the influx of organic material. Similarly, Smith (1986) provides experimental evidence to suggest that low-intensity natural disturbance in the form of nekton falls can contribute significantly to community structure. Anthropogenic disturbance is likely to increase in the immediate future as waste disposal to the deep sea floor escalates and resource extraction is undertaken. Thiel (1992) discussed the potential large scale disturbances caused by manganese nodule mining activities.

The deep ocean benthos exhibits unexpectedly high species diversity which has been attributed to the previously perceived long-term stability. It is now thought more credible that the disturbances described earlier create a patchiness which maintains high diversity (Grassle, 1989). An understanding of the variability of deep-sea benthos may reveal the structuring role of disturbance. Disturbance undoubtedly exists in the deep-sea and its effects remain more evident than in shallow water environments where the effects of one disturbance on a community are often obliterated by subsequent events (Grassle and Morse-Porteous, 1987). It would thus be very useful to quantify the effects of such disturbance on deep-sea organisms. Sampling in the deep sea is often haphazard, both in time and space, and so it would be especially useful to construct an index of "well being" for a

given species so that any individuals examined can be assessed and compared with this index. Information on past disturbances could then be inferred and changes at the population level could be predicted and monitored. If such an index could be applied to material that has been fixed and preserved (in formaldehyde) then there is an opportunity to exploit fully the data gained from deep-sea sampling. Almost all the material collected exists in this form and many museums and laboratories have archives of preserved specimens. It would be possible to combine material from different collections to give a global view, literally and in terms of community evolution, of disturbances and community responses that have occurred. Such retrospective information would enhance our knowledge of this relatively inaccessible environment and enable future studies to be more clearly defined.

1.2 Physiological and Biochemical Indices in Ecological Studies

It is of vital importance when making physiological measurements to be aware that biological systems within animals do not operate in isolation. Even a specific external change leads to alterations in innumerable cellular processes, and so no single index can truly reflect all the interactions of its vital processes. Historically, research has been directed towards those indices which measure generalized stress responses, an expression of the animals well-being. Probably the most comprehensive work to date is that set out by Bayne *et al.* (1985), who stated that seasonal and laboratory-induced stress could be detected via a number of parameters; the ratio of oxygen consumed to nitrogen excreted (the balance between catabolic processes), "scope for growth" (a measure of the energy status of the organism), and changes in the growth efficiency (the conversion of food into somatic tissue growth) and body condition. They proposed that each of these factors can be considered as a stress index, particularly scope for growth because it is an integration of the whole organism's basic responses, incorporating feeding, food absorption, respiration and excretion, and can be expressed in terms of potential survival of the organism. Although this rationale is undoubtedly valid it relies on laboratory experiments with living animals to measure these parameters over time and such a methodology is not suitable in the current investigation.

These workers also suggest that measurements of molecular components of

the cells and extracellular fluids may also be useful stress indices. Indeed, since responses to an environmental change, at whatever level of cellular organization, are fundamentally biochemical, then such indices should be the most sensitive to environmental change and provide the earliest detection of a decline in the animal's health. Biochemical components can be measured enzymatically or chemically and so the methodology required is often a straightforward assay. This approach was thought to suit best the aims of the current project.

Free amino acids are components of the tissue fluid that have attracted attention over the last twenty years. Several workers have reported on the correlation of changes in the tissue levels of certain of these metabolites with environmental stress. Jeffries (1972) suggested that stress in the hard shell clam *Mercenaria mercenaria* could be quantified by variations in the molar ratio of taurine:glycine; a mean value of less than three was found in an unstressed population, values between three and five indicated chronic stress and those greater than five were found in animals subjected to acute stress. Bayne *et al.* (1976) performed similar studies on another bivalve, *Mytilus edulis*, and proposed that the taurine:glycine ratio offered some potential as a generally applicable indicator of stress. Studies on the polychaete *Neanthes virens* by Carr and Neff (1984) record significant differences in alanine concentrations between normal and oil-contaminated populations. There is thus a body of evidence to suggest that free amino acid measurements may provide valid indicators of environmental disturbance.

There are certain biochemical components within an organism's tissues that are concerned with the production of chemical energy and they can be considered as an hierarchy of energy availability. The top tier, the primary energy supplier in all living organisms, is the adenylate system of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP). The potentially available chemical energy is determined by the ratio of these components to each other and an index of assessing this available energy was first proposed by Atkinson (1968). The adenylate energy charge (AEC) is described by the following relationship;

$$AEC = \frac{ATP + \frac{1}{2}ADP}{ATP + ADP + AMP}$$

It has a value between zero and one, where, if all of the components exist as ATP, there are the maximum number of high energy phosphate groups and the AEC will have a value of one. If the system is composed entirely of AMP, then there are no high energy phosphate groups and the AEC will be zero. The application of AEC as a biochemical index of sublethal stress was proposed by Ivanovici (1980) and Ivanovici and Wiebe (1981), further experimentation revealed that the AEC of normally metabolising cells is between 0.75 and 0.95. It was also suggested that values of AEC consistently between 0.5 and 0.7 are found in organisms in situations that are limiting or non-optimal but the organisms remain viable and if returned to normal conditions the AEC values will recover. Under severe stress situations AEC's of 0.5 and below were noted and these animals did not recover when placed in normal situations. The AEC would seem an ideal index - it can be applied to all organisms, the response time of the adenylate system to environmental change is very fast, minutes in the case of micro-organisms (Chapman *et al.*, 1971), and variation between individuals is less for AEC than for the concentrations of the individual nucleotides, an obvious experimental advantage.

However, the AEC has received criticism and exceptions to the above findings can be found in the literature; some species remain viable despite AEC values below 0.5 (Ball and Atkinson, 1975) and high AEC values have been measured in moribund organisms under lethal conditions (Chapman and Atkinson, 1973). Some of these exceptions may be attributable to questionable methodologies - ATP is a highly labile compound and if insufficient care is taken to inactivate enzymes that degrade ATP a lower AEC will result (Ivanovici and Wiebe, 1981). It can be suggested that AEC provides a sensitive general indicator to detect the initial effects of stress but it should be used in conjunction with other indices to quantify responses to environmental change. This approach was adopted by Hawkins and Hutchinson (1990) in their work on the stress responses of *Ostrea edulis*. Additionally, AEC says nothing about the potential energy stored in other forms, such as the phosphagens creatine and arginine phosphate or storage carbohydrates and lipids.

The phosphagens are used to rephosphorylate ADP to ATP when energy demands on the system are high. As such they can be considered as a second tier

of metabolites in the energy availability hierarchy. At this level also we can consider the metabolic elements of glycolysis, an almost universal pathway in biological systems of metabolic energy production. In glycolysis, glucose is converted to pyruvate (with the production of ATP) which then enters the Kreb's (tricarboxylic) cycle and, under aerobic conditions, leads to the production of succinate. If conditions are anaerobic lactate accumulation will occur, although there are exceptions, for example the oyster *Ostrea edulis* in which anaerobic respiration halts the Kreb's cycle at succinate. This then accumulates in preference to lactate (Hammen, 1969; Mustafa and Hochachka, 1971), which is potentially more harmful to the oyster in large quantities. By measuring the concentrations and ratios of these key elements, the changes in the functioning of the enzymes responsible for the transformations between metabolite pools can be inferred.

At the base of the hierarchy are the long term storage products glycogen and lipids. The levels of these change slowly, in response to seasonal effects and are often linked with reproduction. As such, they cannot be used to measure immediate environmental effects but will respond to long term changes where conditions would be expected to draw on energy reserves. Once the reproductive and seasonal cycles are understood the superimposition of fluctuations resulting from disturbance can be detected. Gabbott and Walker (1971) found that a simple condition index correlated well with measurements of glycogen content and such long term reserves have also been used as reproductive condition indices (Gabbott, 1975).

Biochemical components that are unaffected immediately by environmental change but which, with time, exhibit disturbance responses would be likely to be useful in the study of deep-sea organisms. The drastic changes in environment that occur as the samples are caught and brought to the surface result in an inevitable change in the physiology of the organism, usually culminating in rapid death. The measurement of metabolites unaffected by this process will reveal information on the physiological well-being of the animal pre-capture.

The aims of this investigation were to include and extend the methodologies used by Bayne *et al.* (1985) and Hawkins and Hutchinson (1990) to examine the effects of environmental disturbance on an integrated range of metabolic levels. In this way the responses of the organism's physiology as a whole can be studied and

those elements that reflect the changes most closely can be isolated.

1.3 The Study Site and Species

The validation of the use of biochemical techniques to give ecological information required a readily accessible study site so that comprehensive time series data could be obtained to test the methodologies. The site chosen in this case was the Baie de Somme estuary on the northern French coast (fig. 1). The ecology of the estuary had been studied for ten years prior to the commencement of this investigation in 1990, consequently there was a great deal of data containing both population and environmental observations.

The intertidal areas of the estuary covered a total area of over 70 km^2. There was a macrotidal regime characterized by harsh hydrodynamical conditions and sedimentary dynamics. The tidal amplitude was particularly large, reaching a maximum of 9.8 m which ran in a north-east to south-west direction once inside the bay. The tidal flow was asymmetric because of the phenomenon of a double low water, the ebb flow lasting over twice as long as the flood. Consequently the flood was significantly faster and stronger than the ebb. Pollution within the estuary was derived from agricultural land run-off, the nitrates causing planktonic blooms and possible eutrophication (Desprez *et al.*, 1992).

The benthic macrofauna of the estuary comprised a relatively limited species pool (a full species list is given in tables 2 and 3, chapter 3) and was dominated by molluscs and polychaetes. The dominant species occurred throughout the intertidal areas although relative levels of dominance differed from region to region and with time. The bivalve *Cerastoderma edule* (Linnaeus) occurred in very large numbers and supported a fishing industry which was of local economic importance. The polychaete *Pygospio elegans* (Claparède) occurred in dense aggregations (up to 500,000 per square metre) and its tubes formed raised beds 10-15 centimetres high which altered the local small scale current regime. This phenomenon of bed formation was well expressed in the Baie de Somme. Crustaceans such as *Bathyporeia pilosa* (Lindström) and *Corophium volutator* (Pallas) have been recorded in very large numbers but the occurrences were sporadic. Other dominant species included *Nereis diversicolor* (O.F. Müller), *Eteone longa* (Fabricius), *Macoma balthica* (Linnaeus) and *Hydrobia ulvae*

(Pennant).

The species chosen for biochemical studies were *C. edule* and *N. diversicolor* as both occurred in reasonably large numbers and had been consistently present in the estuary for at least the last twelve years. The relatively large size of both species was conducive to individual biochemical study.

There was an archive of preserved specimens collected over this period and there is a body of literature on the ecology of both species. Studies on the reproduction and gametogenic cycles of *N. diversicolor* were related by Dales (1950), Olive and Garwood (1981) and Möller (1985) and ecophysiological studies have been described by Schöttler *et al.* (1984) and Verschraegen *et al.* (1985). The significant potential of polychaetes for use in marine environmental quality monitoring has been proposed by Pocklington and Wells (1992). The reproduction of *C. edule* has been outlined by Newell and Bayne (1980), Yankson (1988), Navarro *et al.* (1989) and specifically in the Baie de Somme by Ducrotoy *et al.* (1991). The metabolism and ecophysiology of the cockle has been described by Ahmad and Chaplin (1984), Nossier (1986) and Hummel *et al.* (1989). Such information has enabled comparisons to be made with the present study.

Two sites were selected within the estuary for comparative monitoring, LCS to the north of the estuary and HHS in the south (fig. 1). The northern half of the estuary was directly exposed to the prevailing south-westerly waves, the southern half was somewhat protected by the shore-bar at Pointe de Hourdel (Ducrotoy and Sylvand, 1991). The sites also differed in proximity to, and influence of, the River Somme. HHS was much closer and the prevailing winds and currents ensured that the fresh water flowed out in the south of the bay. LCS had a more stable, more marine, salinity regime. Salinity at HHS varied from 6‰ to 25‰ and at LCS only between 26‰ and 31‰ (Ducrotoy *et al.*, 1991). The riverine influence at HHS was also noticeable in the sediment. Fine particles settled out in this region, as the fresh river water met and mixed with the marine water. In calmer months some silt accumulation occurred (Desprez, pers. comm.). The remainder of the sediment at both sites was primarily fine sand. The sites were at the same height on the shore, so tidal exposure times were virtually identical.

Samples for faunal and biochemical analyses were taken on a monthly basis

from each site for a two year period. This intensive sampling coupled with the data available for the previous ten years allowed simultaneous correlations of biochemical, community and environmental fluctuations.

1.4 General Aims of the Project

The rationale described in sections 1.1 and 1.2 has highlighted the necessity for a procedure to predict ecological changes and it has been reasoned that biochemical indices would be the basis of such a procedure. The components that were measured were key compounds in the metabolic energy production pathways of glycolysis, the tricarboxylic acid cycle and the adenylate system. Structural and storage macromolecules were also determined. The correlation of fluctuations in these indices with subsequent population changes and/or environmental perturbations was intensively performed on communities in the Baie de Somme to validate the methodologies. When the most suitable of these indices had been determined they were applied to organisms and communities where the causative environmental processes may not have been measurable, principally the deep-sea environment. The adaptation of the methods for analysis of formalin preserved material would greatly enhance the knowledge of past and present deep sea macrofaunal communities, their responses to disturbance and hence the structuring processes.

The structure of the study was such that each validation process had to be completed before the next could be commenced. The structure of the thesis reflects this, consequently each chapter has an integral discussion section. The final chapter is concerned with discussing the feasibility and application of this approach.

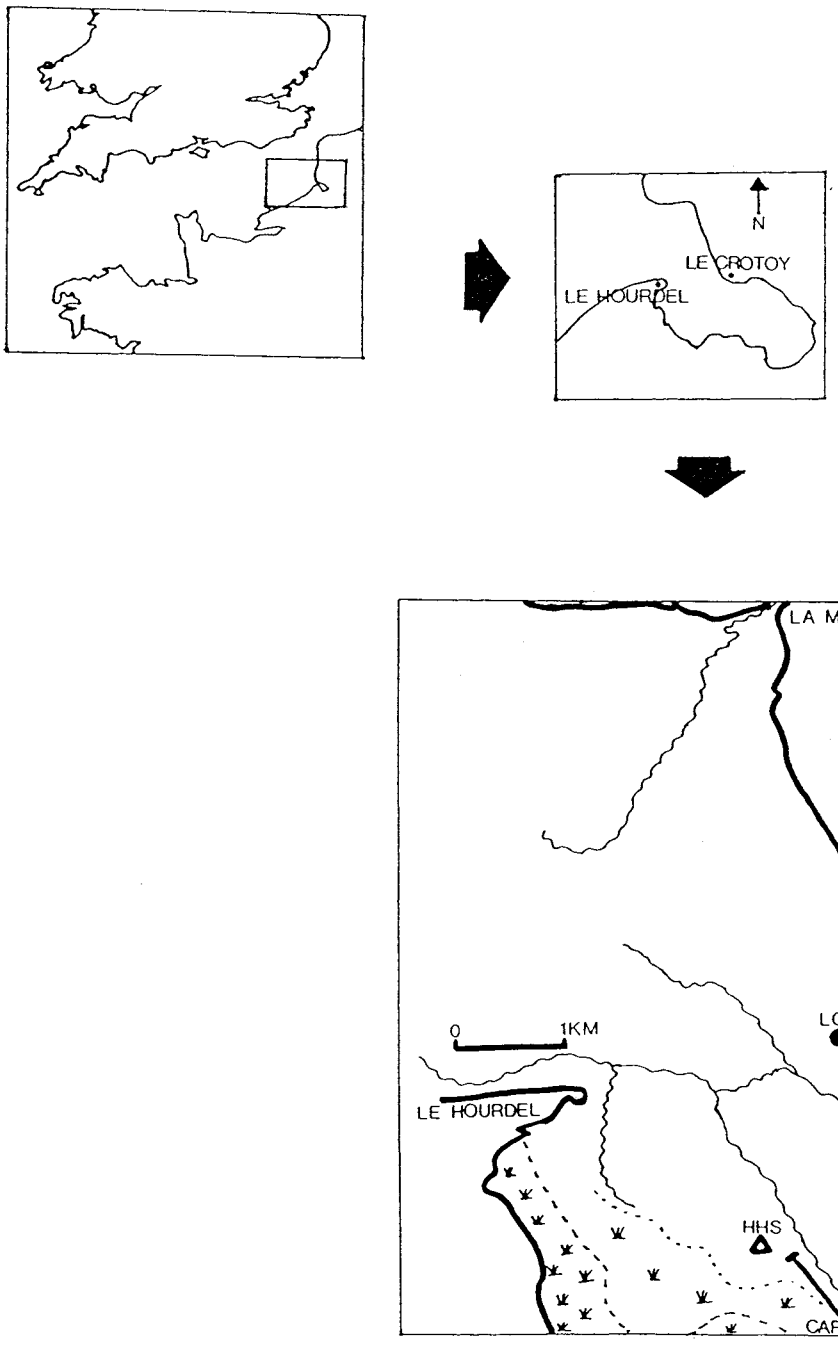


Fig 1. Map to show the location of the Baie de Somme and the selected sampling sites LCS and HHS.

CHAPTER 2

Biochemical studies of Baie de Somme Species

2.1 Introduction

The primary step in the validation exercise involved the biochemical investigation of the two representative Baie de Somme species *Cerastoderma edule* and *Nereis diversicolor*. The components that were measured were the adenylates (ATP, ADP and AMP), arginine phosphate in cockles and creatine phosphate in *Nereis*, lactate, succinate and pyruvate in the cockle only, glucose, glycogen and total carbohydrates, lipids and proteins.

2.2 Methods

2.2.1 Collection of Samples

The biochemical techniques required that the samples be frozen as soon as possible after collection to ensure that the biochemical components measured within the tissues were as near as possible to *in vivo* levels at the time of capture. This was achieved by freezing animals *in situ* by immersion in liquid nitrogen ensuring almost instant freezing. At each site a minimum of six *C. edule* and six *N. diversicolor* were obtained (six was the optimum number for the capabilities of the assay equipment and statistical analysis), washed briefly in the surrounding sea water, individually placed in small plastic bags and then frozen in the liquid nitrogen. On return to the laboratory these samples were maintained at -20°C and transported between laboratories in polystyrene boxes containing dry ice.

2.2.2 Preparation of Samples

Each of the frozen animals was weighed (wet weight) prior to further treatment, in the case of *C. edule* the shell length was measured to the nearest millimetre with callipers and the shell was then removed before weighing. In both cases any remaining sand or debris was removed first. The animal was then ground to a fine powder in a stainless steel pestle and mortar (previously cooled in liquid nitrogen). The animal was kept frozen at all times in the procedure, if

necessary reimmersing in liquid nitrogen, and all implements were cooled before contact with the animal. Portions of the powdered animal were transferred to two preweighed, acid-washed glass vials, these were then reweighed and stored in a freezer for further biochemical analyses. The weight of the material remaining in the pestle was calculated and noted and 1ml of 6% v/v perchloric acid was added. The material was reground and then stored in a labelled Nalgene® centrifuge tube at -20°C for further enzymatic biochemical analyses.

In all of the following biochemical methods reagents have only been described by name and quantity. The full composition of each reagent is given in Appendix 1.

2.2.3 Enzymatic Analyses

Approximately 5x the tissue weight (in grammes) of perchloric acid (in millilitres) was added to the samples stored frozen in Nalgene® centrifuge tubes. The samples were then centrifuged at 4000 r.p.m. for 10 minutes. A drop of bromothymol blue indicator was added to each tube and the contents neutralized with potassium hydroxide added drop-wise until a pale blue colour was obtained. The tubes were again centrifuged on the same settings as before, the supernatant was then decanted into graduated centrifuge tubes and the final sample volumes noted. The resultant solutions were processed immediately since the more labile compounds begin to break down at room temperature.

In all of the following analyses the absorbances of the test solutions were read on a spectrophotometer at 340 nm. Both 1 cm^3 and 3 cm^3 cuvettes were used but were always new, and test solutions were read against a reagent blank for each analysis. This corrects for any changes in absorbance that might be caused by various additions of reagents and enzymes.

(i) *Adenylates and Adenylate Energy Charge (AEC)*

Adenylate Tri-, Di- and Monophosphate (ATP, ADP and AMP) tissue concentrations were measured using the procedures described by Bayne *et al.* (1985) which are based on the following reactions:

(1) ATP + Glucose $\xrightarrow{HK}$ Glucose-6-phosphate (G6P) + ADP

(2) G6P + $NADP^+$ $\xrightarrow{G6PDH}$ 6-phosphoglucon-δ-lactone + NADPH + H^+

(3) AMP + ADP $\xrightarrow{MK}$ 2 ADP

(4) 2 ADP + 2 phosphoenolpyruvate $\xrightarrow{PK}$ 2 ATP + 2 pyruvate

(5) 2 pyruvate + 2 NADH + 2 H^+ $\xrightarrow{LDH}$ 2 lactate + 2 NAD^+

G6PDH = glucose-6-phosphate dehydrogenase
HK = hexokinase
LDH = lactate dehydrogenase
MK = myokinase
NAD = β-nicotinamide-adenine dinucleotide, oxidised form
NADH = β-nicotinamide-adenine dinucleotide, reduced form
$NADP^+$ = β-nicotinamide-adenine dinucleotide phosphate, oxidised form
NADPH = β-nicotinamide-adenine dinucleotide phosphate, reduced form
PK = pyruvate kinase

ATP was measured by following reactions (1) and (2). ADP and AMP were found from reactions (3)-(5).

ATP was analysed by itself in one set of cuvettes, while ADP and AMP were analysed sequentially in a separate set of cuvettes.

ATP Assay

2.8 cm^3 of ATP assay buffer was dispensed into each cuvette followed by additions of 0.2 cm^3 portions of perchlorated samples. 10 μl of G6PDH was added to each cuvette, it was stirred well, and ensuring that there were no air bubbles inside the cuvette on the transparent surfaces, the absorbance (A1) was read after 5-10 min. This allowed sufficient time for oxidation of any endogenous G6P that may have been in the sample. 20 μl of glucose, 10 μl of HK were added and again

the solution was stirred. 10-20 minutes at room temperature was allowed for the reaction to reach completion (i.e. it was observed that there were no further changes in absorbance on the recorder), then A2 was read.

ADP-AMP Assay

2.8 cm^3 of ADP-AMP assay buffer was dispensed into each cuvette. 0.2 cm^3 of sample, and 0.2 cm^3 of water for the blank were added. Then 5 μl LDH was added to all cuvettes, the solutions were mixed well, bubbles were removed, and absorbance (B1) read after 5-10 min. 5 μl PK was added and B2 was read after the reaction was complete (5 min). Finally, 5 μl MK was added, and B3 was read after 15-20 min.

Calculation of Results

Nucleotide Concentrations: The concentrations of ATP, ADP and AMP were calculated as follows:

$$\text{ATP } (\mu\text{mol g}^{-1} \text{ wet wt tissue}) = \frac{\Delta A^{ATP} \times AV \times EV}{6.22 \times SV \times TW \times 1.0}$$

$$\text{ADP } (\mu\text{mol g}^{-1} \text{ wet wt tissue}) = \frac{\Delta A^{ADP} \times AV \times EV}{6.22 \times SV \times TW \times 1.0}$$

$$\text{AMP } (\mu\text{mol g}^{-1} \text{ wet wt tissue}) = \frac{\Delta A^{AMP} \times AV \times EV}{6.22 \times SV \times TW \times 2 \times 1.0}$$

where:

ΔA^{ATP} = A2 - A1

ΔA^{ADP} = B1 - B2 If $\Delta A < 0$, use 0, not the negative number.

ΔA^{AMP} = B2 - B3

AV = assay volume (cm^3), i.e. volume of solution (buffer and

sample) in cuvette.

EV = extract volume (cm^3), i.e. volume of neutralized supernatant measured after extraction procedure.

6.22 = extinction coefficient, i.e. absorbency at 340 nm and pH 7.6 of a solution of NADPH or NADH

SV = volume of sample supernatant used in cuvette (cm^3).

TW = tissue weight (g).

1.0 = length of light path inside cuvette (cm).

(ii) *Glucose and Glycogen*

Glucose and glycogen were measured using the methods based on reactions (1) and (2) (above) but in the presence of excess ATP to measure glucose. Glycogen is determined from measurement of the glucose produced by hydrolysis of glycogen by amyloglucosidase.

Glucose: 0.93 cm^3 of the ATP buffer system was placed in a 1 cm^3 cuvette with 10 μl of ATP solution and 5 μl G6PDH then 50 μl of the perchlorate extract was added. This was mixed and after 5 minutes the initial absorbance value was read. 5 μl of HK was added and after 10 minutes, when the reaction had reached completion, the final absorbance was read and ΔA, the change in absorbance, was calculated.

To calculate the tissue glucose concentration:

$$\text{Glucose conc. } g^{-1} \text{ wet weight} = \frac{EV \times \Delta A \times 3.2154}{TW}$$

Glycogen: The extract had to be hydrolysed to convert the glycogen to glucose. This was achieved by adding 0.2 cm^3 of extract to a mixture of 0.1 cm^3 phosphate buffer (adjusted to pH 7.0), 0.4 cm^3 of amyloglucosidase solution and 1.6 of distilled water. This was incubated for 1-2 hours at 40°C in capped tubes. After hydrolysis a 50 μl sample was removed and its glucose concentration determined using the method given above.

Tissue glycogen levels were calculated with reference to a glycogen

standard which was also hydrolysed. A standard was necessary because of the presence of small amounts of glucose in the amyloglucosidase from the manufacturer (Sigma Chemicals).

To calculate the tissue glycogen concentration:

$$\text{Glycogen conc. g}^{-1}\text{ wet weight} = \frac{\Delta A^{Glycogen} - (\Delta A^{Glucose}/23)}{\Delta A^{Standard}} \times \frac{EV}{TW}$$

(iii) *Arginine phosphate/Creatine phosphate*

Arginine phosphate concentrations were found using the procedure given by Gäde (1985), based on the following reaction;

$$\text{Arginine} + \text{pyruvate} + \text{NADH} + H^+ \overset{ODH}{\rightleftharpoons} \text{Octopine} + NAD^+ + H_2O$$

ODH= Octopine dehydrogenase

The arginine phosphate was converted to arginine by hydrolysing 0.2 cm^3 perchlorate extract with 0.2 cm^3 hydrochloric acid. The solutions were mixed in a capped tube, placed in a boiling water-bath for 1 minute and then rapidly cooled in crushed ice. This was then neutralized with 0.1 cm^3 sodium hydroxide.

0.2 cm^3 of this hydrolysed extract was added to a 1 cm^3 cuvette containing 0.79 cm^3 ADP-AMP buffer system. A1 was noted and then 10 μl ODH was added and the solutions mixed. A2 was read after 10 minutes.

$$\text{Arginine phosphate } \mu\text{mol g}^{-1}\text{ wet tissue wt.} = \frac{EV \times \Delta A \times 6.029}{TW}$$

Creatine phosphate (the equivalent of arginine phosphate in polychaetes) was measured in the same cuvettes used to measure ATP, according to Heinz and Weisser (1985) and based on the following reactions;

(1) Creatine phosphate + ADP $\rightleftharpoons^{CK++}$ creatine + ATP

(2) ATP + D-glucose $\rightarrow^{HK}$ D-glucose 6-phosphate + ADP

(3) D-glucose 6-phosphate + $NADP^+$ $\rightarrow^{G6PDH}$ D-glucono-δ-lactone 6-phosphate + NADPH + H^+

CK = Creatine phosphokinase

After completion of the ATP assay 5 μl of ADP and 10 μl of the enzyme creatine phosphokinase were added to the cuvette, the solutions mixed and a third absorbance read after 10 minutes. The difference between this and the final ATP absorbance were used to calculate creatine phosphate levels.

$$\text{Creatine phosphate } \mu\text{mol g}^{-1} \text{ wet tissue wt.} = \frac{EV \times \Delta A \times 2.4116}{TW}$$

(iv) *Succinate*

Succinate measurements were based on the method given by Beutler (1985):

Succinate + CoA + ITP $\rightleftharpoons^{SK}$ succinyl-CoA + IDP + P_i

IDP + PEP $\rightarrow^{PK}$ ITP + pyruvate

Pyruvate + NADH + H^+ $\rightarrow^{LDH}$ L-lactate + NAD^+

CoA = coenzyme A
ITP = inosine-5'-triphosphate
IDP = inosine-5'-diphosphate
PEP = phosphoenolpyruvate
SK = succinic thiokinase

2.1 cm^3 succinate buffer was pipetted into each 3 cm^3 cuvette followed by 0.2 cm^3 perchlorate extract, 10 μl LDH and 10 μl PK. The cuvette contents were thoroughly mixed and absorbances A1 were read. 20 μl of succinic thiokinase was added, the solutions mixed, incubated in a 30° C water bath for 25 minutes and then absorbances A2 read.

$$\text{Succinate conc. } g^{-1} \text{ wet weight} = \frac{EV \times \Delta A \times 1.9855}{TW}$$

(v) *Lactate*

Lactate was measured by the method given by Noll (1984) using the coupled reactions:

$$\text{L-(+)-lactate} + NAD^+ \overset{LDH}{\rightleftharpoons} \text{pyruvate} + NAD + H^+$$

$$\text{Pyruvate} + \text{L-glutamate} \overset{GPT}{\rightleftharpoons} \text{L-alanine} + \text{2-oxoglutarate}$$

GPT = Glutamic-Pyruvic transaminase

2.79 cm^3 lactate buffer was pipetted into each 3 cm^3 cuvette, followed by 0.2 cm^3 perchlorate extract and 5 μl GPT suspension. These solutions were thoroughly mixed, and absorbance A1 read. 5 μl of LDH was added, the solutions were mixed and the constant final absorbance A2 was read (usually after 15-30 min).

$$\text{Lactate } \mu\text{mol } g^{-1} \text{ wet tissue wt.} = \frac{EV \times \Delta A \times 2.4116}{TW}$$

(vi) *Pyruvate*

Pyruvate was measured using the method described by Lamprecht and Heinz (1984), based on the following reaction;

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{Lactate} + \text{NADH}^+$$

0.65 cm^3 pyruvate buffer was added to each 1 cm^3 cuvette, followed by 0.3 cm^3 perchlorate extract. The solutions were mixed and absorbances A1 read. 5 μl LDH were added and absorbances A2 read after 5-10 minutes.

$$\text{Pyruvate } \mu\text{mol g}^{-1} \text{ wet tissue wt.} = \frac{\text{EV x } \Delta\text{A x } 0.5118}{\text{TW}}$$

2.2.4 Gross Biochemical Measurements

The components determined were total lipids, proteins and carbohydrates.

(i) *Lipid*

Total lipid content was found using the gravimetric method of Folch *et al* (1956). 4 cm^3 of chloroform-methanol solvent was added to one of the two glass vials containing powdered tissue. The sample was filtered into a graduated centrifuge tube, and the filter and sample were washed several times with small amounts of the solvent. The filtrate was transferred to a separating funnel and 1 cm^3 of potassium chloride solution was added, the solutions were well mixed and allowed to separate into two phases. The lower organic phase was transferred to a third pre-weighed glass vial and the remaining contents of the funnel washed with potassium chloride solution, the lower phase being added to that already run-off. The upper aqueous phase containing non-lipid contaminants and amino-acids was discarded. The vial containing the extracted lipid was allowed to evaporate overnight. It was then reweighed and the results expressed as mg lipid per gram tissue weight.

The tissue in the second glass vial was freeze-dried prior to further analysis to obtain the dry weight of tissue. The ratio of wet:dry tissue was calculated for each animal and the wet tissue biochemical results converted to more accurate dry tissue results. It was assumed that the animals were thoroughly homogenised by the preparatory treatment (indeed the expression of all the biochemical results as

per gram of tissue depends on homogeneous tissue samples). The contents of the vial were then rehydrated with 1 cm^3 distilled water and the resulting solution used for both protein and carbohydrate determinations.

(ii) *Carbohydrates*

The sugar content of the tissues was determined using the colorimetric method of Dubois *et al.* (1956), as modified by Strickland and Parsons (1968). The technique measures the orange/yellow precipitate formed by the condensation reaction of the simple sugars, oligosaccharides, polysaccharides and their derivatives with phenol and concentrated sulphuric acid.

Triplicate 0.1 cm^3 aliquots of each sample were made up to 1.5 cm^3 with distilled water in a large test-tube. To this was added 0.4 cm^3 phenol followed by a rapid addition of 2 cm^3 sulphuric acid reagent. The contents of each tube were mixed, covered, and allowed to stand at room temperature for at least 30 minutes. The absorbance of each sample was measured on a spectrophotometer at 490 nm. The concentration was determined from the regression of glucose standards covering the range 0.1 - 1.0 mg cm^{-3}, and carbohydrate content expressed as mg per g tissue weight.

(iii) *Protein*

The method for determining total protein content was a modification of the Folin and Ciocalteau method described by Lowry *et al.* (1951). Triplicate 0.1 cm^3 aliquots of sample were made up to 0.5 cm^3 with distilled water, 0.5 cm^3 of sodium hydroxide was added to each sample and they were left for 30 minutes. A mixture of 1 cm^3 copper sulphate, 1 cm^3 sodium potassium tartrate plus 100 cm^3 sodium carbonate was prepared and 5 cm^3 of this was added to each sample and left to stand for 10 minutes. 0.5 cm^3 of Folin and Ciocalteau reagent was added, the contents of the tubes thoroughly mixed and allowed to stand for a further 30 minutes. The transmission at 750 nm of each sample was measured on a spectrophotometer. The protein concentrations of the samples were calculated from the regression of standards prepared from bovine serum albumen fraction V in the range 0.02-0.2 mg cm^{-3}. The protein content of the tissue was expressed as mg per g tissue weight.

2.3 Results

The following figures (2 to 10) show the fluctuations with time of the mean values of each biochemical component, at both sites, for both species. Measurements have been converted from wet tissue results to dry tissue results using the wet:dry tissue ratio obtained by the freeze drying of the tissue aliquot for the carbohydrate and protein assays. This conversion reduces the variation in the mean values arising from differing amounts of external water.

The fluctuations of each component will be described separately to highlight seasonal cycles and specific site differences, although it should be remembered that each component is not responding in isolation to the environment. The biochemistry of the cockle will be considered first.

The individual adenylate graphs (fig. 2) showed great variability at both sites, not unexpected in such short-term response metabolites. This variability lessened in the graphs of total adenylates and AEC (fig. 3) and the adenylate pool achieved higher values in the second year, particularly at LCS. AEC values were significantly higher at LCS (table 1). Since LCS is the more stable of the two sites this result is indeed expected. Arginine phosphate also showed higher values at both sites in the second year and some evidence of seasonality with low points in mid-summer. There was an apparently anomalous peak in February 1991 at HHS which can be explained by meteorological factors. That month was particularly cold (see fig. 26) and HHS has little insulating surface water present at low tide. There is some evidence to correlate low temperatures with high arginine phosphate levels in oysters (Hawkins and Hutchinson, 1990). Since samples were taken at low tide the cockles would have been exposed to low temperatures, whereas at LCS the warmer overlying water would have reduced the severity of the exposure.

Fig. 4 shows the respiratory metabolite levels. Overall patterns were similar for all three, being low in summer and peaking in January/February. Significant differences between sites were only evident in lactate

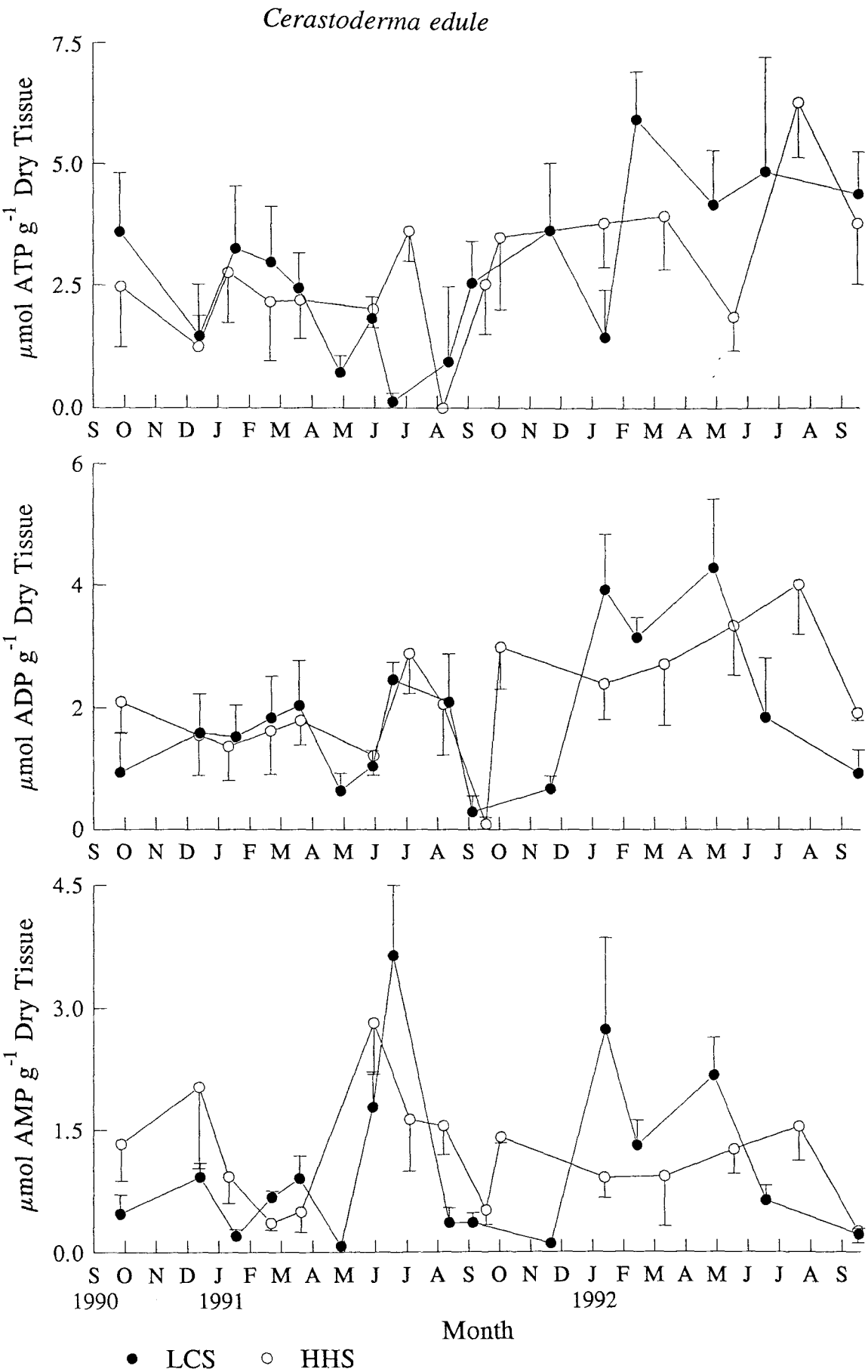


Fig. 2. Mean levels of components with time. Error bars indicate std. dev.

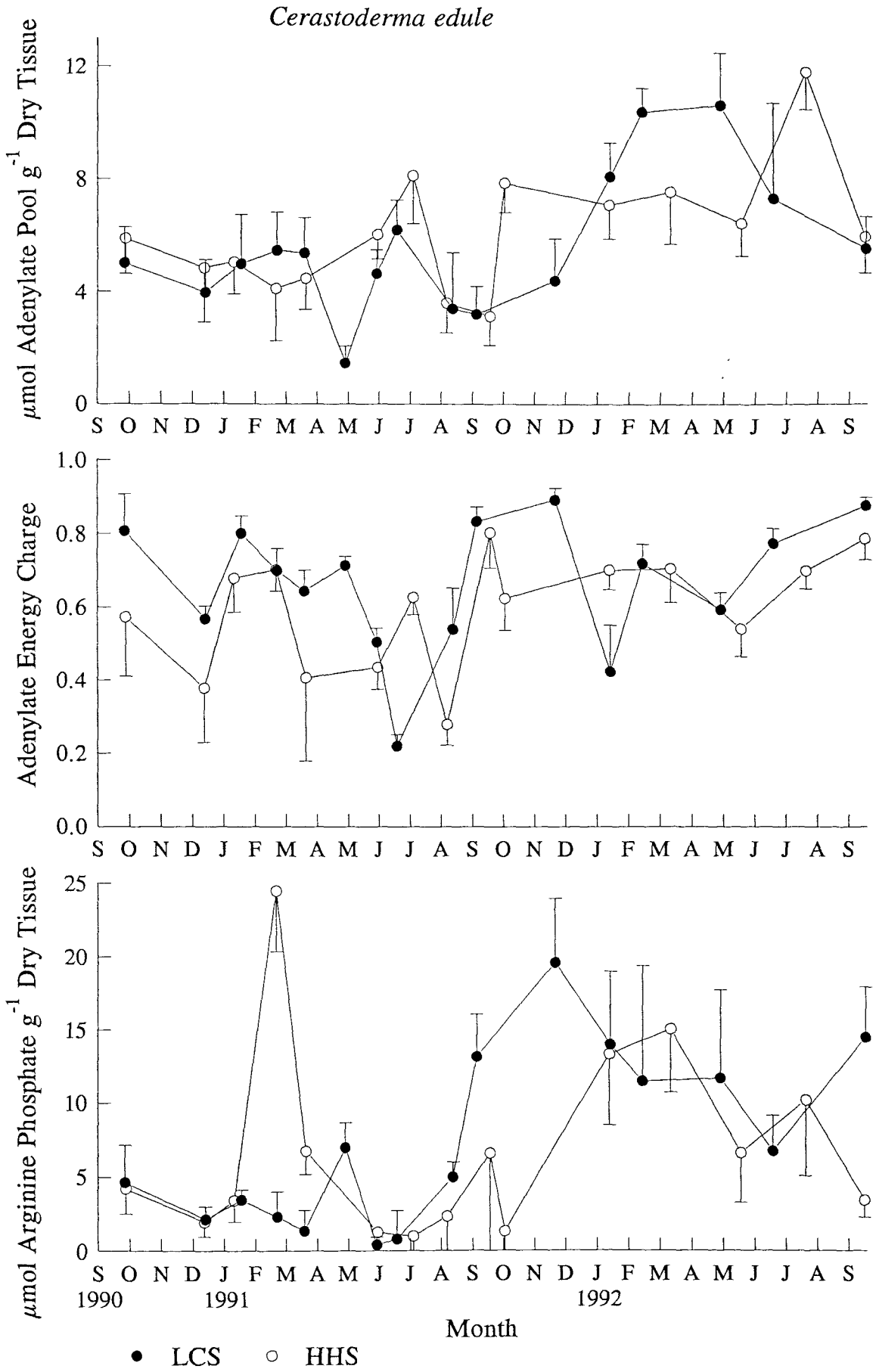


Fig. 3. Mean levels of components with time. Error bars indicate std. dev.

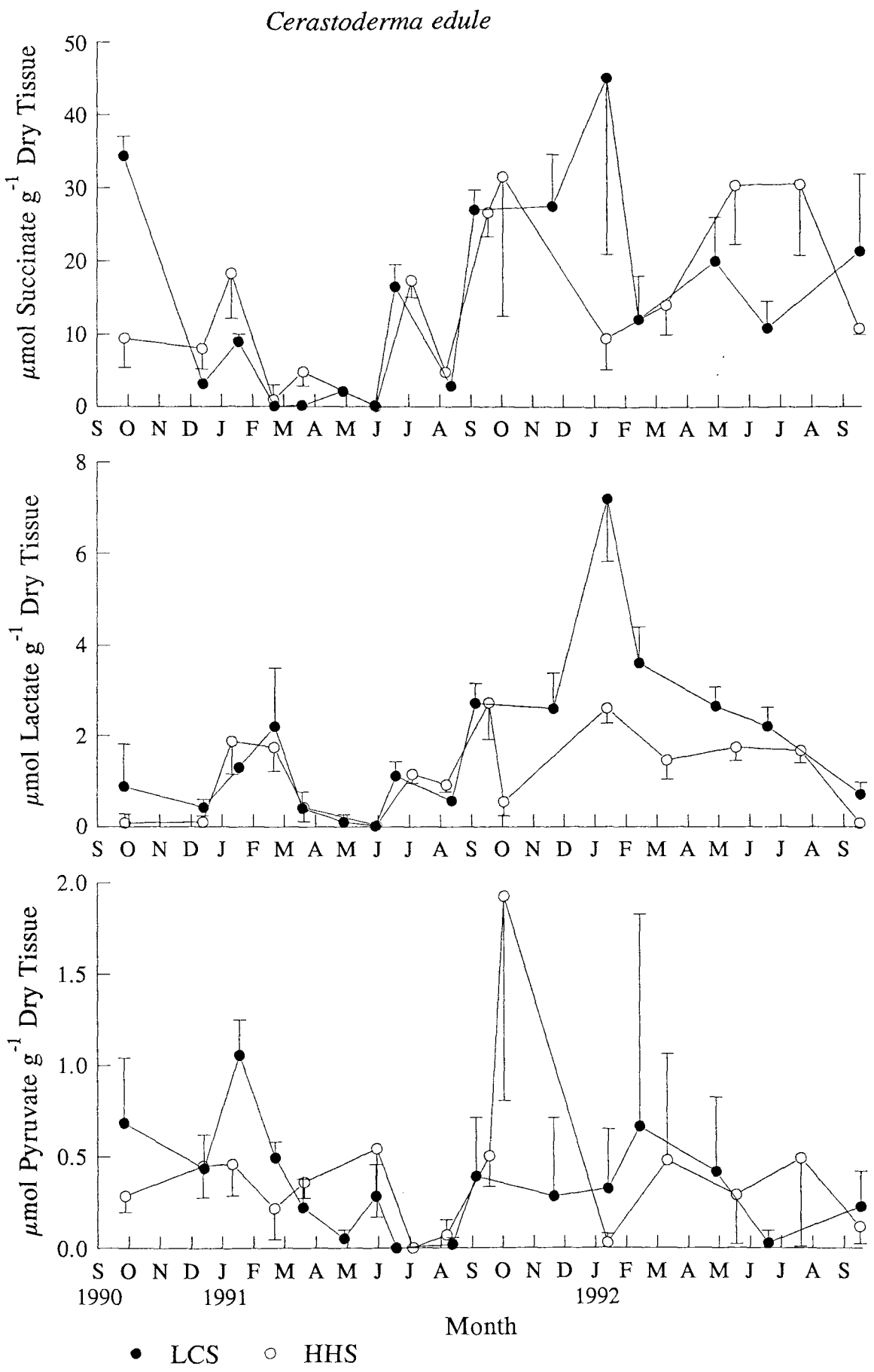


Fig. 4. Mean levels of components with time. Error bars indicate std. dev.

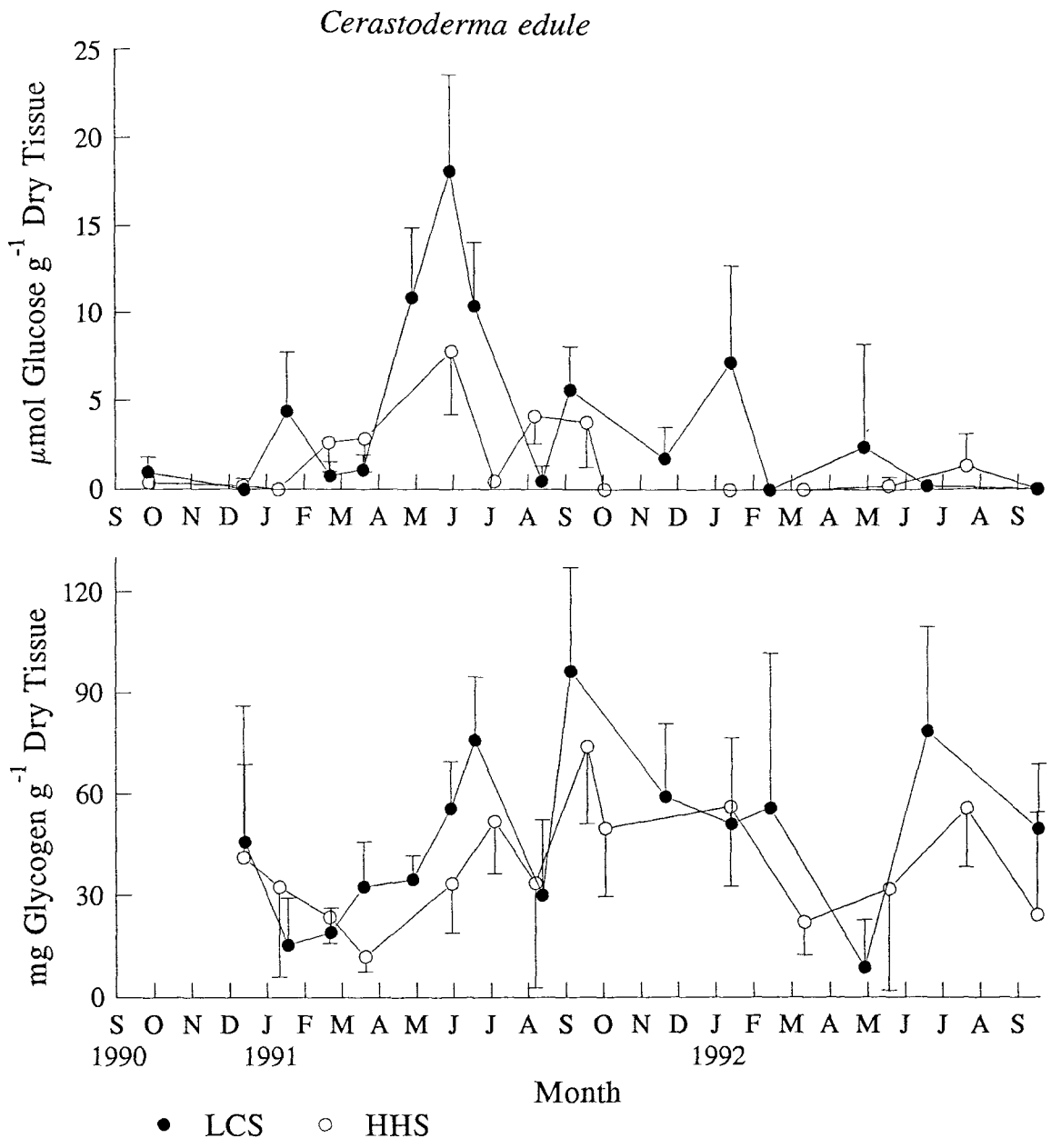


Fig. 5. Mean levels of components with time. Error bars indicate std. dev.

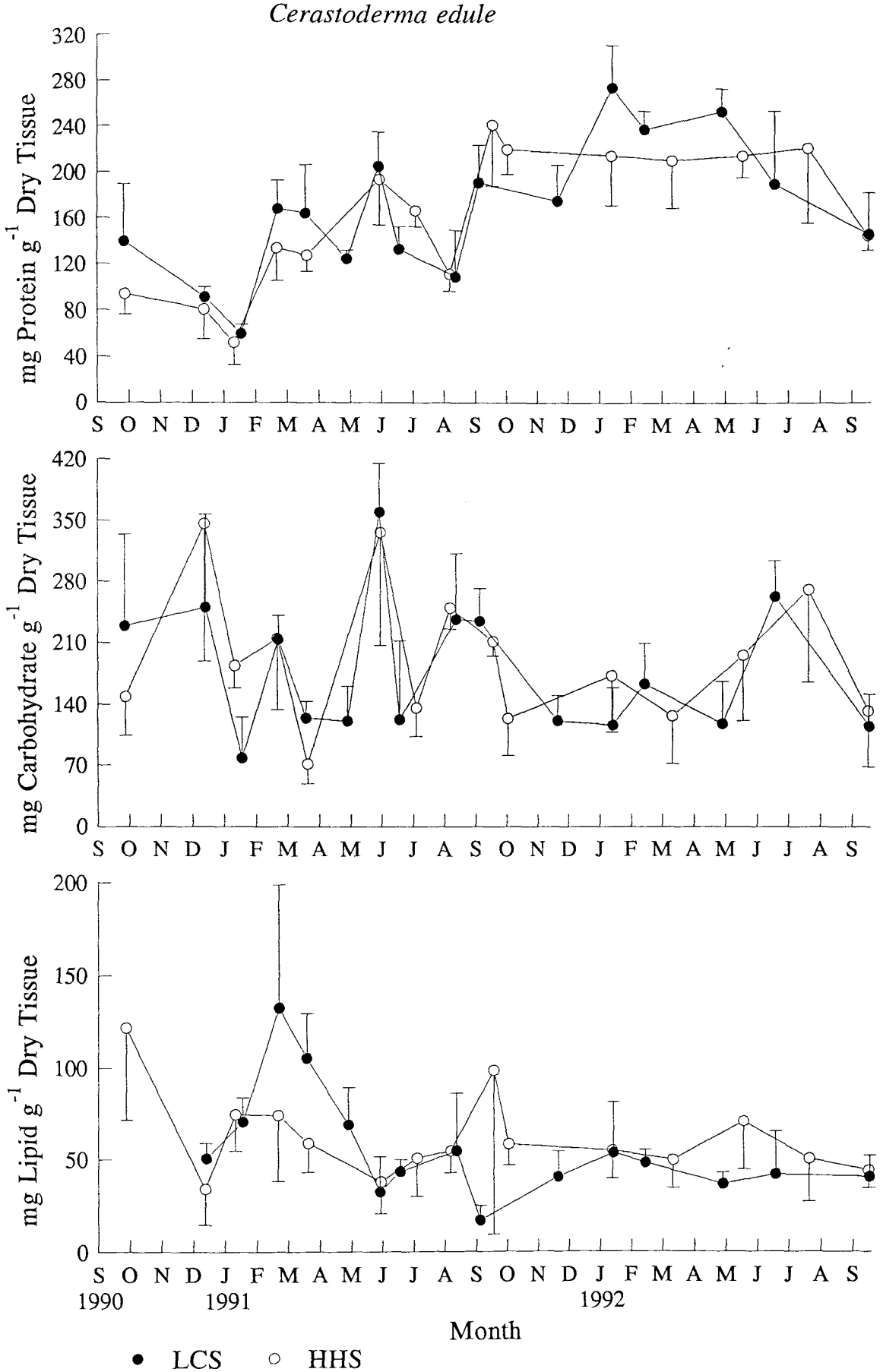


Fig. 6. Mean levels of components with time. Error bars indicate std. dev.

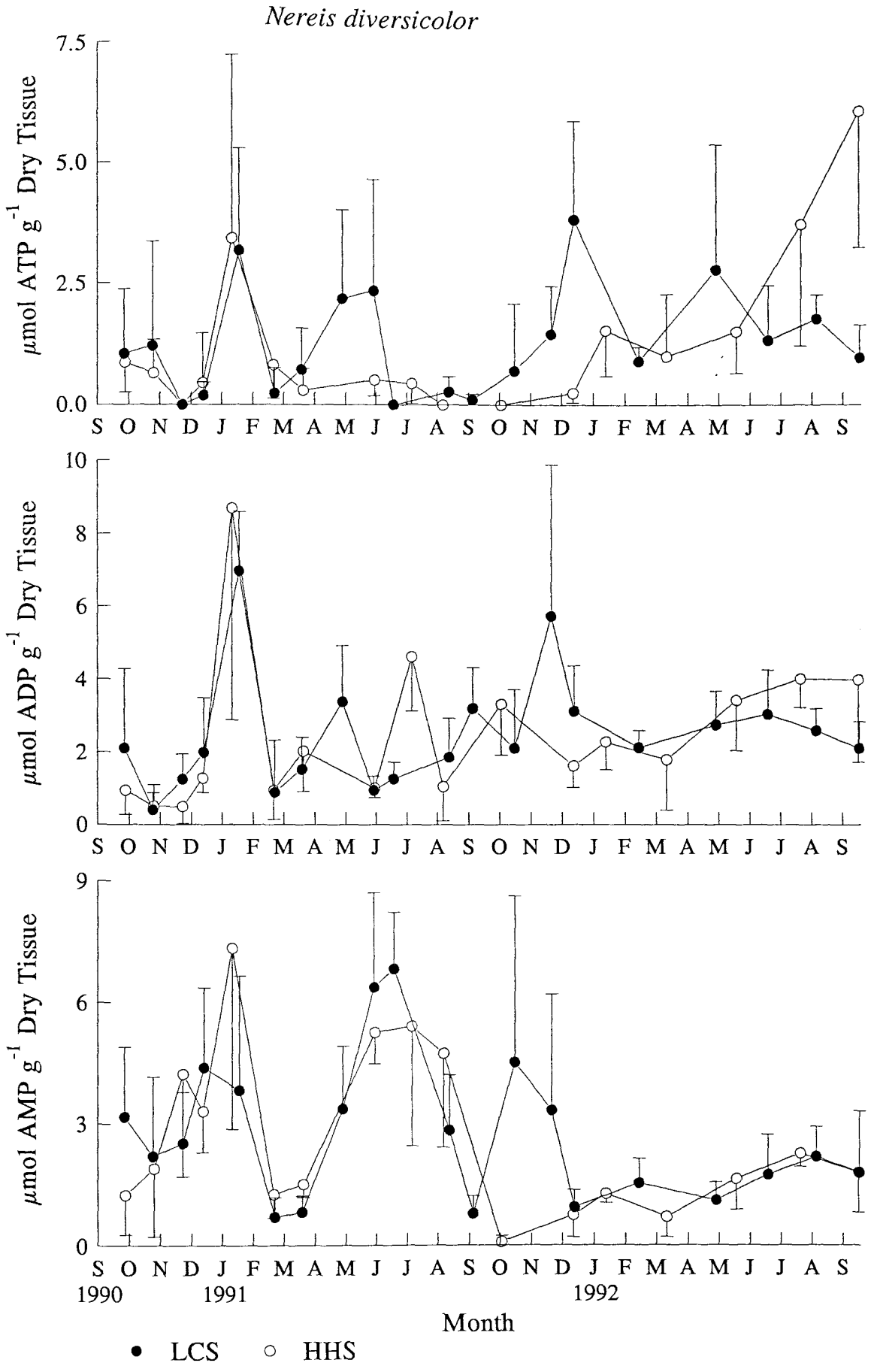


Fig. 7. Mean levels of components with time. Error bars indicate std. dev.

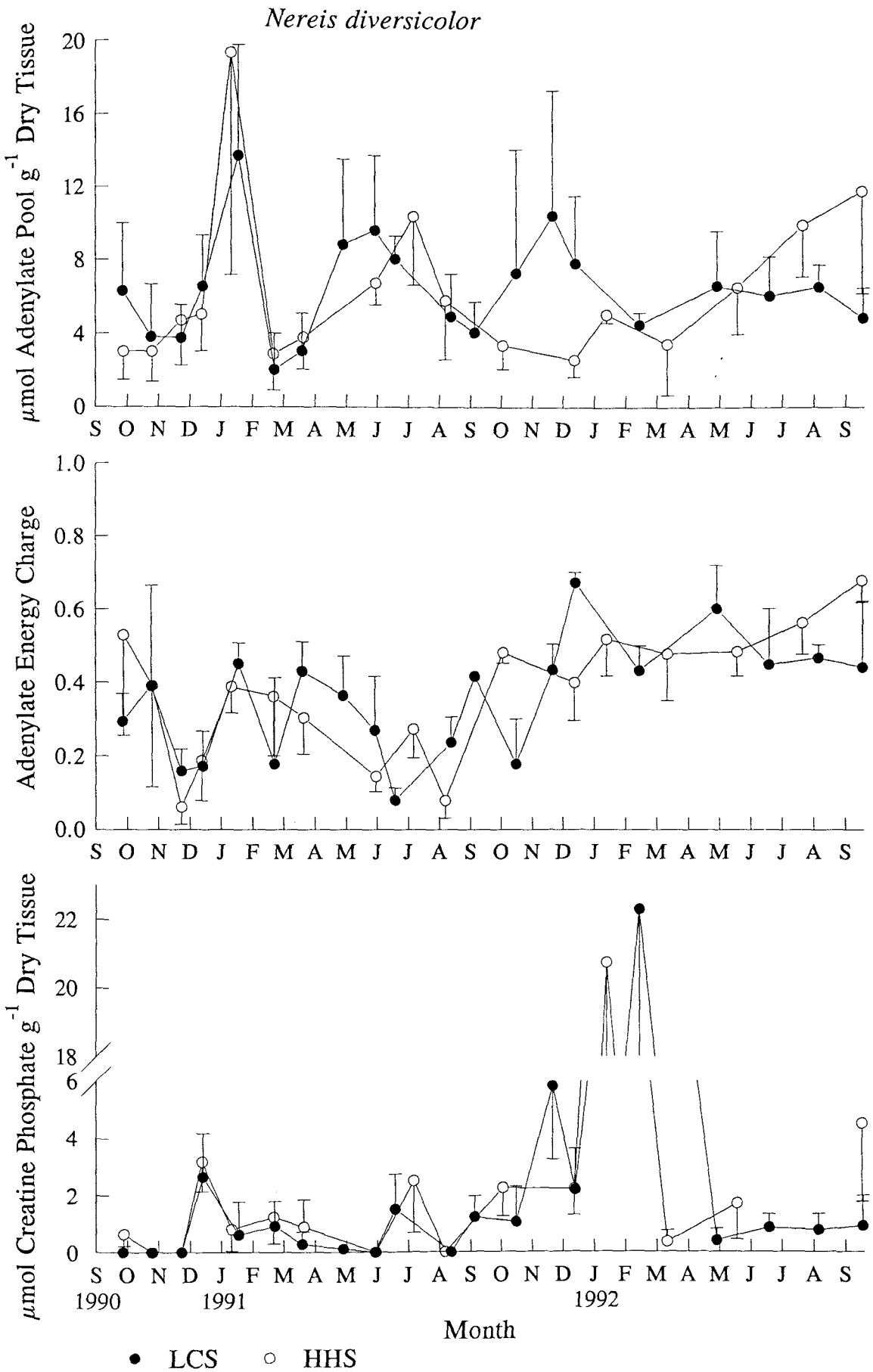


Fig. 8. Mean levels of components with time. Error bars indicate std. dev.

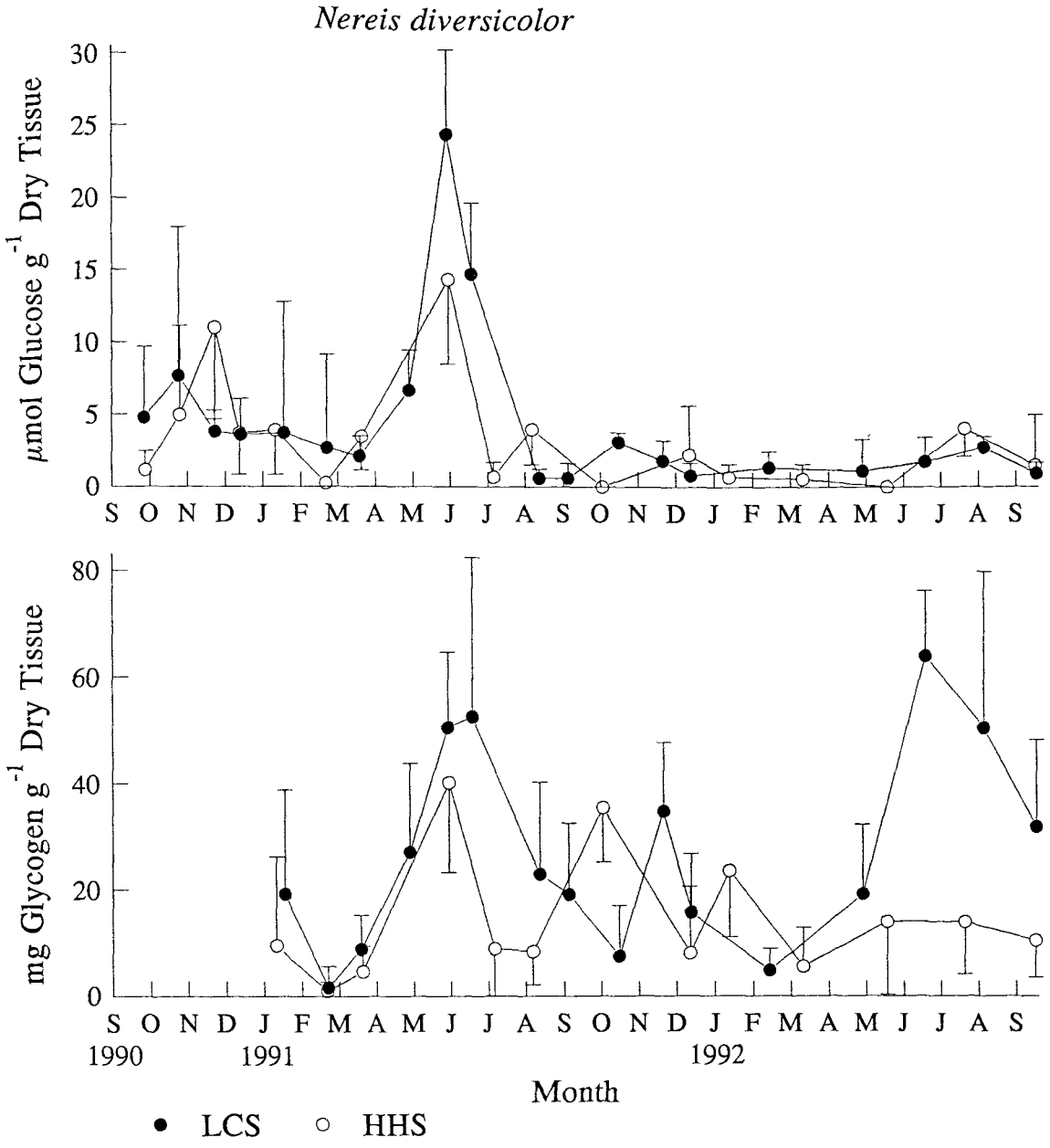


Fig. 9. Mean levels of components with time. Error bars indicate std. dev.

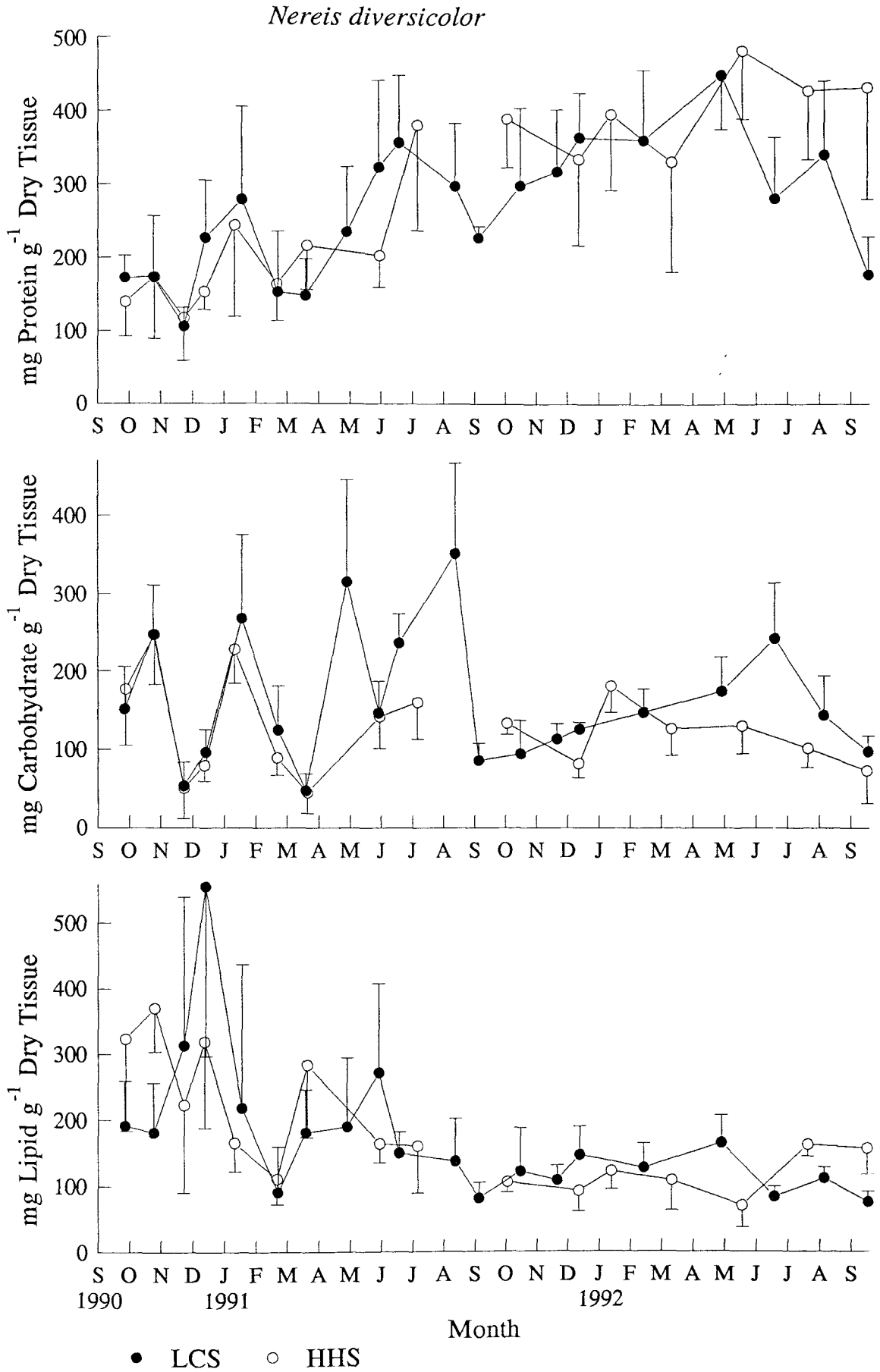


Fig. 10. Mean levels of components with time. Error bars indicate std. dev.

Levels of glucose (fig. 5) were variable at both sites, often very little glucose was present and this was more common at HHS. Glycogen levels changed with a distinct seasonal pattern, evident at both sites but more obvious at LCS. The low point occurred in spring, levels rose through the summer to a peak in early autumn, and then declined through to winter. Although this pattern was visible at both sites there were some significant temporal differences in addition to differing levels. The pattern is undoubtedly related to the reproductive cycle of the animal, which will be discussed in chapter 3.

Changes in the levels of the gross biochemical components are shown in fig. 6. There were few apparent differences between the sites and relatively large fluctuations. Protein showed a general rise throughout much of the two year period, dropping in the latter months. Total carbohydrate exhibited no obvious seasonal pattern but the responses of lipid suggested some seasonality at LCS, highest values for each year being recorded in early spring. This was more obvious in the first year and not apparent at HHS.

The individual adenylate results for *N. diversicolor* (fig. 7) were very variable, as was noted in the cockle. Major peaks were coincidental in all three adenylates but with no detectable seasonality, and no differences between sites. The adenylate pool (fig. 8) did show a cycle of peaks and lows, but it was not the same pattern in both years, nor indeed at both sites. The AEC values were relatively low but similar at both sites. (The lowness of the values has methodological implications which will be discussed in section 2.4). There was an apparent gradual rise in AEC during the course of the study. Creatine phosphate levels were generally low, often there was none present at all. An unusual peak occurred in January/February 1992 but it was of similar magnitude at both sites, at the same time, suggesting a real, if inexplicable, response.

The levels of glucose (fig. 9) followed a cycle apparent in the first year but not in the second. The pattern was similar at both sites but differences in magnitude led to a significant site difference. As with the cockle, glycogen showed a clear seasonal cycle, most obvious at LCS, that was indubitably linked with reproduction. Levels were highest in the summer, falling through autumn and winter to a low in February and then rising again during spring and early summer. There was a second peak in November 1991 at LCS which interrupted the

autumnal decline. This will be discussed in chapter 3 with reference to the reproductive state of the polychaete. Gross biochemistry results are shown in fig. 10. Protein levels showed a gradual rise throughout most of the two year study. Fluctuations in total carbohydrates were large, particularly in the first year. Lipids also showed larger fluctuations and higher levels in 1990/91.

To determine the differing responses of the organisms at the two sites to fluctuations in the environment it was necessary to assess significant differences in the mean values of each biochemical component. A two-way analysis of variance (ANOVA) was considered appropriate, since this test measures the difference between two sets of observations (in this case the individual biochemical measurements for the animals at each site), whilst accounting for fluctuations in a third factor (in this case time). Table 1 gives the p values (where $p < 0.05$ i.e. significant at the 5% level) resulting from the evaluation of differences in the biochemistry of the animals at the two sites.

It can be seen that several components exhibited significantly different responses at the two sites. The phosphagens, glucose and glycogen were significantly different in both species, the adenylates in general showed no differences. It is worth mentioning that the two-way ANOVA also measured the differences in each component with time whilst accounting for site (as opposed to differences between sites, irrespective of month, as shown in table 1). For each component for both species, without exception, differences were significant at the $p < 1\%$ level. This does not of course prove that each component had a seasonal cycle, or even a pattern with time, but it does indicate that none of the components remained stable with time, there were significant monthly differences.

Table 1. Results of 2-way ANOVA to detect differences in biochemical components at LCS and HHS.

COMPONENT	*C. edule*	*N. diversicolor*
Protein	n.s.	n.s.
Carbohydrate	n.s.	$p = 0.007$
Lipid	n.s.	n.s.
ATP	n.s.	n.s.
ADP	$p = 0.029$	n.s.
AMP	n.s.	n.s.
Adenylate Pool	n.s.	n.s.
AEC	$p < 0.001$	n.s.
Arg./Cr. phosphate	$p = 0.028$	$p = 0.027$
Glucose	$p < 0.001$	$p = 0.023$
Glycogen	$p = 0.016$	$p < 0.001$
Succinate	n.s.	
Lactate	$p < 0.001$	
Pyruvate	n.s.	

2.4 Discussion

In each of the preceding graphs it is apparent from the error bars that variation between individuals in the levels of each component were relatively large. This is a common problem when making biological measurements on individual organisms from a population. Nevertheless, significant differences between the sites have been detected, thus vindicating the choice of six individuals as a compromise between essential replication and time constraints.

The biochemical components that have been measured were chosen because they represent key points in metabolic pathways which will respond to environmental disturbance in the short, medium and long term. This enabled the detection of the effects of changes on all scales, from seasonal cycles to one-off unpredictable events. It will then be possible to integrate the responses of all of the components, an important element of this study, to develop the idea that biochemical responses do not occur in isolation. This integration will be discussed in chapter 5, for the moment the individual components will be examined to isolate specific information. As the adenylate system has received much attention in the literature as a general stress index, it would seem pertinent to discuss the contribution of the current investigation in establishing its validity as an indicator of environmental quality. Little of the literature, however, has been directly related to field population studies, most concentrating on specific pollutant experiments. The most noticeable result in this study was that AEC values for *N. diversicolor* were extremely low, both in comparison to values obtained for *C. edule* and considering that values below 0.5 supposedly indicate animals stressed beyond recovery (Ivanovici and Wiebe, 1981). Values for *C. edule* were almost always above 0.5 and often in the expected 0.7+ range. The methods employed for adenylate extraction closely resembled those suggested by Ivanovici (1980). It has also been reported by Karl *et al.* (1978) that cold acid extraction (as utilised in this study) achieved a higher yield of adenylates from multicellular organisms than alternative extraction procedures such as the use of boiling buffers. Furthermore, the methods used here did not differ between the two species. It can therefore be assumed that the methods employed provided adequate extraction and that the efficiency of adenylate extraction was similar for both species. As mentioned in Chapter 1 there are published examples of successful organisms with low AEC

values (Ball and Atkinson, 1975), and additionally, the response of the total adenylate system should be considered. It has been suggested that the adenylate system contains a self-regulating mechanism to oppose sudden changes in energy charge (Chapman and Atkinson, 1973). Under normal metabolic conditions the use of ATP generates equimolar amounts of ADP or AMP and so no change in the adenylate pool would be expected. However, under conditions which make high energy demands and thus a decrease in AEC, there is an increase in the deamination of AMP (catalysed by adenylate deaminase) which removes AMP from the system and so opposes the drop in AEC. The removal of AMP results in a drop in the total adenylate pool (Atkinson, 1977). As the adenylate pool decreases the activity of adenylate deaminase has been found to decrease (Chapman and Atkinson, 1973), presumably a self-limiting response to prevent excessive pool depletion.

Such a regulatory system could account for reported unexpectedly high AEC values if there is a corresponding alteration in total adenylate pool size. Zaroogian and Johnson (1989) however, could not explain increased AEC's in the polychaete, *Nephtys incisa* treated with dredged material. Work by Sylvestre and le Gal (1987) on *N. diversicolor* suggested that such a tolerant and resistant organism was able to maintain a high degree of AEC regulation. Verschraegen *et al.* (1985) discussed the potential of AEC measurements in *N. diversicolor* as pollutant monitors and concluded that they do not provide a good indicator of environmental quality. The AEC regulatory properties of living systems suggest caution in the use of the adenylate system as a general index of the physiological status of organisms in the environment.

In the current investigation specific effects of environmental stress were less relevant than the detection of differences in response to environmental disturbance between sites. The adenylate system did not appear to be sensitive enough to detect these differences, at least not in the format of this study. It should be remembered that the response of these metabolites can be measured in minutes, therefore expecting to detect site differences using monthly measurements (not necessarily taken in the same week) may have been ambitious.

The validity of the use of AEC as a biochemical index may be linked to the type of species chosen. Values of AEC in the cockle in the Baie de Somme were

in the anticipated range and differences between sites were significant (table 1). Picado and le Gal (1990) report significant decreases in AEC and changes in the adenylate pool of *C. edule* for the lowest concentration of sewage pollutant tested. In contrast however, Savari *et al.* (1989) could detect no significant difference in AEC in cockles between a relatively unpolluted and a chronically polluted site, when effects were clearly visible at the population level. *N. diversicolor* is a more tolerant, adaptive and therefore less susceptible species and this is reflected in the apparent insensitivity of its adenylate system. As a method for detecting environmental differences in time series data, AEC and adenylate measurements have been shown to have limited use when considered in isolation. There is an explicit requirement to include other components in the assessment.

The second tier metabolites, the phosphagens and glucose, did show significant site differences in both species, as did lactate in the cockle. Succinate and lactate are the end products of a respiratory cycle that begins with pyruvate. Many marine bivalves shut their valves tightly on exposure to air thus excluding almost all oxygen, and anaerobic respiration occurs. The cockle however, is one exception, its valves gape widely on exposure and it is known to be able to respire aerobically in air as well as water (Boyden, 1972). Consequently, *C. edule* has limited use for the succinate pathway employed by many marine bivalves, whereby the accumulation of lactate is reduced by halting the Kreb's cycle at the less harmful succinate, when in anaerobiosis. The lack of accumulation of succinate in *C. edule* on exposure has been recorded by Widdows *et al.* (1979) and Ahmad and Chaplin (1984). Furthermore, the seasonally related changes in the use of the succinate pathway, noted in bivalves such as *Mytilus edulis*, do not occur in *C. edule* (Ahmad and Chaplin, 1985). If this is taken into account then the seasonal patterns in succinate and lactate levels found in the present study would not be expected. Some seasonality was evident, particularly at LCS; levels were low in spring/summer and higher in autumn/winter. Whilst the pattern was not identical in the two years, it existed nonetheless. An explanation can be gained if one considers the unique tidal characteristics of the Baie de Somme. The estuary experiences a double low water, therefore high shore sites such as LCS and HHS were exposed for great lengths of time. This factor, combined with the neap/spring regime which also altered the duration of exposure, could have led to situations

whereby the cockles were uncovered for most of a tidal cycle. This degree of exposure is greater than in those conditions described by other researchers and it is possible to speculate that at such extremes, seasonal changes in the succinate and lactate pathways were expressed.

Glycogen showed significant site differences in both species but, of the other long-term metabolites, only total carbohydrates in *N. diversicolor* was significant. Since glycogen is the primary polysaccharide in annelids (Scheer, 1969), differences in total carbohydrates may have been enhanced by glycogen differences. It may be expected that structural elements such as protein and carbohydrate are not sensitive enough to detect differences between sites in this case. In both species protein showed no seasonal fluctuations, a result also expressed by Ivell (1981) working on *C. edule* in the Limfjord, Denmark, stressing the mainly structural nature of this component. Total carbohydrate levels in both species fluctuated greatly. This was most probably caused by a conflict of cycles between the structural carbohydrates and those concerned with reproductive cycles and storage. Such variability obscured any site differences. Lipid is also known to be related to reproduction. Ivell (1981) found that lipid levels in *C. edule* paralleled the reproductive cycle, with highest values in May/June. Similarly, maximum lipid values in cockles have been noted in summer, dropping to a minimum at the onset of winter (Navarro *et al.*, 1989). Polychaetes are known to utilise lipid as well as glycogen during the final stages of sexual maturation (Pocock *et al.*, 1971). However, the seasonal cycles suggested by these investigations do not match those found in the present study. An explanation may be found when the reproductive state of the Baie de Somme animals is considered in chapter 3.

In contrast, glycogen levels did follow suggested seasonal patterns, evident in both species at both sites, though more clear at LCS. Ivell (1981) records glycogen reserves in the cockle rising in spring and autumn and declining during August and winter. Interestingly, the decline in August noted in his study is duplicated in both years, at both sites in the Baie de Somme cockles. He suggested high temperatures increasing metabolic demands as the causative factor. There is some evidence here to support this idea, since August 1991 was considerably warmer than usual, and August 1992 slightly so (fig. 26), corresponding with the

more dramatic decline in glycogen in August 1991. There were however, other months that were unusually warm which did not show drops in glycogen. In any case, other researchers have recorded maximum glycogen in autumn and minima at the end of winter as glycogen is utilised in gametogenesis and reallocated from the soma to the gametes, and lost on spawning (Navarro *et al.*, 1989; Hummel *et al.*, 1989). This is the precise pattern noted in the Baie de Somme cockles.

The peaks and lows in glycogen levels in *N. diversicolor* followed the same cyclic pattern but were somewhat temporally shifted, reflecting the differing reproductive cycle. Minimum levels occurred in February in both years, coinciding with reported early spring spawnings (Dales, 1950; Olive and Garwood, 1981; Möller, 1985). The reproduction of *N. diversicolor* in the Baie de Somme will be examined in the next chapter.

This biochemical investigation has demonstrated that treated individually, some useful information on seasonal cycles and environmental responses can be gained from measuring these components. It has also been possible to detect differences in the responses of the animals at the two sites. At this stage the actual environmental stimulus that they were responding to is irrelevant. The biochemical changes were measured over time, thus the animals at LCS and HHS were either responding differently to the same seasonal conditions, or the conditions were different at the two sites. Both situations can be related to differing levels of disturbance and importantly, these differences can be detected (as proven) at the individual biochemical level. Combining the biochemical data to give an integration of the organisms responses, to be correlated with environmental and population data will be carried out in chapter 5. This will allow the controlling environmental factors to be isolated, and inferences on the processes controlling population structures to be made.

The previous discussion of the metabolites on an individual basis has additionally revealed the potential value of glycogen as a biochemical index. It is a long-term response metabolite that has still remained sensitive enough to detect significant site differences in both species (which have very different modes of life style). It also exhibited the strongest seasonal patterns of all the examined

metabolites, corresponding with the probable reproductive cycles of the two species. These attributes strongly suggest development of glycogen as a suitable index for deep sea specimens, as discussed in the previous chapter.

CHAPTER 3

Baie de Somme Population Studies

3.1 Population Structure

The benthic macrofaunal community of the Baie de Somme has undergone large scale structural changes over the decade preceding this study. The most noticeable, and those of greatest economic relevance, have been the changes in population density of the cockle. From a density of several thousand per square metre prior to 1982, numbers fell by two orders of magnitude, until successful recruitment restored the levels in 1987. Further mortalities occurred in 1989, but some recovery was apparent by 1991. Within the periods of depleted cockle densities the polychaete *Pygospio elegans* increased dramatically in numbers, rising from less than 100 individuals per square metre in 1983 to densities close to 200,000 per square metre in 1986. Numbers declined as *C. edule* became re-established in 1987. The interactions of these two species are not fully understood but it is likely that the opportunistic *P. elegans* was able to exploit the space made available by the cockle mortalities, which have been attributed to interacting climatic and eutrophication events (Desprez *et al.*, 1992). Such an inverse correlation between numbers of cockles and *P. elegans* has also been noted in the Waddensee, after successive cold winters severely reduced the cockle population (Clay, 1967a).

There have also been redistributions of species assemblages within the Baie de Somme throughout this period. Ducrotoy and Sylvand (1991) identified a decline in true estuarine-type species coincident with salt marsh advancement, and the increase of marine animals on lower tidal levels concurrent with greater marine water intrusion.

The fluctuations described above were attributed to the high-energy environmental characteristics of the estuary. A combination of anthropogenic (channel stabilization, for example) and natural perturbations led to opportunistic responses by the dominant species and subsequent instabilities in population structure. The monitoring of population changes was continued in this investigation

to attempt to discern the variability within the Baie de Somme.

3.1.1 Methods of Collection of Population Data.

The benthic faunal populations of the two sites described in chapter 1 were sampled monthly for a two year period. Samples for faunal analysis were taken at the same time as the animals that were collected for biochemical assay. Cores were taken at low tide using a corer with a mouth diameter of 0.02 m^2 penetrating to a depth of 25 cm, this was well below the deepest occurring macrofauna. Ten cores were taken at each site to give a total sampling area of 0.2 m^2. Each core sample was separately sieved *in situ* through a 0.5 mm mesh sieve and the material retained by each sieving fixed with 4% formaldehyde solution and stained with Phloxine B (C.I. No. 45410).

In the laboratory each fixed sample was re-sieved through a 0.5 mm mesh sieve to remove any remaining sand or mud. The sample was then washed into a white tray, so that the stained animals could be easily removed. These animals were identified to species level, counted and stored in 70% ethanol.

In addition to the numerical fluctuations of individual species with time, the faunal composition of the two sites was compared using the Bray-Curtis similarity classification. A review of the use of similarity coefficients by Burd *et al.* (1990) suggested that the Bray-Curtis coefficient most closely resembled the predicted similarities with benthic faunal abundance data. The analysis produced a dendrogram showing the similarities of LCS and HHS monthly samples, derived from the numbers of individuals of each occurring species. It was necessary to transform the raw data, before applying the ordination, in order to reduce the bias caused by the extreme dominance of *P. elegans* at HHS. The transformation used was root(root x).

3.1.2 Results

Tables 2 and 3 show the numbers of individuals of each species at the two sites, and additionally the fluctuations of the dominant species are displayed graphically in figs. 11 to 14.

Table 2. Species abundance data at LCS from September 1990 to May 1991.

SPECIES	NUMBER 0.2 m^{-2} $MONTH^{-1}$								
	9-90	10-90	11-90	12-90	1-91	2-91	3-91	4-91	5-91
Cerastoderma edule	244	315	294	150	348	104	210	148	68
Macoma balthica	64	33	47	40	33	5	66	82	278
Mya arenaria									
Hydrobia ulvae	1212	4728	1500	135	2508	1262	977	1055	1736
Nereis diversicolor	197	137	254	119	128	75	116	134	380
Pygospio elegans	200	404	589	11	720	261	220	3573	554
Arenicola marina	6		2	1					
Heteromastus filiformis	27	6	20	15	6	14	45	30	24
Eteone longa	23	41	43	1	6	1	3	978	336
Capitella capitata (agg.)	1			62					
Anaitides maculata		4	3						
Scolelepis foliosa									
Nephtys hombergii									
Cirriformia tentaculata									
Oligochaeta	308	550	945	768	646	1175	2260	2016	3102
Bathyporeia pilosa				17	9	8			
Corophium volutator				4	1	6	2	1	6
Carcinus maenas		2							
Eurydice pulchra			1			1			
Crangon crangon									
Cyathura carinata									
Nemerteans									

Table 2 continued, from June 1991 to September 1992.

SPECIES	NUMBER 0.2 m^{-2} $MONTH^{-1}$								
	6-91	8-91	9-91	11-91	1-92	3-92	4-92	8-92	9-92
Cerastoderma edule	352	365	268	608	163	122	75	35	70
Macoma balthica	3182	2603	1253	1157	433	187	150	205	223
Mya arenaria									
Hydrobia ulvae	11492	8813	5613	488	53	2332	4403	13622	24295
Nereis diversicolor	748	530	178	65	32	105	170	933	387
Pygospio elegans	477	1013	187	240	32	55	18	108	365
Arenicola marina		2	2				3	3	3
Heteromastus filiformis	65	28	32	13	20	13	30	13	17
Eteone longa	178	278	290	415	80	72	127	47	55
Capitella capitata (agg.)	85	25		5	13	7	25	38	32
Anaitides maculata									
Scolelepis foliosa									
Nephtys hombergii						2	3		
Cirriformia tentaculata									
Oligochaeta	6090	1952	4918	2297	2010	3145	2198	1992	1860
Bathyporeia pilosa				3		3		5	
Corophium volutator							2		2
Carcinus maenas	48	30		2				17	2
Eurydice pulchra									
Crangon crangon									
Cyathura carinata									
Nemerteans					3				

Table 3. Species abundance data at HHS from September 1990 to May 1991.

	NUMBER 0.2 m^{-2} $MONTH^{-1}$								
SPECIES	9-90	10-90	11-90	12-90	1-91	2-91	3-91	4-91	5-91
Cerastoderma edule	366	197	7	16	16	27	18	10	74
Macoma balthica	110	94	1	1	2	3	1		1354
Mya arenaria	3				1			2	
Hydrobia ulvae			42		1		1		2
Nereis diversicolor	14	3	12	15	17	12	12	24	10
Pygospio elegans			306	231	99	175	1986	10522	21176
Arenicola marina	1								
Heteromastus filiformis	97	54	7	16	12	4	11	2	2
Eteone longa			1				5	852	1252
Capitella capitata (agg.)					1				
Anaitides maculata									
Scolelepis foliosa						3			
Nephtys hombergii									
Cirriformia tentaculata									
Oligochaeta	1322	887	173	482	160	595	924	236	390
Bathyporeia pilosa	12	103	491	294	147	123	93		
Corophium volutator				4	2	6	2	6	4
Carcinus maenas				3	1				58
Eurydice pulchra				1					
Crangon crangon									
Cyathura carinata									
Nemerteans									

Table 3 continued, from July 1991 to September 1992.

SPECIES	NUMBER 0.2 m^{-2} $MONTH^{-1}$								
	7-91	8-91	9-91	11-91	1-92	3-92	5-92	8-92	9-92
Cerastoderma edule	780	932	47	48	87	65	40	4700	3722
Macoma balthica	4647	2693	655	1265	762	300	527	887	683
Mya arenaria			2					3	7
Hydrobia ulvae	128	10		2	7		2	1067	35498
Nereis diversicolor	8	17	3	25	30	32	62	625	658
Pygospio elegans	6437	22540	18912	30147	27018	39933	958	62740	21020
Arenicola marina				2			2	15	2
Heteromastus filiformis	2		5	5	8	12	17	270	130
Eteone longa	1448	1318	225	295	117	222	382	342	242
Capitella capitata (agg.)			18			18	7	18	
Anaitides maculata									
Scolelepis foliosa	2								
Nephtys hombergii						3	2	5	
Cirriformia tentaculata					2				
Oligochaeta	363	1148	1090	3885	445	4453	1052	5	1343
Bathyporeia pilosa				50	57				
Corophium volutator	5	8		2	5	15	80	5	
Carcinus maenas	113	23	3	3	2			85	35
Eurydice pulchra									
Crangon crangon								10	
Cyathura carinata						2			
Nemerteans					2	2	3	7	3

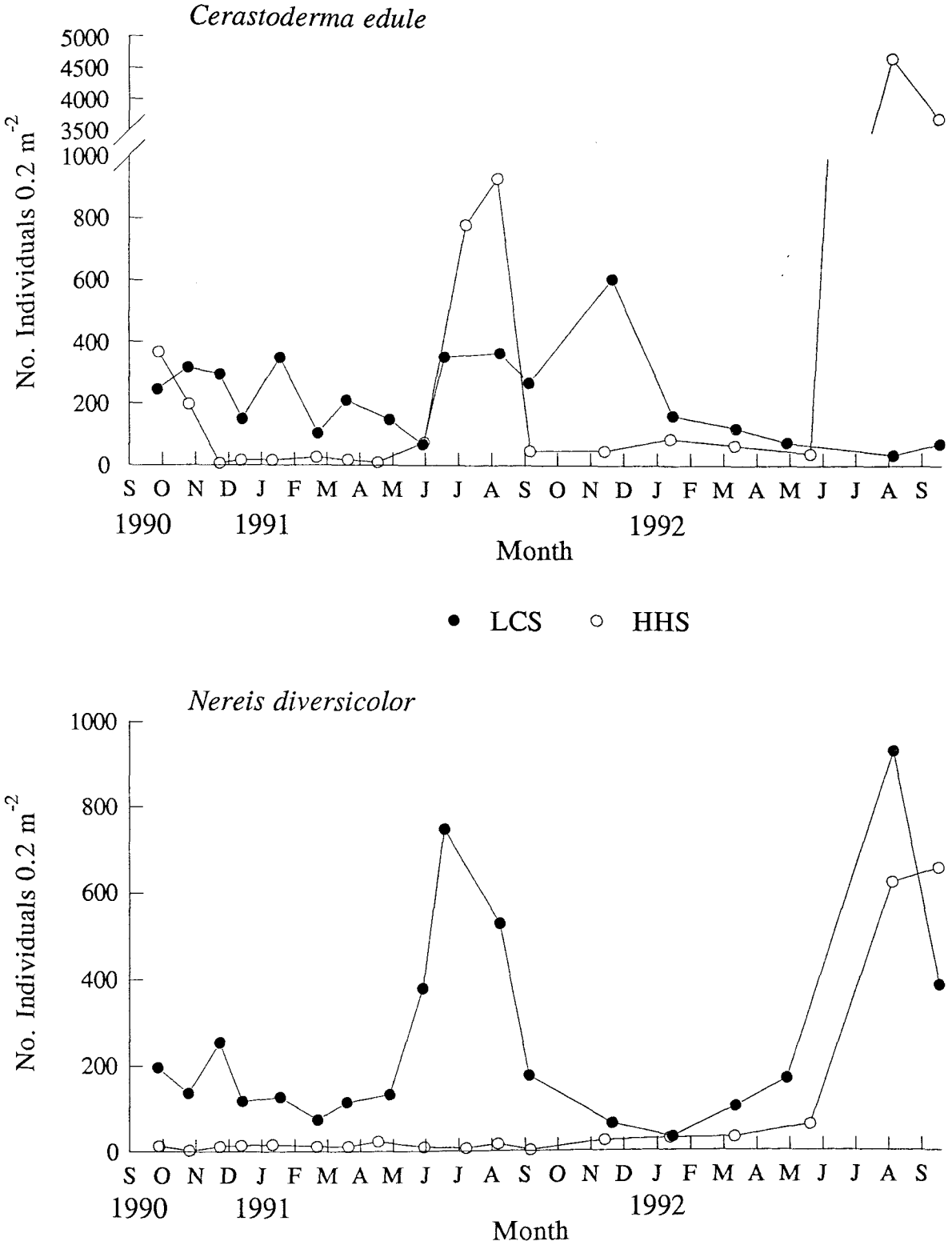


Fig. 11. Population fluctuations of the key species *C. edule* and *N. diversicolor* with time.

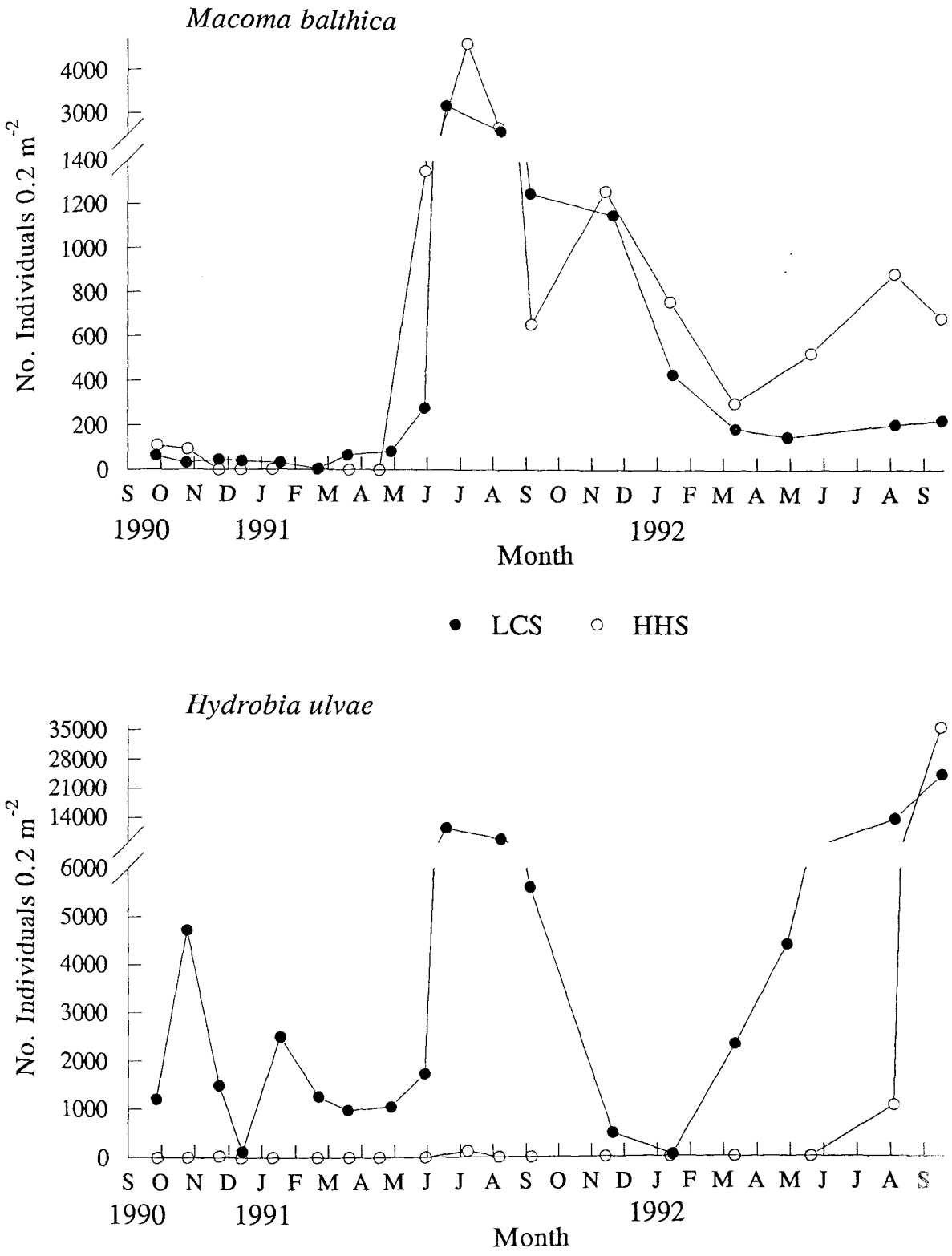


Fig. 12. Population fluctuations of the molluscs *M. balthica* and *H. ulvae* with time.

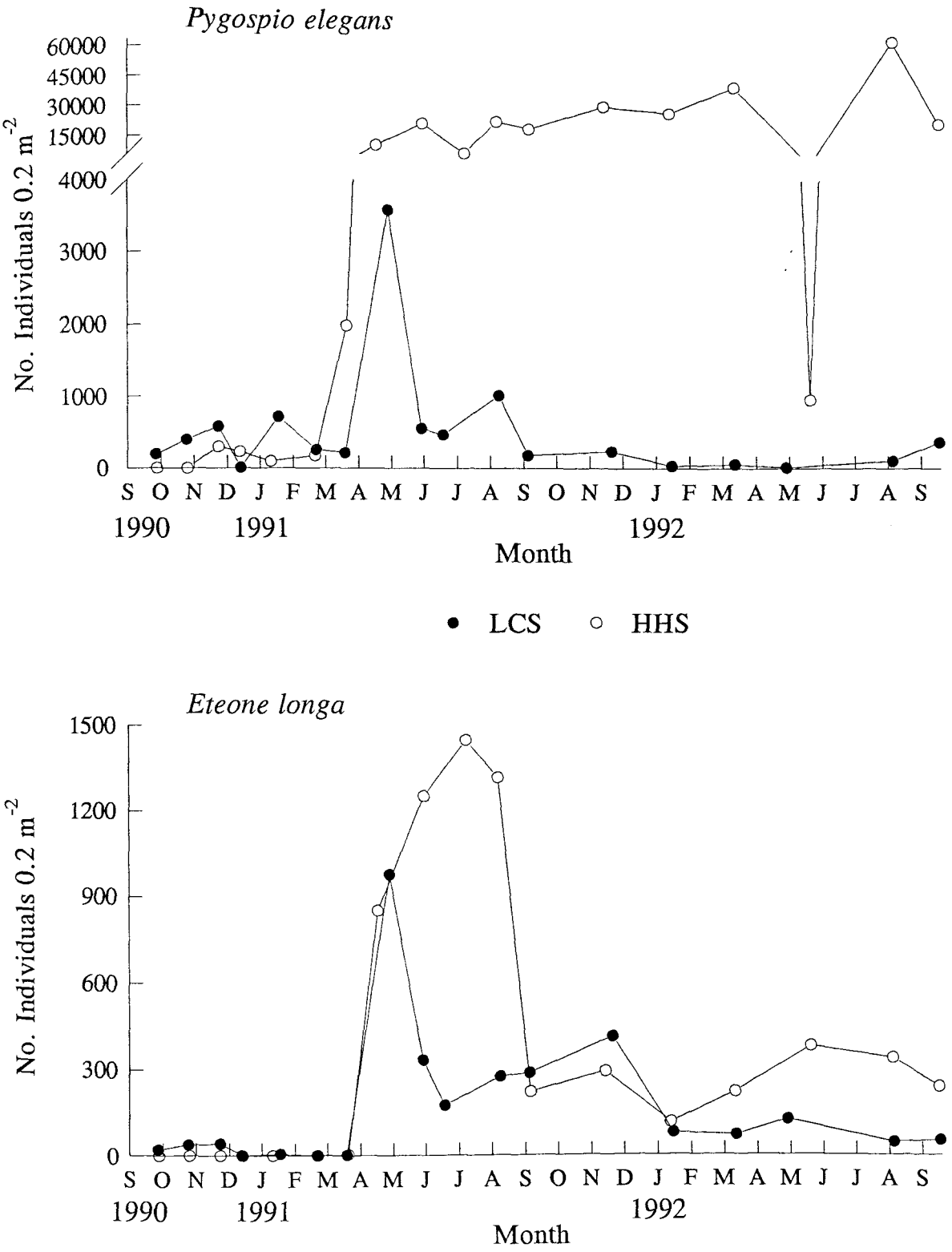


Fig. 13. Population fluctuations of the polychaetes *P. elegans* and *E. longa* with time.

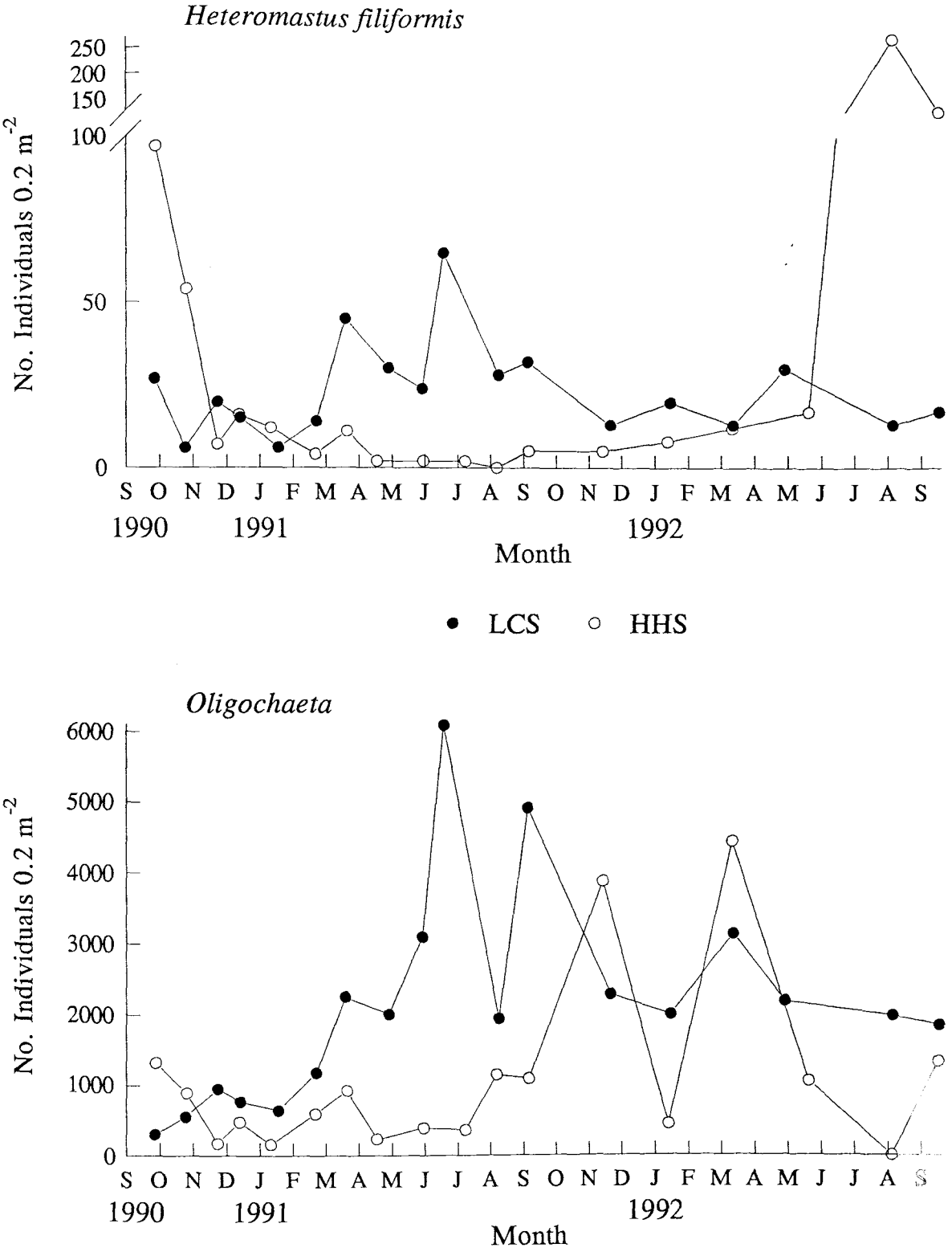


Fig. 14. Population fluctuations of the polychaete *H. filiformis* and total oligochaetes with time.

It is evident from tables 2 and 3 that the faunas at both sites were mainly composed of a few species that were almost always present in relatively large numbers, with sporadic occurrences of a few, rarer species. The two sites shared a common pool of species but with differences in dominance. The most notable differences were the absence of *H. ulvae* from HHS for the majority of the study period (fig. 12) and the extremely high numbers of *P. elegans* at HHS (fig. 13).

The densities of *C. edule* at the two sites remained very different throughout the two years (fig. 11). The HHS population showed obvious recruitment events in both midsummers, between June and August 1991 and June and September 1992 but numbers dropped almost immediately to the pre-recruitment low levels. Numbers at LCS fluctuated throughout the time series, with no clear recruitment pulse. There were normally more cockles present at LCS than HHS, although numbers were seen to decline at LCS towards the latter part of the study.

In contrast, recruitment of *N. diversicolor* (fig. 11), was much more obvious at LCS, occurring in both summers as opposed to only in the second summer at HHS. Numbers of worms at HHS were generally very low, only a dozen or so per square metre, except for the latter two months.

The bivalve *M. balthica* is known to occur in large numbers throughout the estuary (Desprez *et al.*, 1992). At the commencement of the study numbers were very low at both sites (fig. 12), but a successful recruitment in summer 1991 raised the densities. Numbers at LCS then gradually declined, with little recruitment the following year. Densities at HHS remained higher and some recruitment was evident in summer 1992.

The gastropod *H. ulvae* was almost entirely absent from HHS, with the exception of the last two months (fig. 12). A recruitment pulse was evident at LCS in summer 1991 between June and September, with a more extended period of recruitment from May onwards in 1992.

P. elegans also showed very different densities at the two sites from spring 1991 onwards (fig. 13). Numbers at LCS remained relatively low, with the exception of a peak in summer 1991. Recruitment at this time at HHS led to extremely high densities which were then maintained throughout the duration of the study. At these high densities the worm tubes were densely packed and formed

raised beds. The beds did not occupy the whole site, and so core samples were taken within beds and from the surrounding sediment in a ratio corresponding to the coverage of the site by the beds. Densities within the beds were estimated from within-bed core samples and found to be in excess of 500,000 individuals per square metre.

The polychaete *E. longa* showed similar densities at the two sites (fig. 13). Numbers were initially low until the summer 1991 recruitment, thereafter constant numbers were maintained. The recruitment of summer 1992 was much reduced, particularly at LCS.

The capitellid polychaete *H. filiformis* occurred in relatively low densities but these were maintained throughout the study period (fig. 14). Recruitment was not obvious, with the exception of August 1991 at HHS.

Total densities of oligochaetes are also shown in fig. 14. They were not identified to species level because of taxonomic difficulties but were predominantly of the family Enchytraeidae, with some occurrences of *Tubificoides* sp.. Densities were variable at both sites, showing a general increase in summer 1991 at LCS and in autumn 1992 at HHS.

It is also worth noting that sporadic high densities of the amphipods *B. pilosa* and *C. volutator* were found, particularly at HHS (tables 2 and 3).

In addition to comparing the fluctuations of individual species at the two sites it was also possible to compare the entire species assemblages on a month by month basis. Fig. 15 shows the results of a Bray-Curtis similarity plot from the transformed (root(rootx)) species abundance data (all species were included). The algorithm used was group averaging. The dendrogram divides into three main clusters. Cluster A consists of the early HHS samples, autumn and winter 1990/91. Cluster B contains all of the LCS samples, which can be further divided into B1 (autumn 1990 to spring 1991) and B2 (summer 1991 onwards). The sample for December 1990 does not fall into either of these clusters. Cluster C contains HHS samples from spring 1991 onwards, with the last two months samples somewhat separate. The species distributions that coincided with these clusters will be discussed in section 3.4.

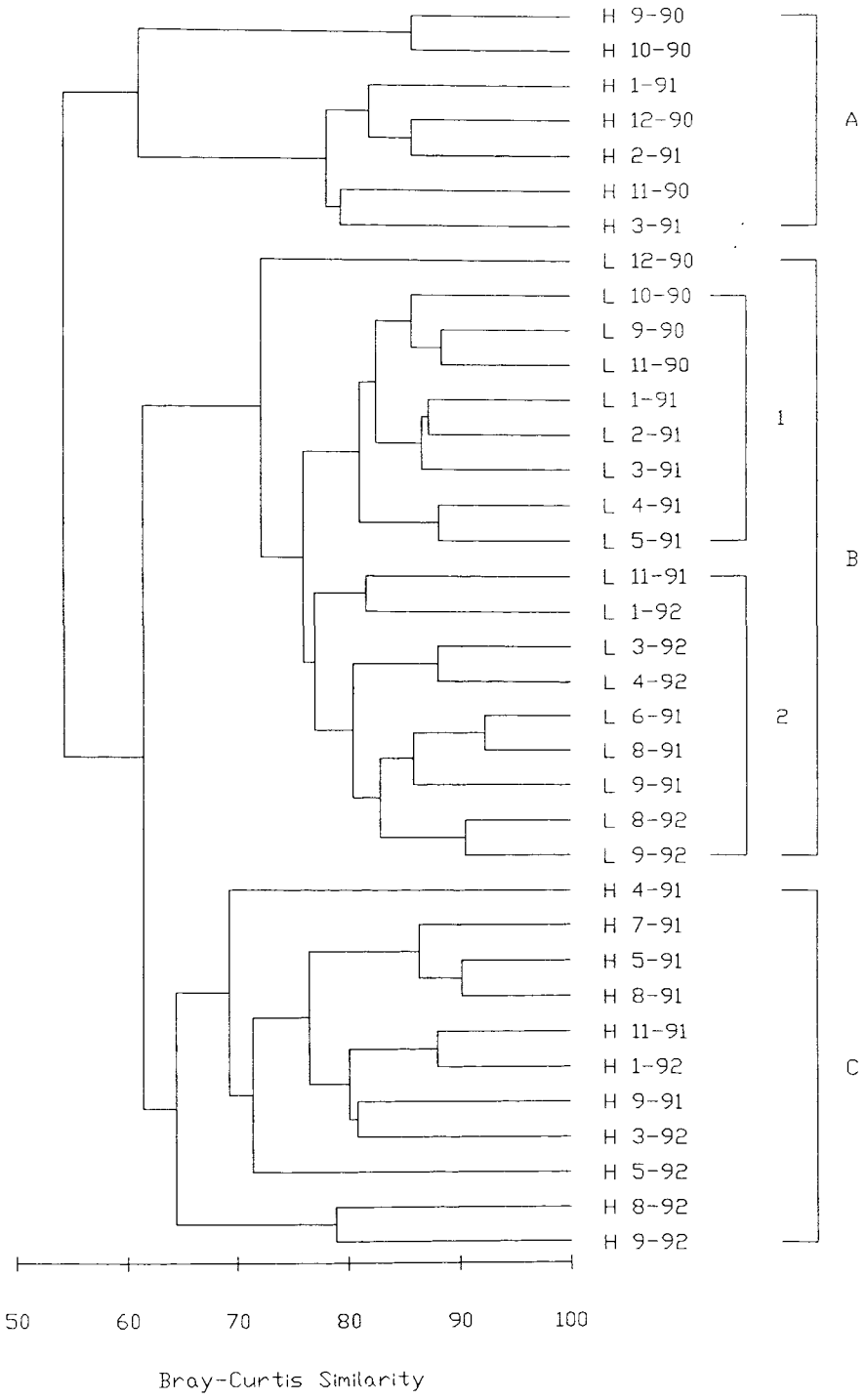


Fig. 15. Dendrogram showing similarity of monthly samples, using transformed species abundance data. L= LCS, H= HHS.

It was also decided to compare the distribution of individual species at the two sites to contrast the degree of patchiness. The protocol applied to measure such distributions was that of the variance:mean ratio of the individual core samples that comprised the monthly data points. The higher the ratio the greater the variability of each species within the core samples and hence the patchier the distribution of the individuals. The statistic loses validity however when numbers of individuals are small (less than ten per core for example) and it would be invalid to compare values for each site when the species occurs at disparate densities at the two sites. Of the seven dominant species shown graphically in figs. 11-14 only two fulfilled these necessary criteria for the application of the variance:mean ratio, *M. balthica* and *E. longa*. These were relatively numerous for the majority of the study period and their densities were of the same order of magnitude at LCS and HHS. Fig. 16 shows the variance:mean ratio for these two species. The period from September 1990 to May 1991 was characterised by low numbers of both *M. balthica* and *E. longa* and can be discounted from this assessment. It is apparent that both species had a more variable distribution at HHS (note log scale).

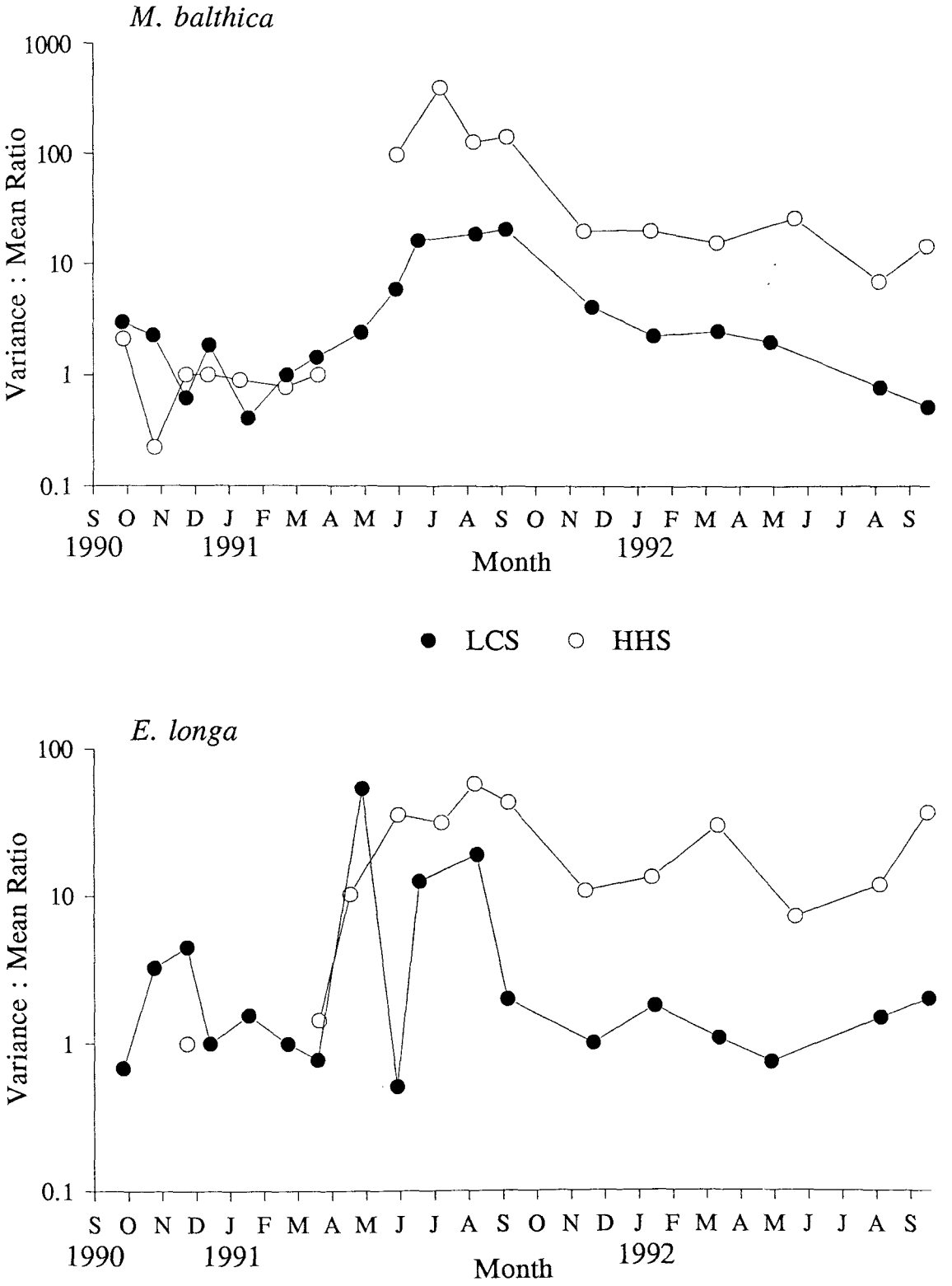


Fig. 16. Variance:mean ratios of the numbers of individuals in the monthly core samples for the species *M. balthica* and *E. longa*.

3.2 Size Frequency of Key Species

The size frequency distributions of *C. edule* and *N. diversicolor* were calculated to provide additional information on the population structure of these two key species. The number and growth of cohorts could then be monitored in addition to the timing of recruitment and the contribution of juveniles to the whole population. The section of the population affected by any mortalities could be determined to complement the biochemical data described in chapter 2.

3.2.1 Methods

The shell length of *C. edule* was measured to the nearest millimetre using vernier callipers. The biometry of *N. diversicolor* was based on peristomium width. It was necessary to select a variable from the anterior end of the worm since many individuals fragmented with the field sieving. As counts of abundance were based on the number of heads present in the sample, the use of an anterior size variable maximised the number of available measurements. Peristomium width was measured to the nearest 0.1 mm using a travelling microscope. All individuals extracted from the core samples were measured.

3.2.2 Results

Figures 17 and 18 show the size frequency distributions of the two species at both sites.

It can be seen from fig. 17 that, although fewer in number, cockles at HHS achieved a greater maximum shell length than cockles at LCS, some approaching 40 mm. This was not however a result of higher growth at HHS. The LCS site was on the edge of the cockle fishery area and the larger cockles were included as part of the catch, thus limiting the maximum size of cockles found at this site (Desprez, pers. comm.). This will also have reduced the maximum age of cockles at this site as the older, larger individuals were removed.

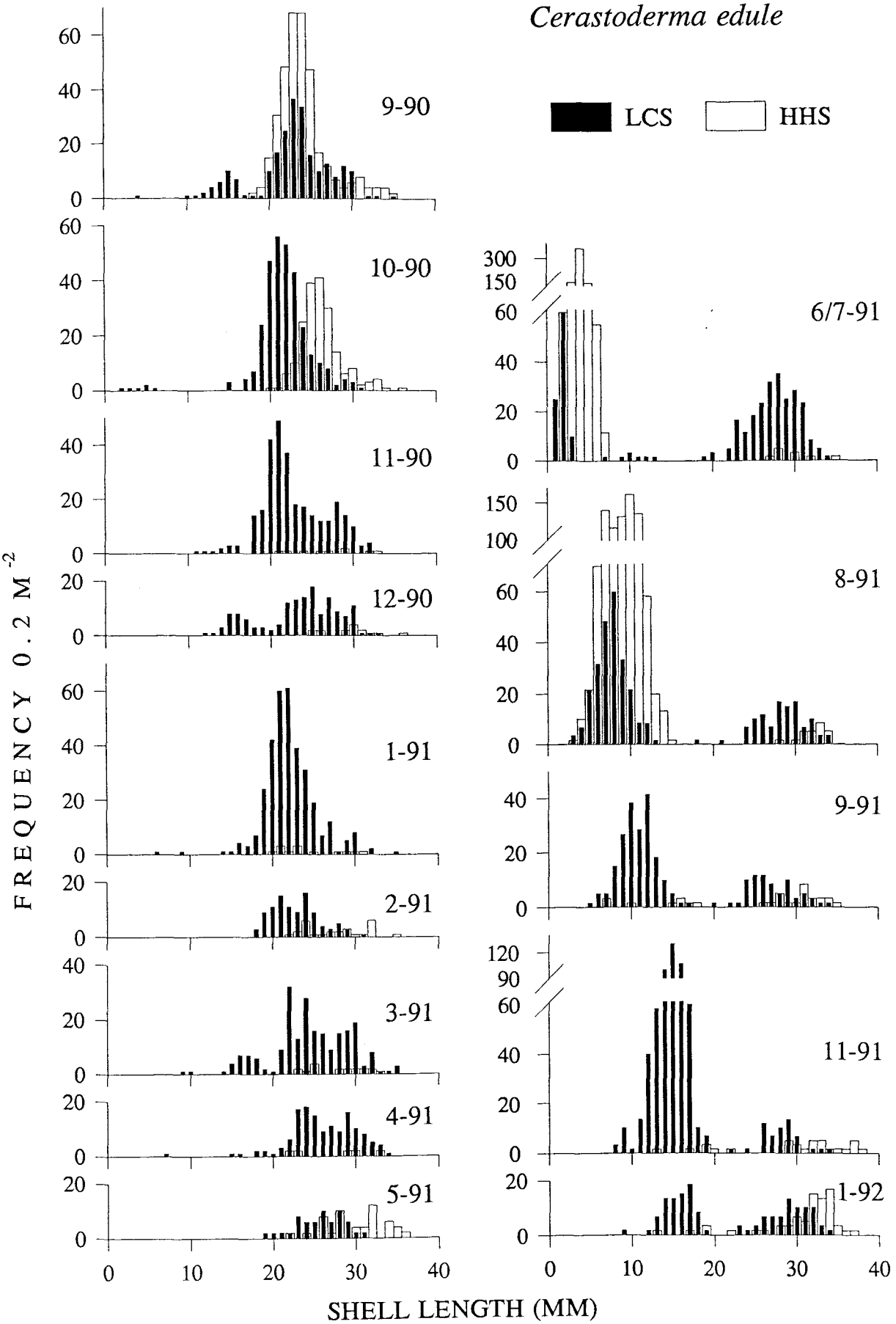


Fig. 17. Size frequency of *C. edule*.

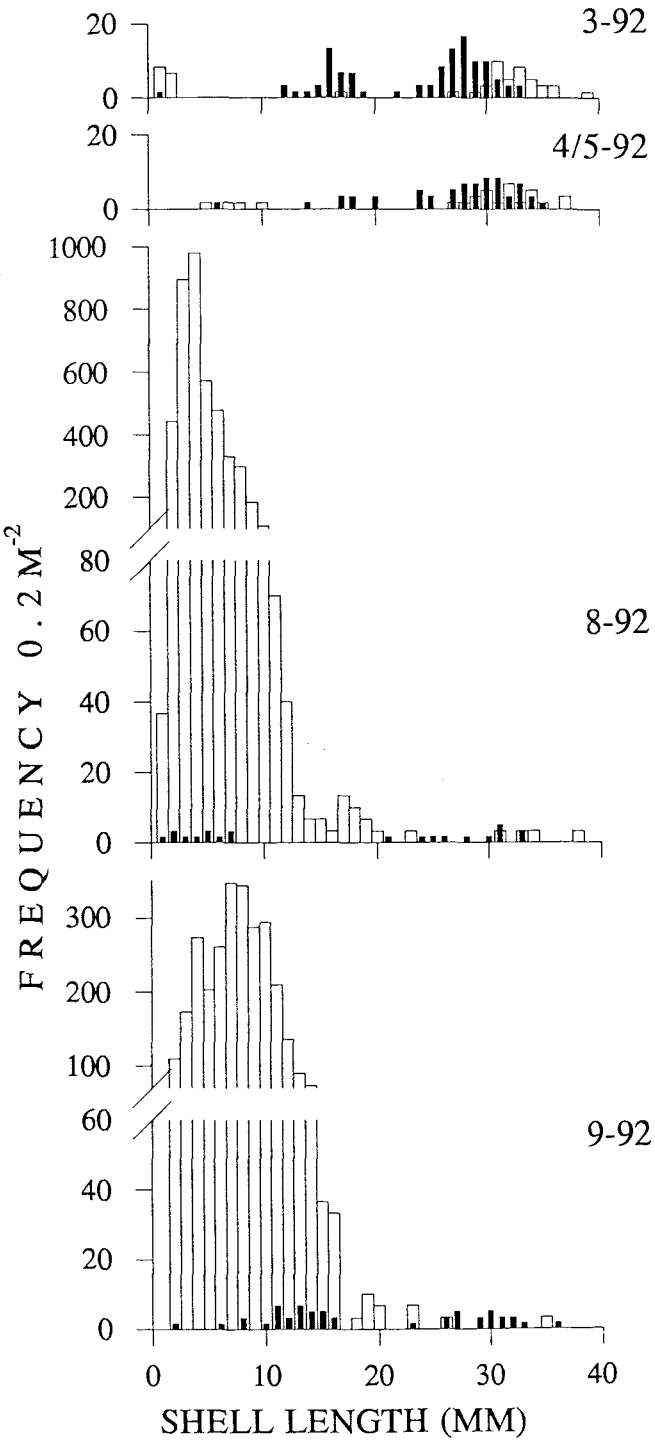


Fig. 17 continued.

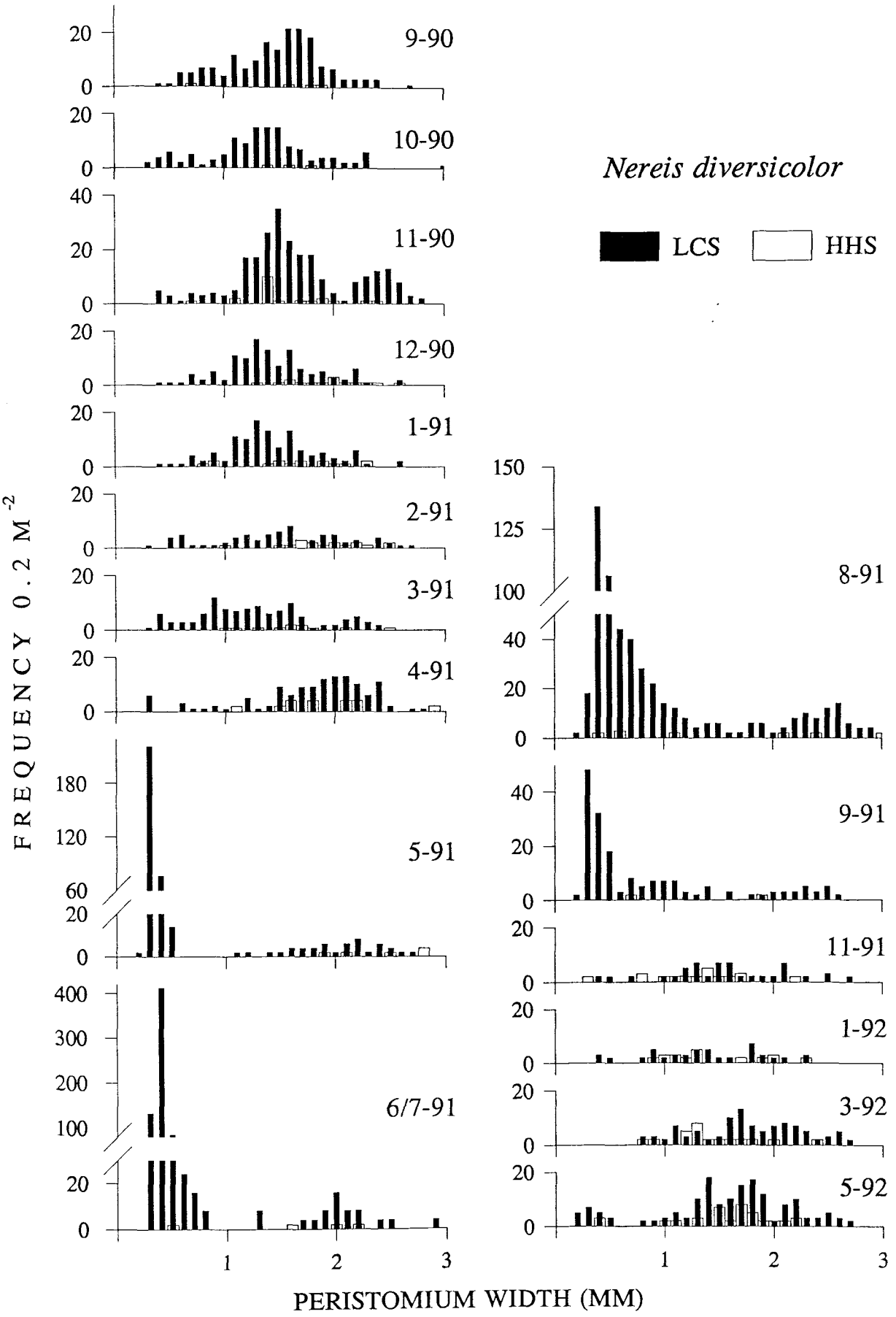


Fig. 18. Size frequency of *N. diversicolor*.

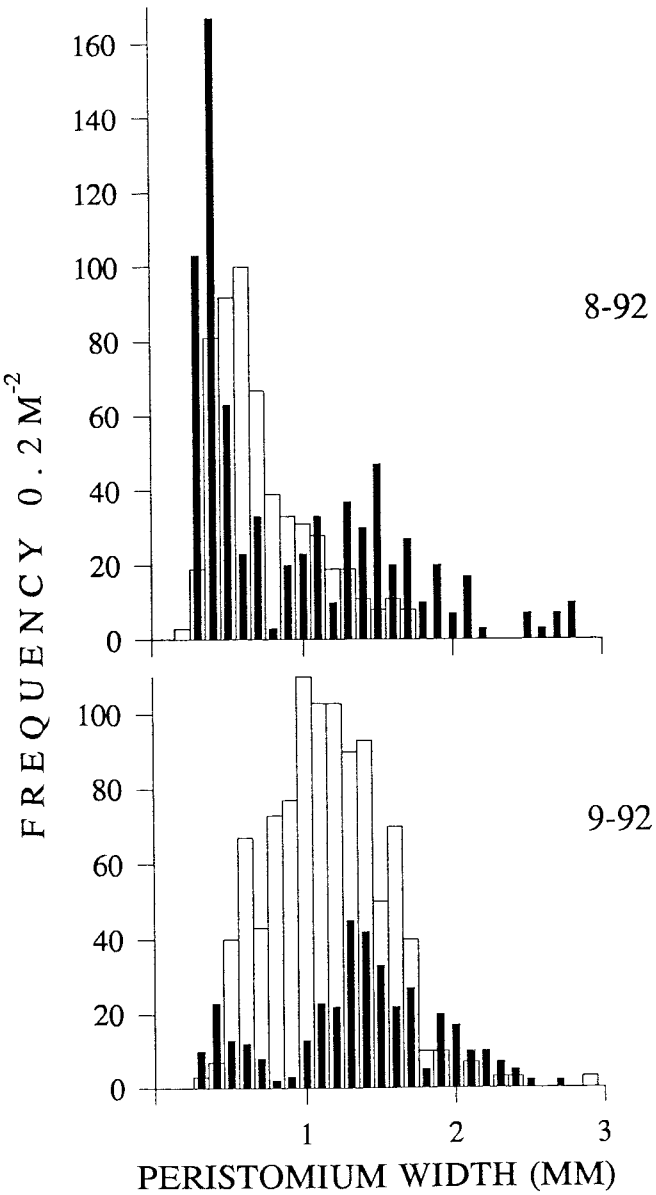


Fig. 18 continued.

There were two well-defined cohorts of cockles present at LCS for the majority of the study period, although from growth ring observations the larger cohort probably contained individuals from two or more years. There was presumably little recruitment in summer 1990 since very few cockles smaller than 10 mm were recorded at LCS at the start of the study, and none evident at HHS. The size of the initial cohort at HHS was dramatically reduced after the first two months, remaining so until the following year. Recruitment was evident at both sites in summer 1991, the first juveniles being recorded in June/July. Numbers of juveniles were initially higher at HHS, approaching 1000 per 0.2 square metre but by September had waned to only tens per 0.2 square metre. The recruits at LCS maintained high densities throughout autumn and winter but by spring 1992 only one or two individuals of this cohort remained. This disappearance was not found in the older cohort, where numbers remained constant throughout the winter, before declining as a result of fishing and/or natural mortalities in summer 1992. Recruitment in the summer of 1992 was very low at LCS but dramatically high at HHS. Unfortunately the study came to an end before it could be seen whether or not this new cohort survived the winter. At the end of the study period numbers of adult cockles (2+ years of age) were very low at both sites, in contrast to the commencement of the study when both populations were composed almost entirely of adults.

Growth rates could not be determined fully for cockles at HHS because of the paucity of animals. However, between September and October 1990 the adult cohort showed an increase in size of some 2-3 mm. The new recruits of 1991 showed growth rates of 5 mm in four weeks, a rate also found in the recruits of 1992. The initial adult cohort at LCS showed no apparent growth in the autumn of 1990. However growth of 8-9 mm occurred between January and August 1991. The new recruits of 1991 reached shell lengths of 15-18 mm in their first six months.

Figure 18 shows the size frequency distribution of *N. diversicolor*. Numbers at HHS were very low with the exception of the last two months, but most size classes were represented. Discrete cohorts were difficult to identify at HHS however, because of low numbers. Cohorts were also unclear at LCS, individuals initially appearing to be well spread over a large size range. The

polychaete has been recorded as living for one to two years (Hartman-Schröder, 1981) and two to three years (Dales, 1950; Essink *et al.*, 1985). It would appear that *N. diversicolor* in the Baie de Somme lives for two to three years, since the recruitment of 1991 can be followed through the following year and individuals of this cohort did not reach the maximum recorded size in one year.

The onset of recruitment in 1991 is hard to identify with certainty. Small individuals were recorded from February, with a major peak at LCS occurring in May. Few recruits were recorded at HHS and not until July. The juveniles at LCS suffered high mortalities and by November few remained to contribute to the population. The second years' recruitment was evident from May 1992, with major peaks occurring at both sites in August. Once again, the termination of the study did not allow speculation on the survival of these juveniles.

Growth rates were difficult to determine because of the obscurity of precise cohorts. The semelparous nature of this polychaete must also be considered. The adults die on spawning, therefore during the spawning period the larger individuals will be removed, decreasing the median size. This explains the apparent "negative growth" of the larger LCS cohort from January to March 1991. The reproductive cycle and its effects on population structure will be considered later in this chapter.

3.3 Reproductive Studies

The reproductive cycles of many species cause changes in their biochemistry, physiology and, in some cases, morphology. For this reason it was judged necessary to investigate the reproductive cycles of the two representative Baie de Somme species, to support or explain the biochemical results of chapter 2 and also to substantiate the population information already revealed in this chapter. Whilst providing useful information, however, this aspect of the Baie de Somme study was not part of the principal aims. The reproductive investigations have not been exhaustive because of the limited time that could be devoted to them.

The economic importance of the cockle fishery in the Baie de Somme has encouraged reproductive studies of the populations by other researchers (Lemoine *et al.*, 1988; Ducrotoy *et al.*, 1991; Desprez, unpublished data). Data from these investigations have been included in the present comparisons.

The reproductive cycle of *N. diversicolor* was simple to investigate. Females have been found to form the major part of populations; 80% in N.E. England (Olive and Garwood, 1981), and 90-99% in the Thames estuary (Dales, 1950), although this paper also mentioned occurrences where males comprised 30%. It was estimated from the individuals sampled in this study that the Somme populations consisted of at least 90% females. The examination therefore of comparatively few individuals could give a reasonable assessment of the reproductive condition of the majority of the population. An additional facilitating factor was that the gametes of *N. diversicolor* can be found floating freely in the coelomic cavity, so that location and extraction of the relevant reproductive material was straightforward.

3.3.1 Methods

Data on the reproductive cycle of *C. edule* were obtained from Desprez (pers. comm.). The method used involved the removal of a small piece of tissue containing gametes, examination under a microscope and assessment of the reproductive state. The reproductive cycle of *N. diversicolor* was derived from individuals collected during the course of the present study.

Five of the larger worms were removed from each monthly sample, although at HHS it was not always possible to find five worms, numbers were so scarce. Peristomium width was measured to ascertain any possible relationship between size of worm and size of gametes. Three segments were removed from the anterior portion of the worm using a scalpel, approximately twelve segments behind the head. The anterior section was chosen to maximise the number of examinable worms, particularly important in obtaining data for HHS. The segments were opened and the contents released. Flushing of the segments and teasing open the tissues of the parapodia with fine forceps, ensured the extraction of all of the oocytes. Any males encountered were ignored and another worm examined in its place. Males were too rare to contribute to the data with any validity and, in any case, the gametogenic cycle of males was much shorter than that of females, spermatozoa only being evident in the coelom just prior to spawning (Olive and Garwood, 1981).

The extracted oocytes were stored in 70% ethanol until analysis, as were

the entire worms before examination, therefore there could be no changes in oocyte size resulting from dilution or concentration of the storage medium. The oocytes were placed on a cavity slide and viewed using a microscope linked to the JAVA™ video image analysis system. The dimension measured was the maximum diameter of each oocyte to the nearest micrometre. All oocytes seen were measured, up to a maximum of approximately seventy, although in several cases the number was less than thirty.

3.3.2 Results

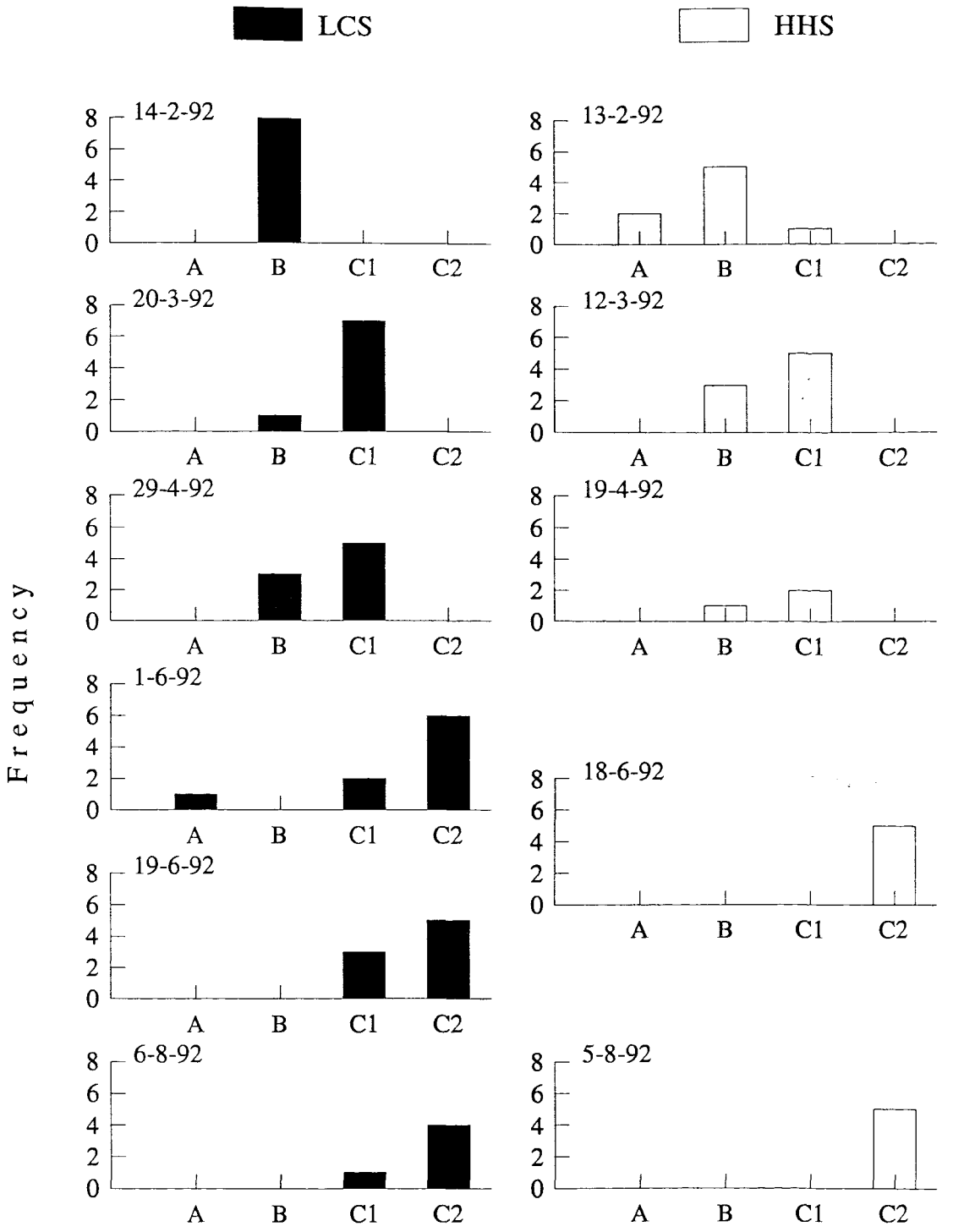
Figure 19 shows the reproductive state of cockles examined for a six month period within the two year study. Cockles at both sites showed a similar reproductive state at any one time. From March onwards cockles were ready to spawn and from June onwards they were either spawning or had just done so.

Figures 20 and 21 display the changes with time of oocyte size in *N. diversicolor*. The oocyte diameters for all five worms in each monthly sample were compared. Those worms which did not have significantly different oocyte sizes (using a one way ANOVA) were combined to reduce the number of data points on the graph, without removing any information. Many of the months are therefore represented by fewer than five data points. The data for LCS were more comprehensive than those for HHS, because of the greater number of worms available for examination. Three "cohorts" of oocytes are clearly visible in fig. 20, which overlap to some extent. At the commencement of the study two cohorts were evident. The larger cohort (A) continued to grow throughout the autumn and winter whilst the smaller cohort (B) remained relatively constant in size. By July 1991 cohort A had disappeared, a result of spawning, and growth in cohort B began, with an increased rate through autumn 1991. This then became the larger cohort, with a new, small cohort (C) evident from September 1991. Cohort B was not evident after June 1992, and growth in C had begun at the latter part of the study.

It may be noted that worms could not be separated into cohort A or B (in 1990/91) on the basis of their peristomium width. Some of the smaller worms in the first winter had oocytes in cohort A and would spawn in spring 1991, whilst some of the largest worms had oocytes in cohort B and would not spawn until

spring 1992. This emphasises the lack of precise age cohorts evident in the size frequency graph of fig. 18.

The data available for HHS (fig. 21) correspond to the patterns suggested for LCS, although it would be difficult to isolate this pattern from the few data points, were the cycle not so well described at LCS.



A- Gametes unrecognisable B- Gametes visible

C1- Mature Gametes, ready to spawn C2- During/post spawn.

Fig. 19. Reproductive condition of *C. edule* during the first six months of 1992. Data from Desprez, pers. comm.

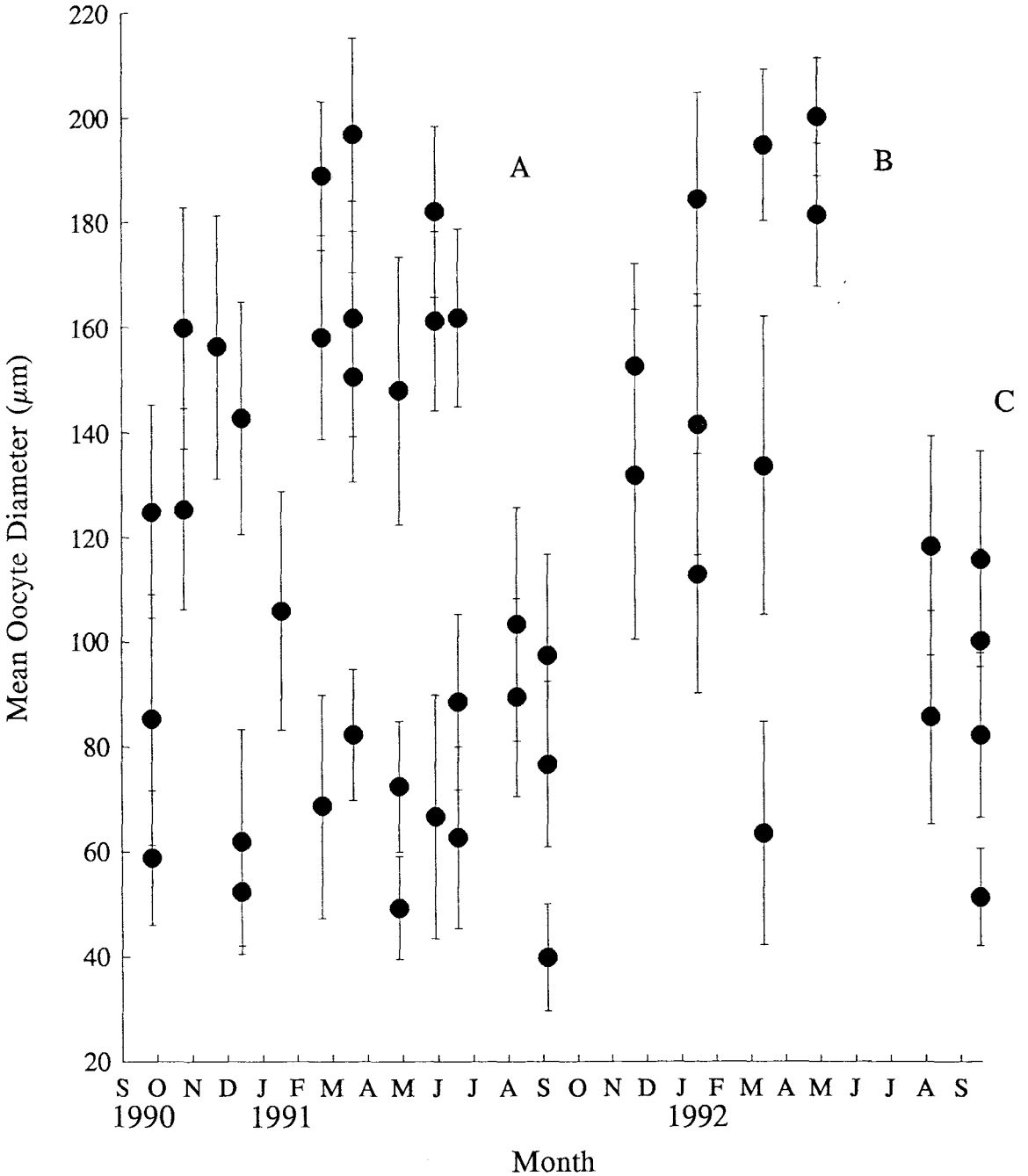


Fig. 20. Changes in oocyte diameter with time in *N. diversicolor* at LCS. Cohorts A, B and C, described in section 3.3.2 are indicated.

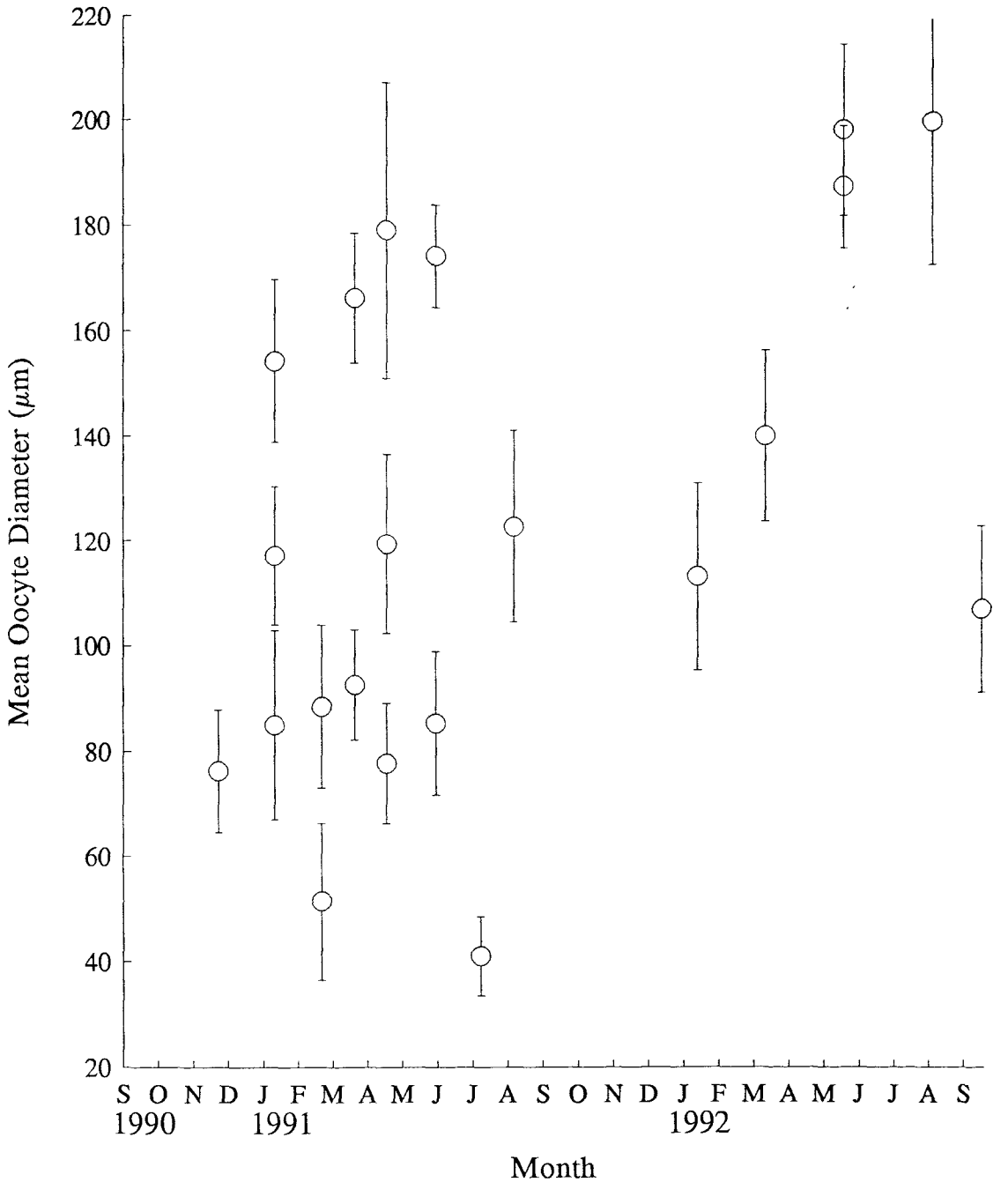


Fig. 21. Changes in oocyte diameter with time in *N. diversicolor* at HHS.

3.4 Discussion

3.4.1 Community Structure

Species diversity within the Baie de Somme was relatively low compared to some other estuaries along the English Channel coast. A littoral estuarine site in Southampton Water, for example, had 73 macrofaunal species in 1990/91 (Rowe, pers. comm.), compared with the 22 species recorded in the Somme. This paucity of species within the Somme estuary is a reflection of the macrotidal system and the associated harsh hydrodynamical regime discussed in chapter 1. The Seine estuary supported 28 species when monitored over a similar period of time, and experienced moderate environmental variability (Ducrotoy *et al.*, in prep.). Despite the low numbers of macrofaunal species in the Somme, differences in faunal assemblages occurred throughout the estuary, in accordance with differing environmental conditions. The sites LCS and HHS were only some two to three kilometres apart yet their faunal compositions, dominated by only seven or eight species, remained distinct throughout the two year study. As the cluster diagram of fig. 15 revealed, all of the monthly samples from LCS were more similar to each other than to any HHS samples. This was the case despite similar recruitment events at both sites. The maintenance of this difference was the result of differing responses of the individual species to the specific features characterising each site. HHS has been implicated as a more perturbed site, on the basis of its hydrodynamic features (chapter 1). This higher level of perturbation can be verified when the species responses are considered.

Both *C. edule* and *N. diversicolor* were found at lower densities at HHS throughout the study period (fig. 11), suggesting a higher degree of environmental stress. In the case of the cockle this was despite much higher recruitment at HHS. However, the majority of the newly settled spat only survived for three to four months. There are at least two possible explanations for the higher initial spatfall at HHS, both of which may have applied. HHS is known to be a region where settling out of material suspended in the water column occurs; a result of the protection afforded by the Hourdel shore bar (Ducrotoy and Sylvand, 1991) and being a region where fresh and marine water meet. *C. edule* has planktonic larvae which would be deposited in this area, together with the particulate material. Evidence for this hypothesis is given in chapter 4 (fig. 24) where the surface

sediment samples from the later months at HHS showed a huge rise in fine material, coincident with large spatfall of *C. edule*.

The second factor governing spatfall at HHS was the presence of adult cockles, since these are known to be major predators of their own larvae (Brock, 1980). Negative relationships between the number of adult *C. edule* and *Mya arenaria* and newly settled bivalve larvae were recorded by André and Rosenberg (1991) and Jensen (1992) also reported high recruitment success in cockles in the absence of adults. Densities of adults were much higher at LCS and in the seaward region where the main cockle fishery was situated, therefore more larvae would have been extracted from the water column, reducing the numbers able to settle. Once settled, the apparently more unfavourable conditions at HHS ensured that few survived for more than three or four months. *Macoma balthica* also showed higher recruitment at HHS (fig. 12) but the numbers were subsequently maintained and did not suffer the same high mortalities that *C. edule* displayed. *M. balthica* also has planktonic larva, hence the higher spatfall at HHS, but this species prefers a muddier sediment (Clay, 1967b). Sedimentary data in chapter 4 show that sediment at HHS was consistently finer than at LCS (fig. 22) which may explain the greater numbers of this bivalve at HHS. *Hydrobia ulvae* also has planktonic larvae but there was no evidence of settlement at HHS until the last two months. Assuming that the current regime led to similar patterns of larval settlement that occurred with the other molluscs, it would seem that the site conditions were too unfavourable for the juveniles to maintain themselves, so as to reach a size where they would be retained by the sieving procedure.

Although physical factors play a large role in determining larval settlement and subsequent juvenile success, biotic interactions must also be considered. As previously mentioned, *C. edule* inadvertently predates its own larvae, and presumably also the planktonic larvae of other species. The enormous numbers of *Pygospio elegans* at HHS will undoubtedly have exerted an influence on larvae. This will have occurred in the physical sense, as the raised beds of tubes altered the small scale current regime, thus influencing the settlement distribution of the larvae, as well as biotically. There is evidence of an inverse relationship between cockle and *P. elegans* densities in the Somme estuary (Desprez *et al.*, 1992) and in the Waddensee (Smidt, 1944 in Clay, 1967a). There are reports that suggest

high densities of *M. balthica* can coexist with high densities of spionid polychaetes (Bachelet, 1986). Some spionids such as *Polydora ligni* are known to ingest bivalve larvae (Breese and Phibbs, 1972). Such biotic interactions may have dictated the disparate densities of bivalves at HHS and LCS, suggesting that *C. edule* appeared not to be able to co-exist with *P. elegans* whilst *M. balthica* was relatively unaffected. It is impossible to say whether the almost total absence of *H. ulvae* from HHS was a result of the presence of *P. elegans* or some physical factor. Densities of *H. ulvae* and *P. elegans* at LCS were positively correlated (Paterson, pers. comm.) but densities of the polychaete were not as high as at HHS. It may be that the small size of this gastropod increased its vulnerability to the higher sedimentation rates present in the HHS region.

P. elegans clearly found HHS more favourable. It may be that the shelter provided by the shore bar reduced erosive currents that would inhibit bed formation, and prevent the massive densities that have been recorded. *P. elegans* has been found across the entirety of the estuary but bed formation was limited to the southern areas (pers. obs.). This polychaete has been recorded as having a remarkable flexibility in reproductive strategy. Many researchers have concluded that two types of larval development may occur, pelagic and also development within the parental tube (reviewed by Clay, 1967a). Additionally, asexual reproduction has been noted by Rasmussen (1953) and Wilson (1985). Such a diversity of reproductive strategies undoubtedly explains the opportunistic nature of this polychaete, which allowed it to increase rapidly in numbers; for example between February and April 1991 densities rose from 200 to 10,000 per 0.2 square metre at HHS. This also supports the suggestion that it was the physical nature of LCS that determined the reduced density of *P. elegans*. Its reproductive plasticity would surely have allowed it to colonize LCS to a greater extent, had the conditions proved amenable.

Of the other dominant polychaetes, *N. diversicolor* clearly favoured LCS. This species produces benthic larvae and although few were detected at HHS, it must be remembered that sampling was based on a 0.5 millimetre mesh sieve. Larvae would have to have been about six weeks old before they could have been retained, thus it is impossible to rule out the occurrence of very young larvae at this site. The reproductive investigation revealed that females at HHS were

producing oocytes with a similar cycle to those at LCS. Assuming that spawning occurred in a similar fashion it may be that the site conditions were too unfavourable for the larvae to survive. It is also plausible that the low numbers of adults at HHS (and consequently very few males) meant that although females matured as normal, spawning did not occur or was very rare, resulting in the production of few larvae. This in turn will have led to few adults in the following years, a self-perpetuating cycle. It is possible that some larvae will have been introduced to HHS from elsewhere in the estuary via strong currents transporting surface sediment. Detailed larval investigations would be necessary to ascertain the complete story, but clearly LCS was a more suitable habitat for this polychaete.

The interactions between the species and the sites have resulted in very distinct faunal assemblages, highlighted in fig. 15. Whilst the LCS and HHS samples remained separate there was also a strong temporal component to the observed clusters. The HHS cluster C was more similar to LCS samples (cluster B) than to HHS cluster A. The causative factor was the appearance of *P. elegans* beds at HHS in April 1991 which were then present for the remainder of the study. The samples of cluster A contained *P. elegans* densities of the same order of magnitude as at LCS. The samples of cluster C contained spionid densities an order of magnitude greater.

The LCS cluster (B) was divided into temporal groups, B1 contained the early samples up to May 1991 and B2 contained those from June 1991 to the end of the study. The species that caused this separation were *M. balthica* and *E. longa*. Both showed recruitment events in May/June 1991 which increased their densities. Numbers of both species were higher for the remainder of the study than prior to this recruitment.

Evidence for the greater degree of perturbation at HHS is demonstrated in fig. 16. The higher variance:mean ratios at HHS indicate a patchier distribution of individuals and suggest increased heterogeneity in this region. Although *P. elegans* was not considered in this exercise because of the incomparable site densities its' distribution was undoubtedly patchy at HHS. Beds containing huge numbers of the polychaete were interspersed with regions where few, or no individuals of this spionid were present. This pattern of bed formation may have been a result of hydrodynamic conditions, in any case their presence will have influenced the other

species at the site, contributing to the overall heterogeneity.

Although not shown in fig. 16, it may be noted that when *H. ulvae* occurred in large numbers at HHS (comparable to LCS), at the end of the study, the variance:mean ratio was again much higher at HHS, albeit for this short period. There is therefore a sufficiently large amount of evidence to propose that HHS experiences greater perturbations in its environmental regime than does LCS and this is reflected in the distribution of individuals and species.

3.4.2 Population Structure and Reproduction of *C. edule* and *N. diversicolor*

Annual variations in the reproductive cycles of the cockles in the Baie de Somme are well known and cause difficulties in identifying a precise reproductive strategy (Desprez *et al.*, 1986). In the current study, differences in the recruitment pattern of the two years were noted so that the population at the end of the study bore little resemblance to that present at the commencement (fig. 17). Superimposed on this annual variation were differences in recruitment patterns between the sites. The reproductive data available in fig. 19 reveal much similarity however, in the gametogenic state of LCS and HHS cockles. These data are also consistent with cycles reported for cockles in other locations. A population on the south-east coast of England showed increased gamete development from February to April, with spawning at the end of May/start of June (Kingston, 1974). Populations in the Tamar estuary, S.W. England showed initiation of gametogenesis in January, a peak in reproductive condition in April and May, followed by a three month spawning period (Newell and Bayne, 1980). Studies in the Somme in 1983 and 1986 have shown maturation of gametes in winter through to April/May with spawning occurring through the summer months of June to August (Lemoine *et al.*, 1988). Late, second spawnings were also noted in the autumn and early winter of both years. Both sites in 1992 showed gamete maturation through spring and spawning occurring from June onwards. The size frequency data (fig. 17) show that the first juveniles of each season were recorded in June/July of 1991 and August 1992, consistent with early summer spawnings. A few juveniles were also noted at both sites in March 1992 suggesting a second, late spawning in 1991. Extended or repeated spawnings would therefore appear to be a feature of the Somme populations, although similar instances have been

recorded in the S.E. England populations by Kingston (1974). It would seem that population differences were not a result of annual or site specific reproductive variability during the study period.

Cockle populations are known to be affected by severe winters. Yankson (1986) suggested that the cold winter of 1981-82 increased fecundity in adult cockles in a south Wales population as a result of improved feeding conditions. This was coupled with an increase in mortalities of larval predators as a result of low temperatures (including adult cockles) which led to reduced predation and hence greater recruitment. Higher cockle spatfalls after the same severe winter were noted in the Danish Waddensee by Jensen (1992). The present study period experienced a cold end to winter 1991, when February temperatures were some 3°C below the long term mean (chapter 4, fig. 26), scarcely rising above 0°C for the first two weeks. It would appear however that this had no noticeable effect on population structure, since adult mortalities did not increase to any great extent, and subsequent reproduction, although obvious, was not as marked as the reproduction at HHS in 1992.

Growth rates of cockles in the Somme have been recorded as high when compared with other estuaries but duration of the growth period has been annually variable (Ducrotoy *et al.*, 1991). Growth was noticeable in both adults and juveniles at LCS during 1991, although adult growth was slight and occurred only up to August. Removal of the larger individuals through fishing may have occurred however, obscuring true growth rates. Growth was also obvious in the 1991 recruits at HHS and also, for the short time studied, the 1992 recruits. Growth rates in cockles are known to be density dependent (Ivell, 1981; Jensen, 1993) and submersion time dependent (Jensen, 1992), as both factors influence food availability. Reduced growth rates have been measured in suspension feeding bivalves in densities covering 10% of the sediment surface area (Peterson and Black, 1987). Personal observations in the Somme have encountered areas where cockles formed a "pavement", with little space between individuals, covering more than 50% of the sediment surface area. These regions were not at LCS or HHS, but between these sites and the mouth of the estuary. On the flood tide the incoming water would reach these denser areas first and by the time the water covered the cockles at LCS and HHS much of the suspended material would have

been filtered by the more populated regions. Recent work has demonstrated that phytoplankton may be depleted within the boundary layer above dense suspension-feeding populations in shallow water areas (Muschenheim and Newell, 1992). Additionally, as the water flooded over the dense fishery areas, it will have received the nitrogenous waste products of the cockles, and also become depleted in oxygen levels as the newly covered cockles hyper-ventilated to recover from exposure-induced anoxia. This will have been exacerbated in warm weather when the water oxygen saturation is lower. It is likely, then, that by the time the water reached the higher shore cockles at LCS and HHS, it may not have been of optimal "quality". These effects could be observed in the biochemistry of the animals, if comparisons were made between low and high shore cockles.

In addition to these intraspecific interactions, interspecific food competition between suspension feeders will have occurred. This will have been particularly prevalent at HHS, with its higher densities of *M. balthica* and *P. elegans*, and may explain the inability of the 1991 cockle recruits to survive the autumn, post-settlement, when phytoplankton levels would be reduced (see chapter 4, fig. 28).

Nereis diversicolor did not exhibit clearly defined age cohorts (fig. 18) but by following the growth of new recruits it can be deduced that three age classes were normally present; those in their first year (new recruits), those in their second year, and also worms that did not quite reach three years of age before spawning. Growth was probably maximal in the younger cohorts, since the second and third year cohorts are hard to distinguish. A review of the literature suggests that this life span was the most common. Three year classes have been recorded by Mettam (1979), Olive and Garwood (1981), Möller (1985) and Humphreys (1985) in habitats ranging from the Severn estuary, England to western Sweden, although a two year span has been recorded by Kristensen (1984).

The reproductive cycle is also well documented. Spawning in most populations occurs in spring, although in many reports time of spawning has been inferred from the appearance of juveniles. Dales (1950) recorded an observed spawning period of two weeks in late February in an Essex population, and Möller (1985) noted mature oocytes in April in a western Sweden population. Humphreys (1985) and Essink *et al.* (1985) recorded the appearance of juveniles from April

and May, respectively, suggesting spring spawnings. The Severn estuary population however, was reported as spawning in summer (Mettam, 1979).

In the current study, maximum oocyte diameters were recorded from February onwards in both years at LCS (fig. 20) and probably similarly at HHS (fig. 21) although lack of data prevent certainty. The first appearance of juveniles at LCS is difficult to pinpoint from fig. 18, but was probably in March/April 1991 and April/May 1992. Measurements made by Dales (1950) revealed that juveniles of 1 mm in length were four to six weeks old. This was probably the minimum size of worm retained by the 0.5 mm mesh sieve, suggesting that spawning occurred in February/March. Further corroboration on the timing of spawning can be gained from the biochemical data in chapter 2. Minimal glycogen levels were recorded in February (fig. 9), suggesting maximum conversion of reserves to gametogenic products, so that spawning could have occurred soon after.

Spawning is thought to be controlled by temperature. Dales (1950) found spawning was coincident with a sudden rise in temperature from 5°C to 8.8°C. It has also been suggested that the lack of a sudden rise can affect the synchronicity of spawning. Bartels-Hardege and Zeeck (1990) found a later, extended spawning period following a mild winter in the same population where spawning had been confined to February and March after a cold winter. Apparent extended spawning may also result from temperature gradients that exist because of vertical shore position. Optimal spawning temperature will be reached progressively later moving down the shore (Dales, 1950) and this may explain the protracted recruitment period at LCS since a sharp rise in temperature was evident in February 1991 (chapter 4, fig. 26). Water currents will inevitably transport some larvae as the tide floods and ebbs, so the larvae recorded at LCS would also have come from higher and lower shore areas in the LCS region.

It is also apparent from fig. 20 that worms with mature oocytes existed beyond the spawning period, into summer. Möller (1985) also recorded gravid worms until July, but these did not spawn, the oocytes gradually breaking down and the unspawned worms dying. The worms only undergo one gamete maturation cycle, ending in death whether or not spawning has occurred.

The lack of defined adult cohorts in the size frequency graphs of fig. 18, coupled with the fact that the larger females had either mature or immature

oocytes, seemingly independent of size, requires discussion. Olive and Garwood (1981) suggested that *N. diversicolor* may have a variable age at maturity, which may be environmentally determined. Gametogenesis did not occur in worms in their first year of life, as in this study, so that the breeding population was composed of worms at least in their second year. They also could not distinguish age groups within the breeding population from size alone and suggested that some of the two year old females had undergone gamete maturation with the three year old females. It is probable that the majority of breeding females in the Somme populations were in their third year. However it is also possible that some in their second year, having attained a similar size (fig. 18), were also able to undergo gametogenesis. If the worms had found a particularly favourable area, with increased organics perhaps, a greater growth rate would have occurred. It is not possible to say with certainty that those females with immature oocytes in spring 1991 (fig. 20) were two year old worms whilst those with mature oocytes were three year worms, since the size ranges with each oocyte type were indistinguishable.

The oocytes underwent a rapid phase of growth from late summer through autumn, evident in figs. 20 and 21. Garwood and Olive (1981) demonstrated that this rapid growth can occur at a wide range of temperatures, provided that the females had reached a critical nutritional level. This period of rapid growth in oocytes in worms at LCS coincided with a fall in glycogen levels (chapter 2, fig. 9) from their summer peak. This supports the hypothesis that nutritional reserves were built up over summer and then utilised for oocyte development. Summer glycogen peaks were not so apparent in HHS worms, and the oocyte data were insufficient to show whether or not rapid oocyte growth occurred in these females. However, mature oocytes were recorded in females during spring so that it is probable that sufficient nutritional levels were attained.

3.5 Summary

The community structures at LCS and HHS remained distinct throughout the two year study period and were partially determined by the differing physical nature of each site. These faunal differences created biotic interactions which aided the maintenance of these distinctions, probably acting most intensively on the

larval stages.

Cockles at LCS and HHS showed similar reproductive cycles which in turn were consistent with cockle populations elsewhere. The higher spatfall at HHS was attributed to the fact that HHS was in a region where settling out of seston occurred, and the higher adult densities around LCS resulted in predation of the settling larvae. Although recruitment was initially greater at HHS, mortalities were almost 100%, a probable combination of the physical heterogeneity of the site, interspecific interactions with the high densities of *M. balthica* and *P. elegans* and intraspecific interactions between the dense juveniles. Adult mortalities at LCS in the latter part of 1991 were probably a combination of fishing and intraspecific interactions with cockles in the denser, main fishery area.

N. diversicolor also showed similar reproductive cycles at both sites, again correlating well with those found in populations at other locations. The maturation cycle was described and found to correlate closely with the seasonal glycogen changes recorded in chapter 2. The lower densities of worms at HHS may have been the result of a self-perpetuating regime with few spawning adults creating few larvae. Alternatively, it may be that the larvae suffered much higher mortalities at HHS.

CHAPTER 4

Environmental Data

4.1 Introduction

The preceding two chapters have described the accumulation of data on the population structure of the littoral community in the Baie de Somme and the physiological state of two dominant benthic macrofaunal species. In order to relate these two factors to each other and to describe any differences between LCS and HHS it is necessary to consider the environment in which the animals were living. The physical and chemical regime of a habitat will influence the organisms at the biochemical level and this in turn may be observed as changes at the population level. There are an infinite number of physical and chemical parameters that may be used to characterise the environment and it is not possible to predict those deterministic factors that control the organisms. Indeed, it is entirely probable that different species will respond in different ways to any one factor. In the present study all environmental parameters for which data were available were used. The time scale of the project did not allow the inclusion of detailed environmental measurements and so data has been collated from external sources, the French Meteorological Office and other researchers studying the Baie de Somme. As this investigation involved the study of benthic macrofauna, it seemed appropriate to monitor the medium of their habitat. Samples of sediment were taken on a 3-monthly basis from both LCS and HHS.

4.2 Sediment Analysis

4.2.1 Methods

A sample was taken of the surface 1cm to monitor fluctuations in sediment properties at the sediment-water interface and, using a small corer (5 cm diameter), an integrated sample of the surface 10cm was taken to detect large scale sedimentary changes.

Each sample was frozen separately after collection and stored at -20°C. Analyses were performed on all samples simultaneously to maximise

comparability. The parameters measured were granulometry and organic content. Particle size analysis was performed, using the technique described by Buchanan (1984), and given below.

Each sample was mixed with 6% v/v H_2O_2 which breaks down any organic material that may bind the particles together. Once the reaction was complete (no more activity was evident) the sample was filtered and washed with distilled water to remove the peroxide. 20 ml sodium hexametaphosphate (6.2 gl^{-1}) was added and the sample agitated and left to stand for 5-10 minutes. The sodium hexametaphosphate reduced the electrical charges which may have caused the fine particles to aggregate and hence altered the particle size distribution. The sample was then washed through a 63μm mesh sieve, retaining the material passing through the sieve, until reasonably clean. The sieve and its contents were dried in an oven at 70°C and then the sediment passed through a stacked sequence of sieves from 2mm to 63μm mesh in half ϕ intervals (Wentworth scale) using a sieve shaker. The fraction retained by each sieve was weighed and the sediment that passed through the lower 63μm sieve was added to the retained wet sieved fraction. This portion of the sample was made up to 1 litre with distilled water, inverted and agitated to resuspend and mix the sediment and then a 20 ml sample was immediately removed to a pre-weighed vessel. This was allowed to dry at 70°C, it was reweighed and the weight of sediment calculated. Multiplication of this amount by 50 gave the weight of the $< 63\mu m$ ($> 4\phi$) fraction which could then be combined with the data already accumulated for the larger fractions.

Total organic content of the sediment was determined by loss of weight on ignition at 550°C. Portions of sediment (5 replicates per sample) were transferred to pre-weighed crucibles and placed in a muffle-furnace at 550°C for 6 hours. After cooling in a desiccator the samples were re-weighed, and the percentage weight loss of the original sample calculated. This loss was equivalent to the total amount of organic material present in the sediment.

4.2.2 Results

The precise granulometric distributions for each sample are given in appendix 2. The data displayed in this section are the statistical derivations from these raw percentage distributions, which are more pertinent to this type of study.

The following variables have been calculated, from cumulative frequency curves, which describe the particle size distribution of the sediment at the two sites (Buchanan, 1984).

Median ϕ This is a measure of central tendency and is defined as the ϕ value which is > 50% of the ϕ values in the distribution and < the remaining 50%.

$Md\phi = \phi 50$

Inclusive Graphic Standard Deviation This measures the spread of the data about the central tendency and gives an indication of how well the sediment is sorted.

< 0.35	Very well sorted
0.35-0.5	Well sorted
0.5-0.71	Moderately well sorted
0.71-1.0	Moderately sorted
1.0-2.0	Poorly sorted
2.0-4.0	Very poorly sorted
> 4.0	Extremely poorly sorted

It is defined as $\sigma_1 = \frac{\phi 84 - \phi 16}{4} + \frac{\phi 95 - \phi 5}{6.6}$

Inclusive Graphic Skewness This measures the asymmetry of the spread either side of the median, the degree to which the data are skewed.

+1.0-+3.0	Strongly fine skewed
+0.3-+0.1	Fine skewed
+0.1--0.1	Symmetrical
-0.1--0.3	Coarse skewed
-0.3--1.0	Strongly coarse skewed

It is defined as $Sk_1 = \frac{\phi 16 + \phi 84 - 2\phi 50}{2(\phi 84 - \phi 16)} + \frac{\phi 5 + \phi 95 - 2\phi 50}{2(\phi 95 - \phi 5)}$

Graphic Kurtosis This measures the degree of departure from a normal distribution, the "peakedness" or flatness of the distribution.

< 0.67	Very platykurtic (peaked)
0.67-0.9	Platykurtic
0.9-1.11	Mesokurtic (nearly normal)
1.11-1.5	Leptokurtic
1.5-3.0	Very leptokurtic (flat peaked)

It is defined as $K_g = \frac{\phi 95 - \phi 5}{2.44(\phi 75 - \phi 25)}$

Percentage Silt/Clay Fraction This is the fraction of the sediment that passes through a 63 μm mesh sieve.

It is defined as the fraction $> 4.0\phi$

These measurements are displayed graphically in figures 22 to 24. The results of the organic content analysis are shown in fig. 25.

The median ϕ of the Baie de Somme sediment lay within the 2.5ϕ to 3.0ϕ range, indicating that the sediment was primarily fine sand (the higher the Mdϕ, the finer the sediment). It can be seen from fig. 22 that the Mdϕ at HHS, both of the immediate surface and the top 10 cms had a tendency to be higher than at LCS. A t-test however, revealed that this difference was not significant (p = 0.067 for the 1 cm layer, and p = 0.115 for the 10 cm layer). Additionally, the Mdϕ at HHS was seen to increase steadily over the two year period, whilst the sediment at LCS fluctuated within a narrow range.

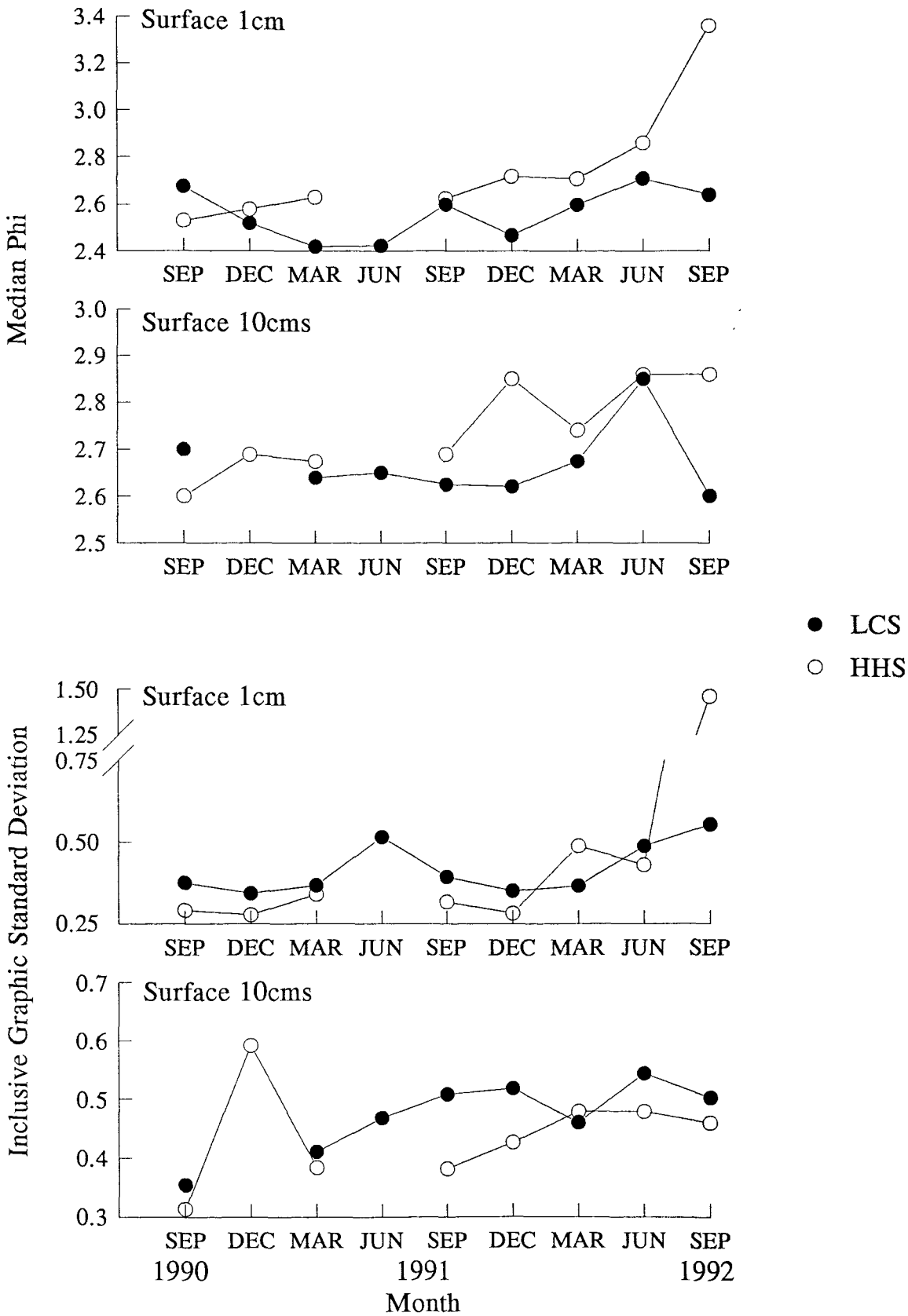


Fig. 22. Fluctuations in the values of Mdϕ and σ_1.

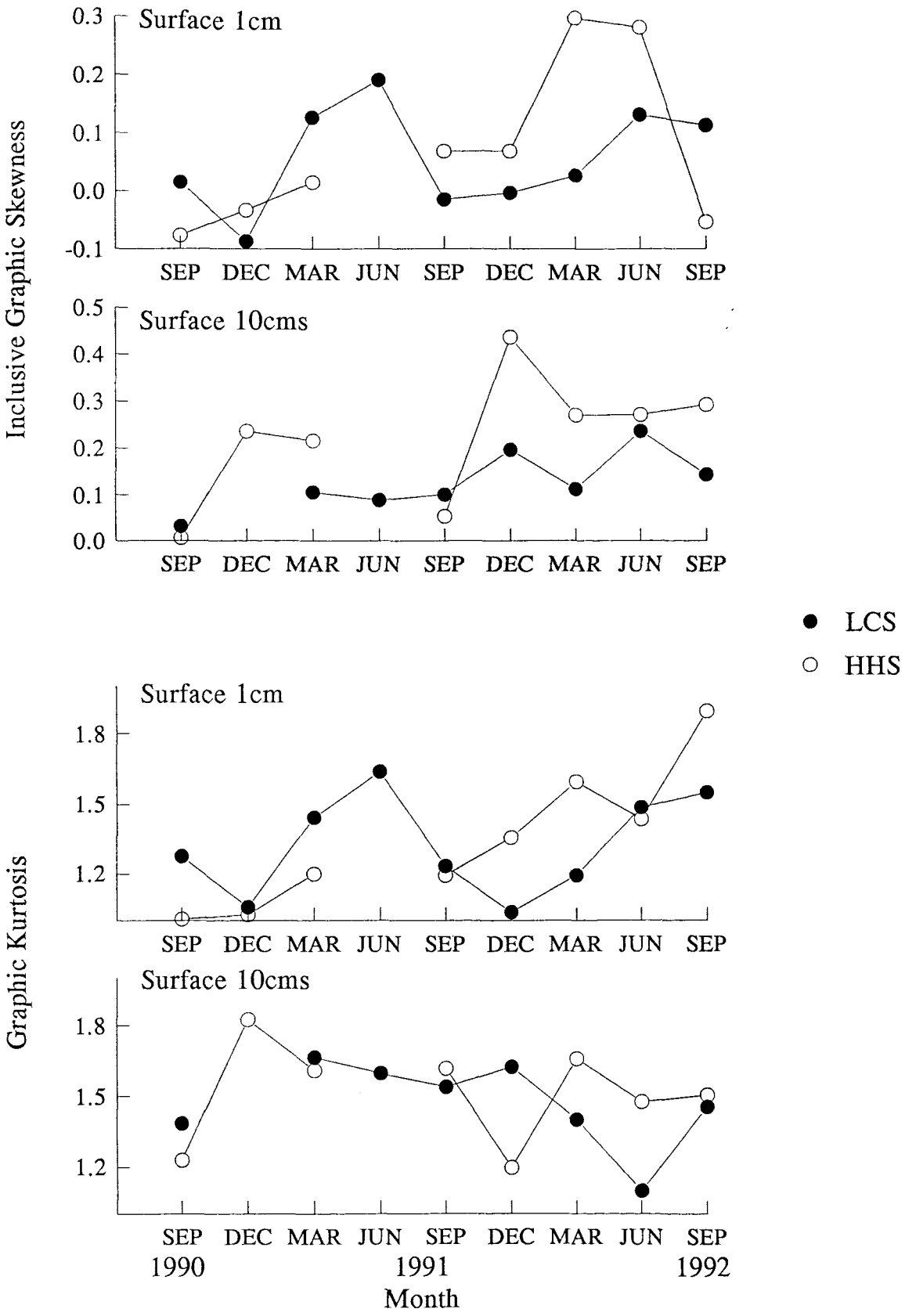


Fig. 23. Fluctuations in the values of Sk_I and K_g.

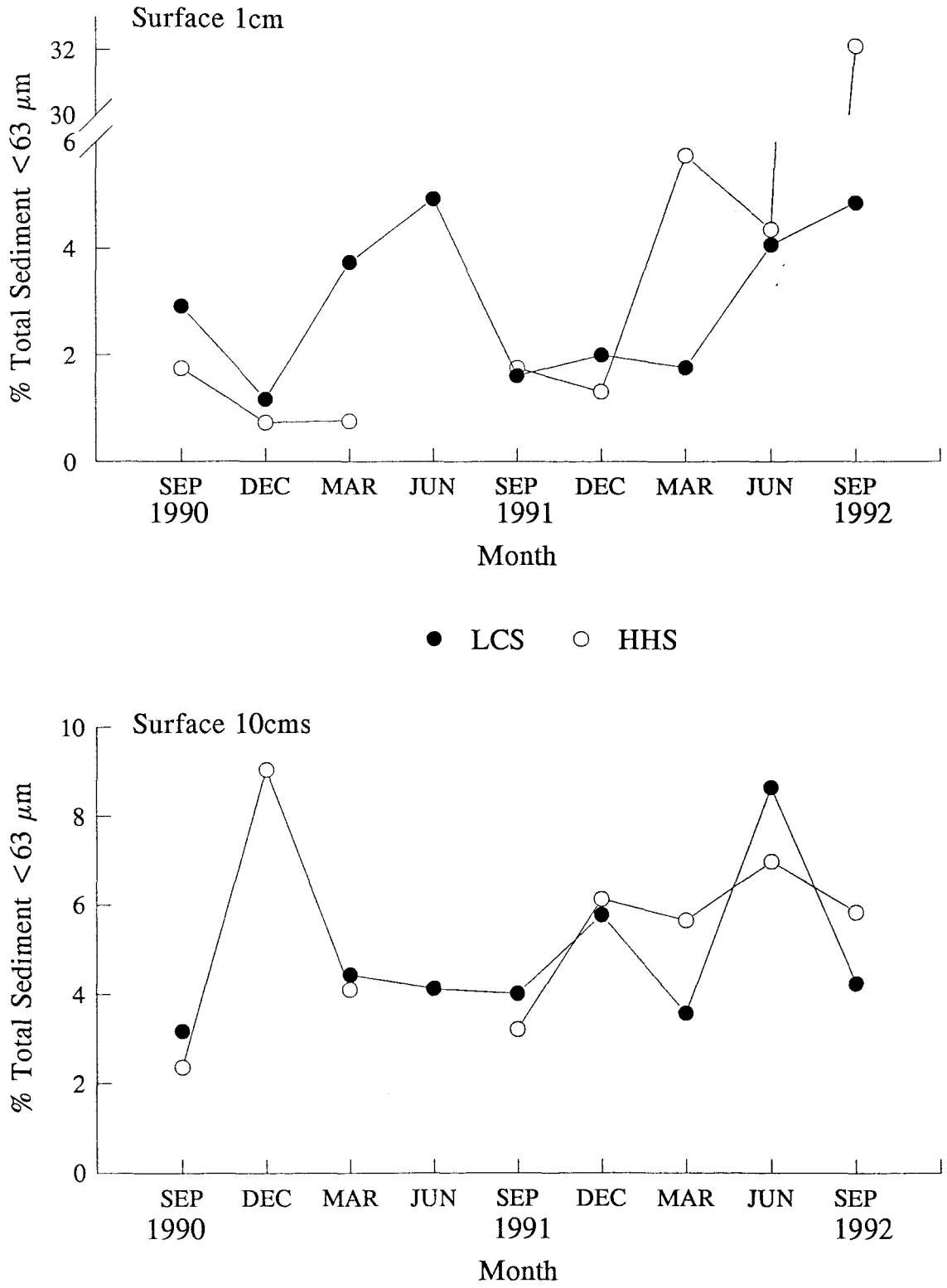


Fig. 24. Fluctuations in the percentage silt/clay fraction.

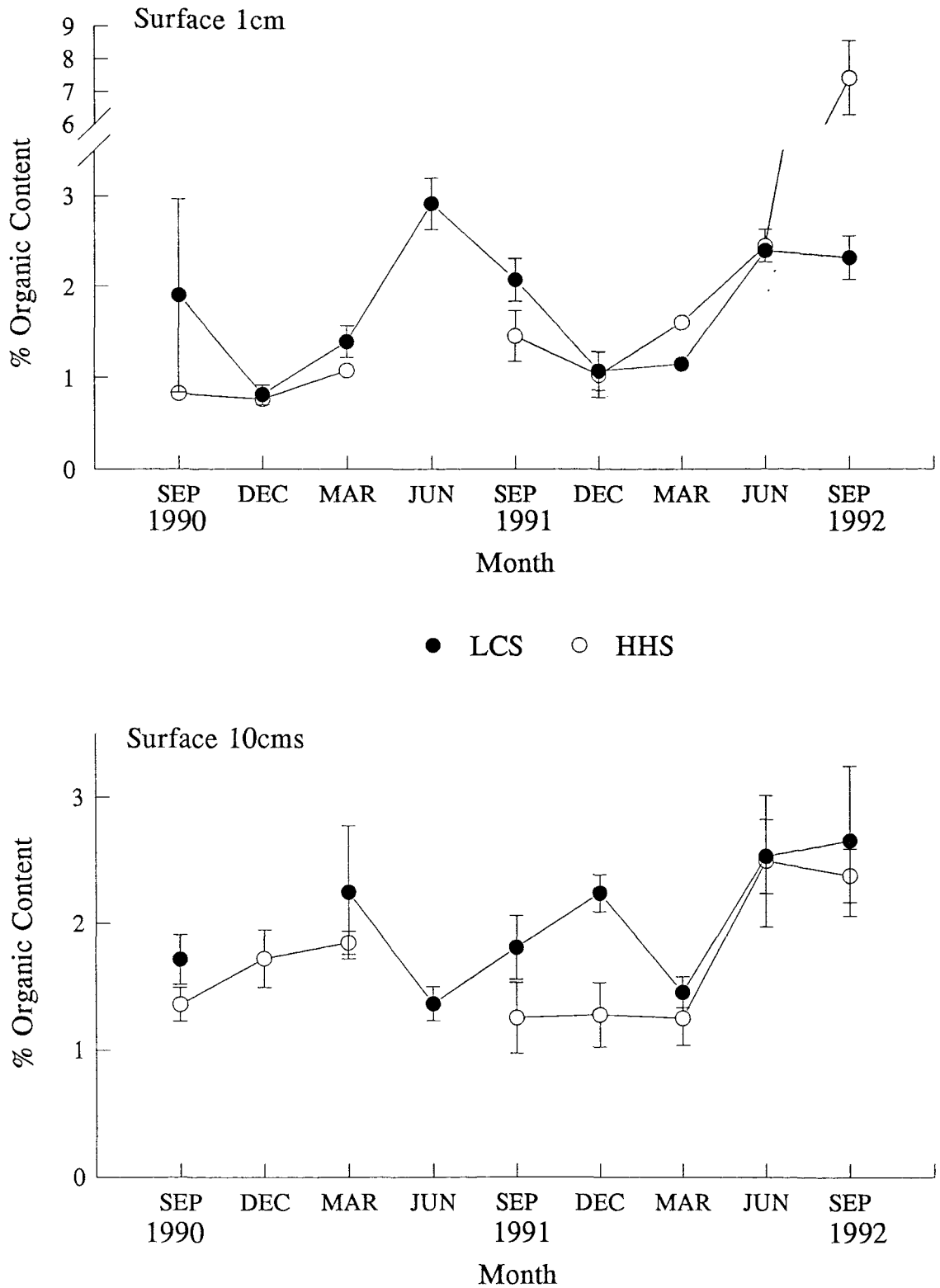


Fig. 25. Fluctuations in the percentage organic content.

Fig. 22 also shows the Inclusive Graphic Standard deviation changes. The values for both sites were generally within the 0.25 to 0.6 range, indicating well sorted sediment, with the exception of the September 1992 sample at HHS, which was poorly sorted. Values for LCS were generally higher than those for HHS, but again not significantly so (t-test, $p > 0.1$ for the 1 cm layer and $p = 0.4$ for the 10 cm layer).

The range of values for Inclusive Graphic Skewness (fig. 23) suggest a generally symmetrical particle size distribution for the immediate surface sediment at both sites, with the 10 cm layer somewhat finely skewed. Both sites showed some degree of fluctuation. The kurtosis values also shown in fig. 23 indicate a leptokurtic distribution at both sites in both the surface 1 and 10 cm layers.

The proportion of the sediment composed of silt and clay can be seen in fig. 24. Values were generally around 5%, except in September 1992 at HHS, when the immediate surface layer showed a sudden increase in fines, to over 30%. This change was not reflected in the 10 cm core sample, suggesting a settling out event involving only the sediment-water interface and not a major sedimentological change. Values were generally higher at LCS for the initial part of the study, then the fines content at HHS increased and was greater for the second half of the study. This concurs with the gradual increase in Mdϕ observed at HHS.

The percentage organic content fluctuations are shown in fig. 25. It can be seen that in both the 1 and 10 cm surface layers the organic content of the sediment was greater at LCS, almost exclusively. The exceptions were the values for the immediate surface sediment in 1992, where HHS values were higher, particularly in September when the dramatic influx of fine material occurred, shown by fig. 24. A two-way ANOVA revealed that the organic content of the sediment at the two sites was significantly different ($p < 0.001$ for both 1 and 10 cm layers).

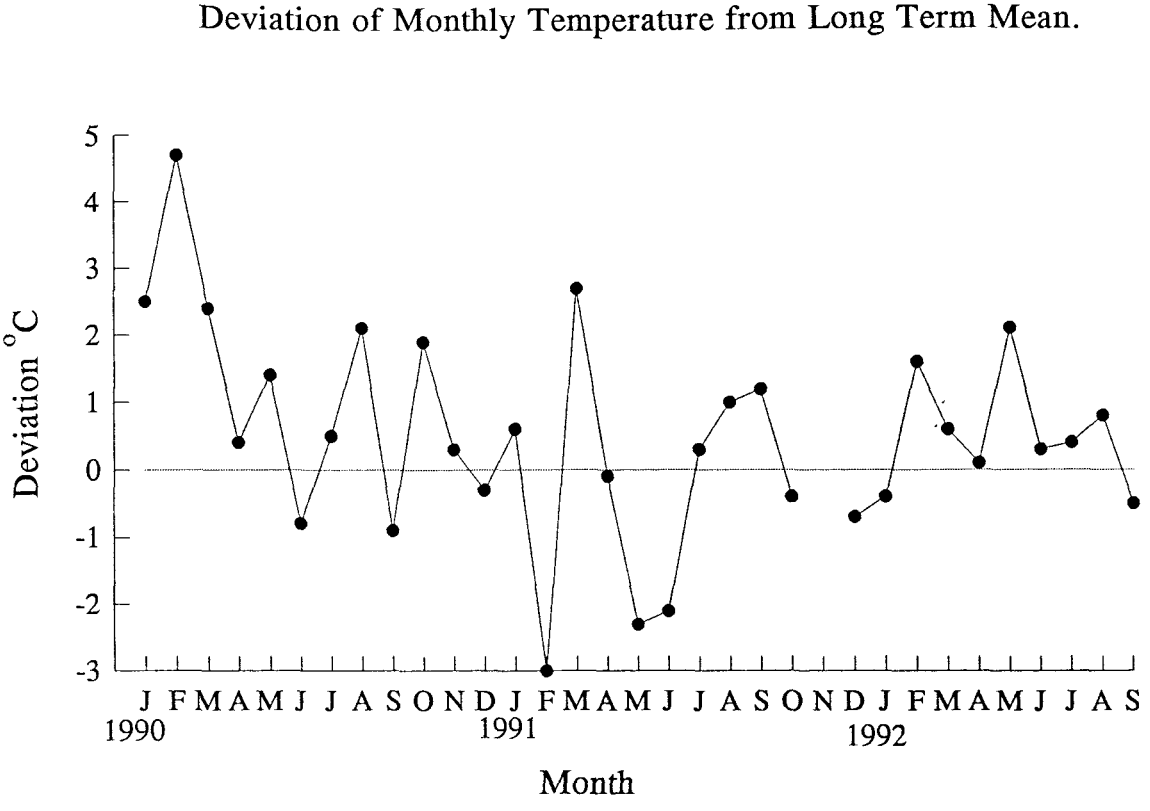
4.3 Meteorological Data

The following meteorological data (figs. 26 and 27) were obtained from the meteorological station at Abbeville, approximately eight miles from the Baie de Somme. Values for the whole of 1990 (prior to the start of sampling) have been included since abnormal seasonal conditions may have had metabolic effects

measurable in the first few samples.

Fig. 26 displays the temperature and rainfall data. Absolute temperatures were not plotted, it was felt that departures from the normal temperature regime would have been more influential in determining the physiological state of the animals. It can be seen that the majority of the study period was warmer than normal, particularly the start of 1990, notable exceptions being spring 1991 and early winter 1991/92. February 1991 was particularly cold and examination of the absolute temperatures revealed a period of almost two weeks in this month where the maximum temperature did not exceed 4°C, remaining below 0°C for six days. The next month, March 1991, was warmer than average however, by the same difference that February was colder. Such a large fluctuation could have been particularly stressful to the fauna.

Rainfall, also shown in fig. 26, fluctuated widely from month to month. Low rainfall occurred at the latter part of 1991, followed by higher than normal rainfall at the start of 1992.



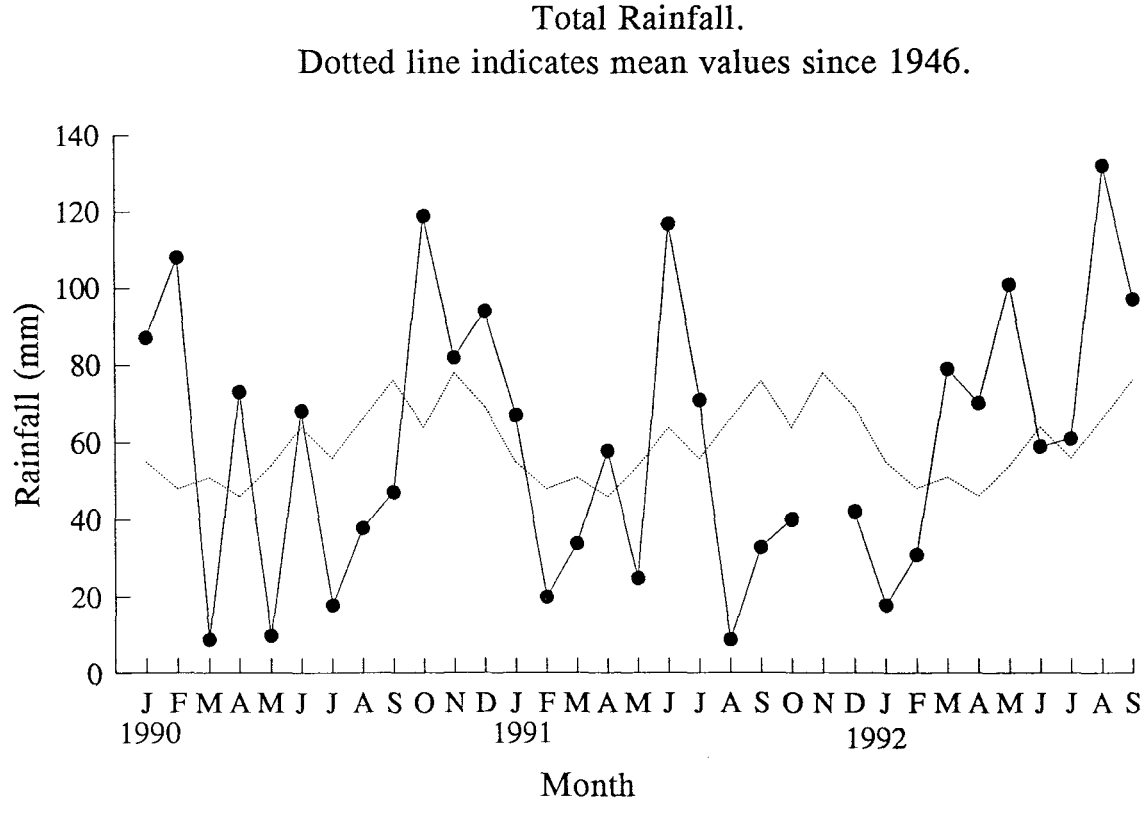


Fig. 26. Temperature and rainfall data from the Abbeville meteorological station.

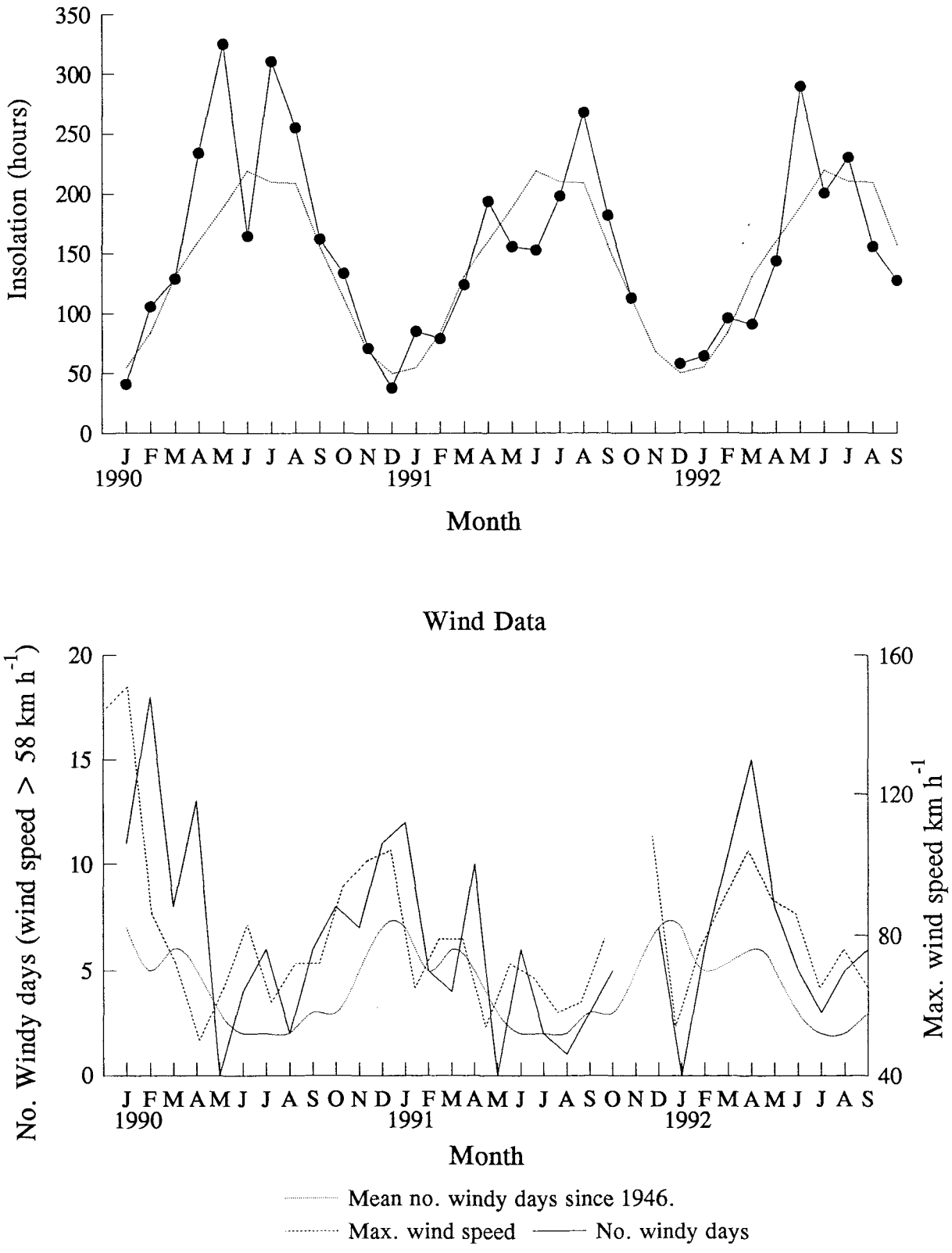


Fig. 27. Insolation and wind data from the Abbeville meteorological station.

Insolation (hours of sun) is shown in fig. 27. This is obviously a seasonally variable factor but levels did not differ noticeably from the long term mean during the study period. The summer prior to the commencement of sampling however, had much higher insolation levels than normal, concurrent with the abnormally high temperatures shown in fig. 26.

Data on wind strengths have also been included (fig. 27) since excessive wind speeds may be expected to have affected the hydrodynamics and sediment movement within the estuary. Once again the period of 1990 before sampling began experienced the highest deviations from normal, with wind speeds around 150 kilometres per hour in February, associated with eighteen days in that month where the wind speed was greater than 58 kilometres per hour. Spring 1992 also showed high wind speeds and numerous windy days. The strongest winds on these two occasions were from the west, which is the direction to which the estuary is most exposed (see fig. 1), exacerbating the effects. Relatively calm periods were also evident, such as summer 1991.

4.4 Phytoplankton Measurements

It can be assumed that the concentration of phytoplankton in the estuary will affect its benthic fauna. The presence of agriculturally derived nutrients introduced to the estuary via the River Somme has suggested that the estuary may suffer eutrophication (Desprez *et al.*, 1992). Notwithstanding this fact the normal bloom cycles of phytoplankton may induce changes in the biochemical and physiological status of the animal, providing a food source for some species (the cockle for example), and altering the chemical composition of the water column. Given these facts, it was thought appropriate to include some measurement of phytoplankton concentration to correlate with the biochemical data. Unfortunately, data were not available for the entire study period but nevertheless an indication of seasonal fluctuations in phytoplankton levels can be gained from fig. 28. Chlorophyll *a* levels were derived spectrophotometrically (Desprez, pers. comm.).

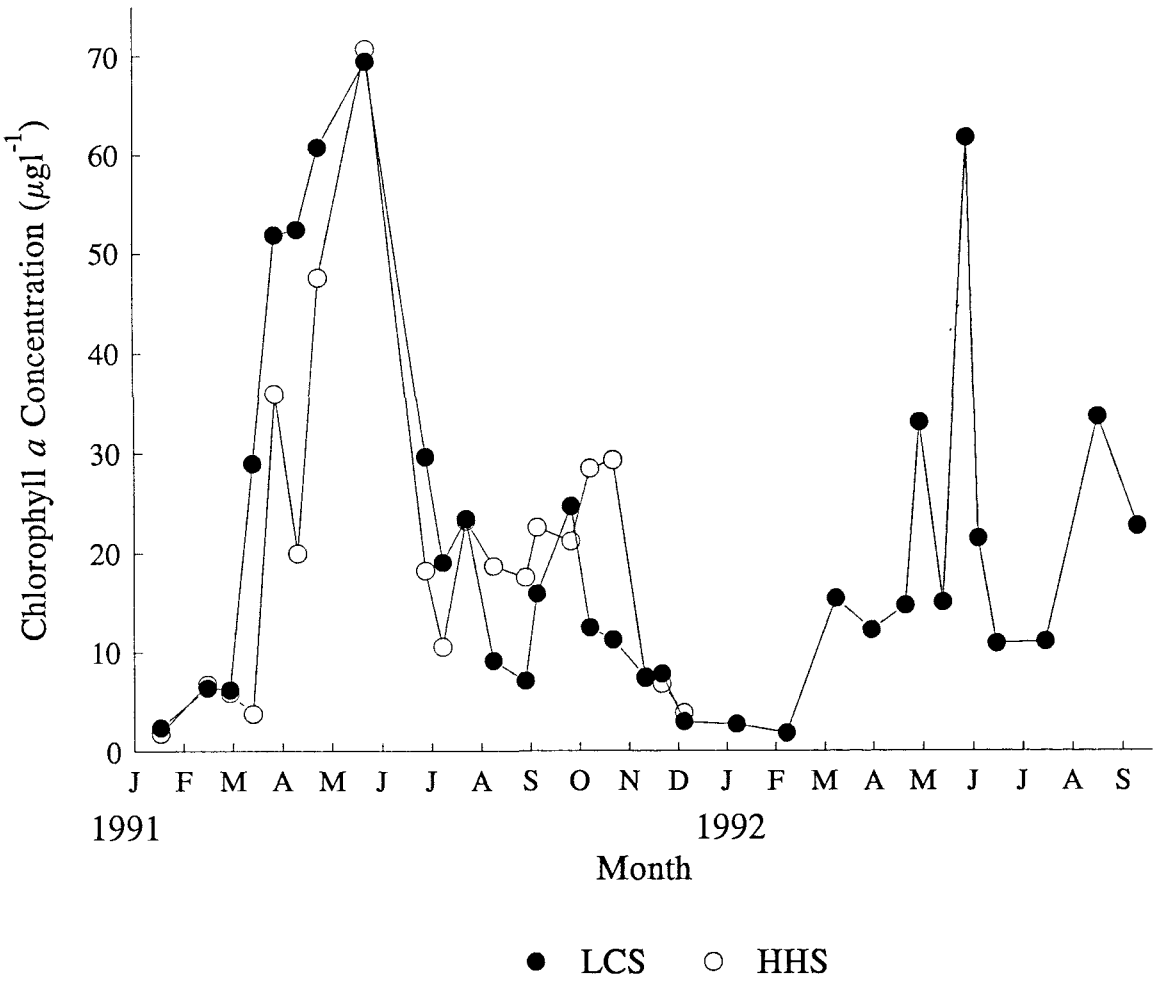


Fig. 28. Fluctuations in the chlorophyll *a* concentrations with time. Data from M. Desprez (pers. comm.).

Although data were only available for HHS for 1991 it can be seen that values did not differ significantly between the two sites, so it can be assumed that phytoplankton populations were homogeneously distributed throughout the upper estuary at high tide. The expected spring bloom was clearly evident in both years, although apparently sustained for a longer duration in 1991. A reduced, late summer bloom was also evident in 1991, and probably also in 1992 although sampling terminated before the autumn.

4.5 Discussion

The sedimentary analysis has detected differences between the two chosen sites in the fundamental habitat of the particular species considered here. The sediment at HHS is generally finer (with an increasing trend towards fineness throughout the study), better sorted and with a significantly lower organic content than the sediment at LCS. A dramatic change in the surface sediment characteristics at HHS was also evident in the latter part of the study (figs. 22, 24, and 25). This phenomenon was coincident with large settlements of molluscan juveniles (chapter 3, figs. 11 and 12) which suggests that the prevailing hydrodynamic and climatic conditions allowed settling out of material held in the water column. The wind speeds for the summer of 1992 were low (fig. 27) which would not inhibit precipitation of suspended seston. In addition there was higher than average rainfall (fig. 26) between March and September 1992 which would have increased river flow and therefore the quantities of riverine silt (although data were not available to verify this). HHS was closer to the river channel, thus such material would be more evident at this site. This is purely speculative but there is no doubt that the sedimentary nature of HHS was altered towards the end of the study.

The meteorological events recorded in figs. 26 and 27 will have affected the populations throughout the estuary more or less equally. These data do show that no extraordinary climatic events occurred that may have been expected to perturb the animals beyond their normal range of experience. A possible exception was the early spring of 1991 when a relatively cold February was followed by a relatively mild March (fig. 26). However, no noticeable effects were detected at the population level, nor even at the biochemical level in the species considered.

The sites have been distinguished from each other on the basis of their sedimentary properties, in addition to the differences evident in the biochemistry data and population structure of the fauna (chapters 2 and 3). A considerable amount of evidence exists therefore to characterise and distinguish the two sites, at several levels.

Notwithstanding the lack of identifiable climatic events it is still essential that environmental measurements be made to define the physical and chemical stressors acting on the organisms. The natural physiological and reproductive cycles of the animals will be determined (at least partially) by the environmental cycles. Correlation of all of the available information will reveal those factors that contributed most strongly to the structuring of the biological assemblages present at the study site. This will be discussed in chapter 5.

CHAPTER 5

Comparison of Biochemical, Population and Environmental Data

5.1 Introduction

The preceding chapters have dealt separately with the key species' biochemistry, population dynamics and environmental characteristics of the two sites, over a two year study period. Whilst useful information was obtained from each of these categories, a specific aim of the project was to correlate biochemical measurements with environmental fluctuations in an attempt to link these with subsequent population changes and so establish a predictive biochemical index. Temporally defined ecological investigations, however, cannot be certain of including a period of population upheaval resulting from a catastrophic environmental change; such events, by their very nature, are unpredictable. Fluctuations within the Somme populations did occur during the study period, but these were most probably normal background fluctuations resulting from reproductive events and natural mortalities. They were not of the same order of magnitude measured in previous years, in the cockle for example (Desprez *et al.*, 1992). Consequently, the detection of these effects at the biochemical level was limited to the measurement of natural cycles. However, the Somme study has also allowed comparison of two sites, of which HHS has been described in the preceding chapters as environmentally heterogeneous and less favourable to the key species. Correlation of biochemical changes with environmental observations in a site-comparative way may enable the basic biochemical responses to be revealed. Some re-definition of the original aim is therefore necessary; rather than correlating biochemistry with environmental variables to predict population changes, the correlation can be used to explain population differences. Both aims are fundamentally the same, since the hypothesis is that population structure is the result of metabolic responses to the environment. The lack of large scale environmental and population changes within the Somme communities has necessitated a change of emphasis.

An opportunity arose during the study to sample other areas of the Somme

estuary where *C. edule* and *N. diversicolor* occurred. The same biochemical procedures described in chapter 2 were applied in an effort to measure the natural metabolic variability at one point in time across the whole estuary. This study also contributed to the previously discussed suggestion that biochemical responses are the cause of population differences. Faunal densities and some degree of environmental characteristics were measured to contribute to the spatial comparisons. The results of this study will be discussed later in this chapter.

Chapter 2 demonstrated that it was possible to detect seasonal patterns and site differences in individual biochemical components. It has also been stated that the biochemical components chosen for measurement correspond to key points in the major energy-producing metabolic pathways and the metabolites are thus inter-linked. Integration of all of the components before correlation with environmental data would therefore be more realistic. These types of multifactorial data require analysis with multivariate methods, such as ordination. Ordination techniques have frequently been used in numerical taxonomy and ecology but not generally for these type of physiological data. However, since ordinations are used to summarize information about many characters in a reduced number of dimensions, it would seem feasible that biochemical components could be treated in a similar fashion to the more usual species/taxonomic groups.

5.2 Time Series

The ordination technique applied to the biochemical data was that of principal components analysis (PCA). From a data set composed of n numeric variables, n principal components can be calculated, each principal component being a linear combination of the original variables. The biochemical data first had to be standardised to eliminate the bias caused by the differing units of measurement. Lipid, for example, was measured as milligrams per gram of tissue giving values in the range of 0 to 500, whereas components such as the adenylates were measured in micromoles per gram of tissue giving values in the range of 0 to 10. The standardization applied was that of dividing each monthly value by the highest occurring value for each component, so that each component had a range from 0 to 1, and the ratio between monthly values was maintained. Both species and both sites were treated separately, thus four analyses were performed. The

performed. The data entered into the PCA were the mean values per month for each biochemical factor. For each species/site combination the first four principal components accounted for at least 75% of the variability. The principal component values for these four principal components are given in appendix 3. The remaining components each accounted for only small amounts of variability, as indicated in table 4, and so were not given further consideration.

Table 4. Percentage variance of each principal component.

Component	Percent Variance			
	C. edule		*N. diversicolor*	
	LCS	HHS	LCS	HHS
1	31.08	33.07	33.12	31.4
2	22.76	22.44	24.68	28.53
3	17.82	14.72	11.15	15.9
4	9.39	8.58	10.28	9.31
5	6.28	6.99	8.75	7.36
6	4.82	4.89	6.64	4.35
7	3.82	3.79	3.65	2.36
8	2.79	2.37	1.09	0.49
9	0.55	1.54	0.46	0.31
10	0.39	0.99	0.18	0.01
11	0.25	0.41	0.00	0.00
12	0.03	0.16		
13	0.00	0.03		
14	0.00	0.00		

The principal component values calculated from the PCA were correlated with the environmental and population variables described in chapters 2, 3 and 4. In this way the factors that were responsible for the patterns in the integrated

biochemistry data extracted by the PCA could be determined. The variables that were correlated are listed below with an explanation of their derivation.

Biological factors

Species density- The density of either *C. edule* or *N. diversicolor* 0.2 $m^{-2}month^{-1}$ at each site.

Total faunal abundance- The total number of individuals 0.2 $m^{-2}month^{-1}$ at each site.

Tidal factors

Neap/spring- The sampling day for that particular month was allocated a value to indicate the state of the neap/spring cycle. For example a neap tide day had a value of 0, a spring tide day a value of 7. One day either side of a neap was given the value 1, one day either side of a spring was given the value 6, and so on. Tidal data were obtained from Admiralty tidal atlases.

Sediment factors

Percentage Fines- The proportion of the sediment $< 63\mu m$. Data were included for both the surface 1 and 10 cms.

Percentage Organics- The organic content of the sediment, again in both the 1 and 10 cm layers.

N.B.- Sediment data was only obtained every three months so the values that were used in the correlation corresponded to the closest month, if not the actual month.

Meteorological factors

In each of the following variables the mean value for the *preceding* month was used in the correlation. In some cases the sampling date was close to the beginning of the month and clearly the animals would not yet have experienced the conditions described by the mean for the actual month. By using the value for the month before it was certain that the conditions had been encountered and would by then have affected the biochemistry, if any effects were to be detected.

Temperature- Mean monthly temperature °C.

Rainfall- Mean monthly rainfall, mm.

Insolation- Mean monthly hours of sunshine.

Wind speed- The maximum recorded wind speed that month, Kmh^{-1}.

Windy days- The number of days per month when wind speed exceeded 58 Kmh^{-1}.

Phytoplankton data

Chlorophyll *a* concentration- The concentration of chlorophyll *a* in the water column (μgl^{-1}). Data were only available for HHS for 1991 and since differences between LCS and HHS during this period were small, the values given for LCS were applied to both sites. These data did not cover the entire study period however, and so the principal component values for the first few months had to be excluded from the correlation.

Values for each variable were site specific wherever possible, but meteorological and phytoplankton data applied equally to both sites. The correlation that was applied was the Spearman rank order correlation, which is a nonparametric test that does not require the data points to be linearly related, and measures only the strength of association.

5.2.1 Results and Discussion

Tables 5 to 8 show the *p* values for the Spearman rank order correlations between the principle component values and environmental factors. Significant relationships ($p < 0.05$) are highlighted.

Table 5. *p* values for *C. edule* at LCS.

Factor	Principal Component			
	1	2	3	4
Cockle density	0.686	0.913	0.954	0.014
Total faunal abundance	0.949	0.892	0.106	0.779
Neap/spring state	0.011	0.985	0.676	0.913
% Fines (surface 1cm)	0.773	0.490	0.221	0.458
% Fines (surface 10cm)	0.863	0.669	0.792	0.067
% Organics (1cm)	0.667	0.259	0.081	0.196
% Organics (10cm)	0.792	0.061	0.906	0.482
Temperature	0.802	0.676	0.002	0.903
Rainfall	0.648	0.734	0.584	0.753
Insolation	0.667	0.621	0.005	0.259
Wind speed	0.686	0.812	0.248	0.753
No. windy days	0.964	0.944	0.130	0.602
Chlorophyll *a* conc.	0.181	0.087	0.221	0.638

Table 6. *p* values for *C. edule* at HHS.

Factor	Principal Component			
	1	2	3	4
Cockle density	0.310	0.124	0.594	0.040
Total faunal abundance	0.471	0.342	0.956	0.499
Neap/spring state	0.382	0.808	0.832	0.426
% Fines (surface 1cm)	0.099	0.210	0.800	0.904
% Fines (surface 10cm)	0.716	0.417	0.543	0.246
% Organics (1cm)	0.147	0.227	0.869	0.939
% Organics (10cm)	0.886	0.667	0.514	0.391
Temperature	0.310	0.473	0.146	0.928
Rainfall	0.181	0.693	0.318	0.062
Insolation	0.325	0.333	0.029	0.785
Wind speed	0.221	0.512	0.382	0.150
No. windy days	0.150	0.750	0.341	0.627
Chlorophyll *a* conc.	0.604	0.541	0.921	0.135

Table 7. *p* values for *N. diversicolor* at LCS.

Factor	Principal Component			
	1	2	3	4
Nereis density	0.490	0.060	0.507	0.307
Total faunal abundance	0.800	0.635	0.904	0.049
Neap/spring state	0.064	0.891	0.101	0.672
% Fines (surface 1cm)	0.519	0.169	0.987	0.978
% Fines (surface 10cm)	0.812	0.541	0.226	0.340
% Organics (1cm)	0.952	0.002	0.814	0.355
% Organics (10cm)	0.173	0.404	0.004	0.893
Temperature	0.648	0.676	0.259	0.106
Rainfall	0.498	0.382	0.086	0.934
Insolation	0.913	0.265	0.466	0.119
Wind speed	0.382	0.148	0.320	0.271
No. windy days	0.474	0.320	0.783	0.019
Chlorophyll *a* conc.	0.295	0.078	0.802	0.389

Table 8. *p* values for *N. diversicolor* at HHS.

Factor	Principal Component			
	1	2	3	4
Nereis density	0.654	0.044	0.503	0.694
Total faunal abundance	0.709	0.313	0.493	0.211
Neap/spring state	0.714	0.286	0.634	0.673
% Fines (surface 1cm)	0.613	0.146	0.613	0.613
% Fines (surface 10cm)	0.229	0.177	0.582	0.644
% Organics (1cm)	0.878	0.411	0.709	0.913
% Organics (10cm)	0.249	0.676	0.291	0.913
Temperature	0.707	0.331	0.632	0.973
Rainfall	0.143	0.218	0.785	0.632
Insolation	0.811	0.632	0.368	0.560
Wind speed	0.838	0.492	0.560	0.865
No. windy days	0.733	0.560	0.785	0.349
Chlorophyll *a* conc.	0.299	0.225	0.614	0.418

The factors that correlated significantly (highlighted in tables 5 to 8) are those that have determined the particular metabolic patterns described by the PCA. For LCS cockles the single most important factor was the neap/spring tidal regime, which correlated with principal component 1 (31% of the total variability). This is not an unexpected result; the relatively high shore position of LCS meant that cockles were immersed for probably close to the minimum duration necessary for physiological tolerance. Therefore any factor controlling the duration of immersion, such as the neap/spring cycle, will undoubtedly control the metabolism to a great degree. As mentioned in chapter 1, respiration modes in bivalves alter on exposure and many of the metabolites contributing to the PCA were intimately involved in biochemical adaptation to anaerobiosis.

Component 2 did not significantly correlate with any factor but both chlorophyll *a* and sediment organics showed some degree of correlation, with *p* values of 0.087 and 0.061 respectively. The data for these correlates were not ideal, in that sediment samples covered a three month period and chlorophyll *a* samples were not taken concurrently with the biochemistry samples and were not available for the entire study period. It is possible that had these data been taken contemporaneously, closer correlation would have been evident. Intuitively, component 2 would appear to be determined by phytoplankton concentration since chlorophyll *a* levels and the sediment organic content both represent, to some degree, the amount of organic material available to the cockle. Chlorophyll *a* is a direct measure of the water column phytoplankton and ultimately water column organics settle out and are detectable in the benthos.

Two factors correlated with component 3 - temperature and insolation, which are partially linked since highest values of both occur in the summer months and are lowest in the winter. Both variables represent seasonality, indicating that component 3 described seasonal metabolic cycles.

Component 4 correlated with cockle density suggesting that some degree of biochemical response occurred with fluctuations in the number of cockles at LCS.

For cockles at HHS the picture is not quite so clear. Neither component 1 or 2 correlated significantly with any factor, nor even showed any degree of agreement with them. Components 3 and 4 did, however, correlate with the same factors that determined components 3 and 4 at LCS, seasonality (in the form of

insolation) and cockle density respectively.

The output from the PCA included the eigenvectors for each biochemical factor (shown in appendix 3). These directional values indicate the contribution, either positively or negatively, of each factor to each principal component. It is interesting to note that glycogen was the most positive factor in component 3 in both LCS and HHS cockles. It was perhaps to be expected that this long-term response metabolite should feature strongly in the principal component that described seasonality. The eigenvectors for the remaining cockle principal components showed no obvious patterns, although in general the adenylates contributed little, which was anticipated since the described cycles were medium- or long-term.

It would seem that cockles at LCS displayed metabolic cycles which were evident as a result of the relative predictability of the surrounding environment. The environment at HHS was more heterogeneous, thus inhibiting the expression of predictable metabolic cycles. Those cycles that were described (seasonality and cockle density) represented only a small proportion of the total variability (23%).

In *N. diversicolor,* principal component 1 remained undescribed for both LCS and HHS samples. It may be supposed that reproductive maturity will have had some control over the metabolic cycles as it has already been shown to correlate with glycogen levels. All worms examined biochemically were sexually mature adults, the vast majority of which would have been female with a great proportion of their body volume occupied by gametes. However, it was not possible to correlate this variable with the principal components. As shown in chapter 3 (figs. 20 and 21) two stages of oocytes were always present, with overlapping maturation cycles, thus a single numerical value to describe reproductive state with time could not be used. It is possible, although not verifiable, that this variable exerted the greatest metabolic influence.

Both components 2 and 3 showed correlation with sediment organics at LCS. Since this polychaete lives in, and ingests the sediment, it is to be expected that fluctuations in the organic content will cause changes in the animals' metabolism. Once again the chlorophyll *a* levels showed a degree of correlation with component 2, although not significant ($p = 0.078$). As with the cockle, it may be that had the data been contemporaneous, a greater degree of correlation

may have been observed. Nevertheless, the apparent association reinforces the suggestion that organic material has a great influence on the polychaete metabolism.

Component 4 correlated significantly with two variables at LCS - total faunal abundance and the number of windy days. It may be coincidental that these two apparently unrelated variables correlate with the same component, however they do have similar, if antiphase, cycles. Faunal abundance was lowest in the winter months, when the number of windy days was highest, and *vice versa* in the summer months. Thus both variables may be representative of a summer/winter cycle, which could be the pattern expressed in the metabolism. Further evidence for this is given by the direction of the two correlations. Faunal abundance correlated with $r = +0.579$, whilst the number of windy days correlated with $r = -0.602$. The opposing direction of correlation is in agreement with the antiphase nature of the summer/winter cycles of these two factors.

Principal components for *N. diversicolor* at HHS showed only one significant correlation, which was component 2 against the density of *Nereis*. This is inexplicable, since the density was generally so low at HHS that it would seem unlikely that the worms could have influenced each other. This variable also showed some correlation with component 2 at LCS ($p=0.06$). It may be that *Nereis* density was representative of some unknown factor, possibly the reproductive cycle. Maximum densities occurred when juveniles were present and minimum densities were coincident with spawning time, thus the cycles of reproduction and density were similar.

The *Nereis* and cockle analyses showed similar results, namely that metabolic patterns were more evident at LCS than at HHS. Thus both key species exhibited the effects of increased site heterogeneity and environmental perturbation in their integrated metabolic cycles.

5.3 Spatial Investigation

The purpose of this study was to describe the biochemical variation within the two key species at one point in time across the estuary. This made it possible to compare the values for LCS and HHS animals with those from other areas, to assess their position in the range of variability across the Somme estuary. It was

also possible to determine the effects of differing physical conditions, faunal composition and density of the key species to assess the contribution of these factors to the animals' metabolism. It has already been shown in this chapter that differing habitat conditions result in the different expression of metabolic cycles. This investigation could not monitor cycles since a temporal component was deliberately excluded. Instead, the aim was to assess the site effects on metabolite levels and ratios.

Fig. 29 shows the position of the sites from which the key species were collected. Cockles were present at all sites but *N. diversicolor* was not found at sites C and E, which were marine in nature rather than estuarine. An additional point of interest was that, as part of the local cockle fishery programme, some cockles had been transplanted from site B to site E six months prior to this investigation. Although of a similar age, ex-site B cockles were easily distinguishable from site E cockles because of their smaller size. In the following discussion E1 refers to cockles indigenous to site E and E2 refers to those relocated from site B.

Samples for biochemical investigation were collected within two days of the September 1992 LCS and HHS samples, using the same procedures for the time series study described in chapter 2. The exception was the sample from site E which was too remote to transport liquid nitrogen to. Instead, a core sample of sediment was taken and left undisturbed in the corer for the return journey. The core was then broken apart, the cockles removed and frozen immediately in liquid nitrogen. Core samples were also taken and sieved *in situ* to assess faunal composition and densities of cockles and *N. diversicolor*. Environmental observations were also noted. The biochemical assays were performed as described in chapter 2. Differences in the animals' biochemistry at each site were assessed using a multivariate analysis. The procedure used was the Bray-Curtis similarity clustering, as used for the population data in chapter 3. As with the PCA the biochemical data first had to be standardised and this was performed as described in section 5.2. The output from the clustering analysis did not allow direct correlation with environmental variables; nevertheless they could be used to assist in the explanation of the site similarities.

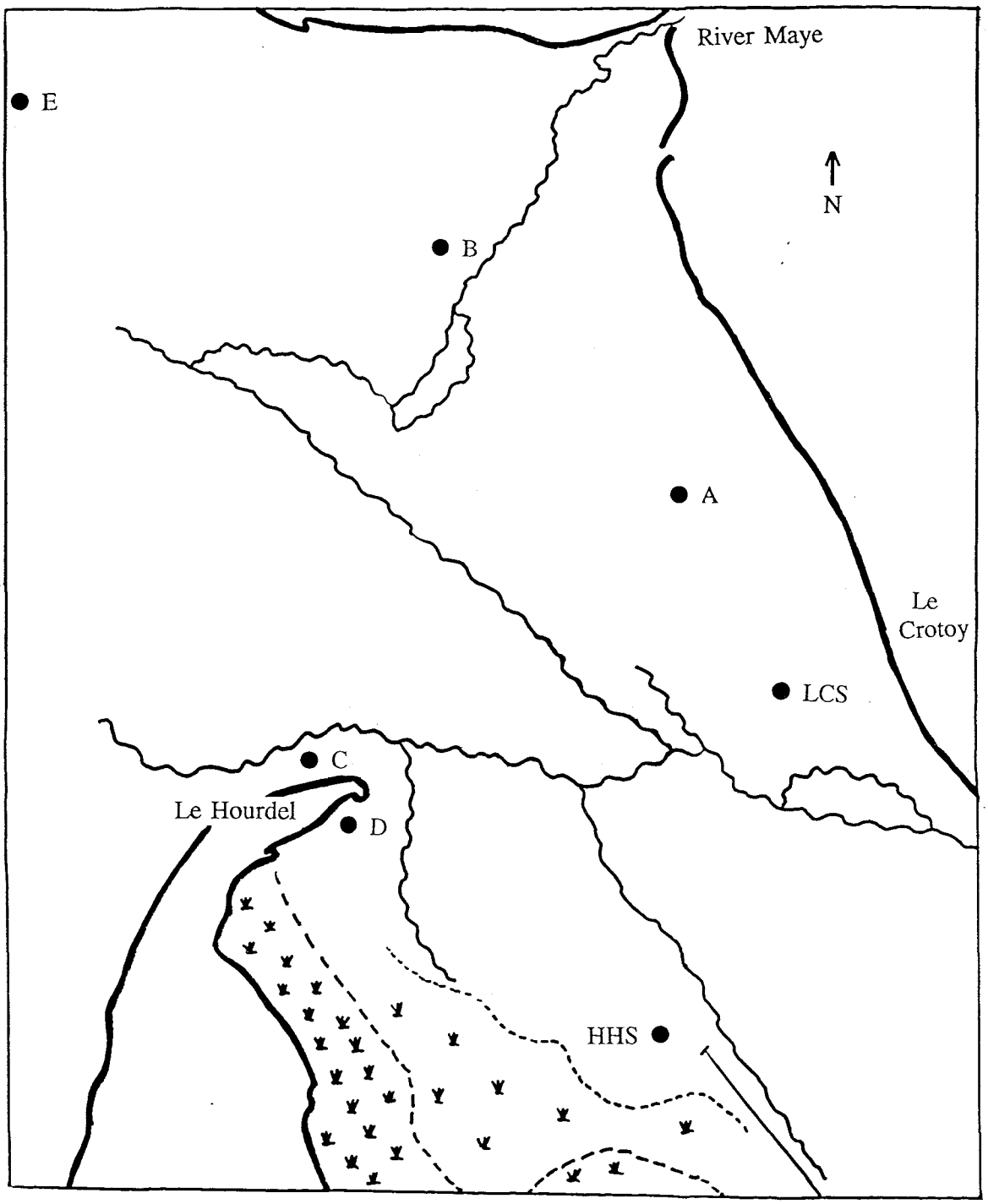


Fig. 29. Map to show the location of the study sites A to E and LCS and HHS.

5.3.1 Results and Discussion.

The following list outlines the nature of the sites visited and faunal compositions are given in table 9.

Site A- The sediment appearance was similar to LCS, with little surface water.
Redox layer 3-4 cms deep.
Salinity 24‰.

Site B- The sediment was muddier and with more surface water than site A.
Quite anoxic, redox layer was 2-3 cms deep, but the deeper sediment was very black.
This was not a cockle fishery although cockle numbers were high. Some dying cockles were evident. It was close to the small river Maye.
Salinity 26‰.

Site C- A marine site just outside the Baie. The sediment contained gravel and clay.
The redox layer was generally 3 cms deep but was very variable. In patches of *Pygospio elegans* and *Lanice conchilega* it could be > 10 cms.
All cockles present were very young (0-1 year).
Unable to measure salinity because of heavy rain, but almost certainly > 30‰.

Site D- Le Hourdel harbour. Two banks were present divided by a brackish-water channel. One bank was sandy containing cockles, the other was muddy and very anoxic and contained *N. diversicolor*.
Salinity 24‰.

Site E- A marine region in the north of the Baie. A low wet area in between two large sand banks. The sediment was sandy with some mud.
Redox layer was 2-3 cms.
It was not a cockle fishery area and indigenous cockles were very large.
Salinity 34‰.

Table 9. Faunal composition at the study sites.

++ Very abundant + Common (+) Some present ? Presence uncertain.

	SITE				
SPECIES	A	B	C	D	E
Cerastoderma edule	213 m^{-2}	1950 m^{-2}	700 m^{-2}	< 100 m^{-2}	810 m^{-2}
Hydrobia ulvae	+	++			
Macoma balthica	+	+	+		(+)
Nereis diversicolor	688 m^{-2}	867 m^{-2}		< 100 m^{-2}	
Arenicola marina	5m^{-2}		+		
Capitella capitata			?	?	(+)
Eteone longa	(+)		+	+	(+)
Heteromastus filiformis	(+)	+	+	+	(+)
Lanice conchilega			+		
Nephtys hombergii			+	+	
Pygospio elegans	(+)		+		(+)
Oligochaeta	(+)	+	?	?	

Figures 30 to 36 show the results of the biochemistry assays, including the LCS and HHS results for September 1992, for comparison.

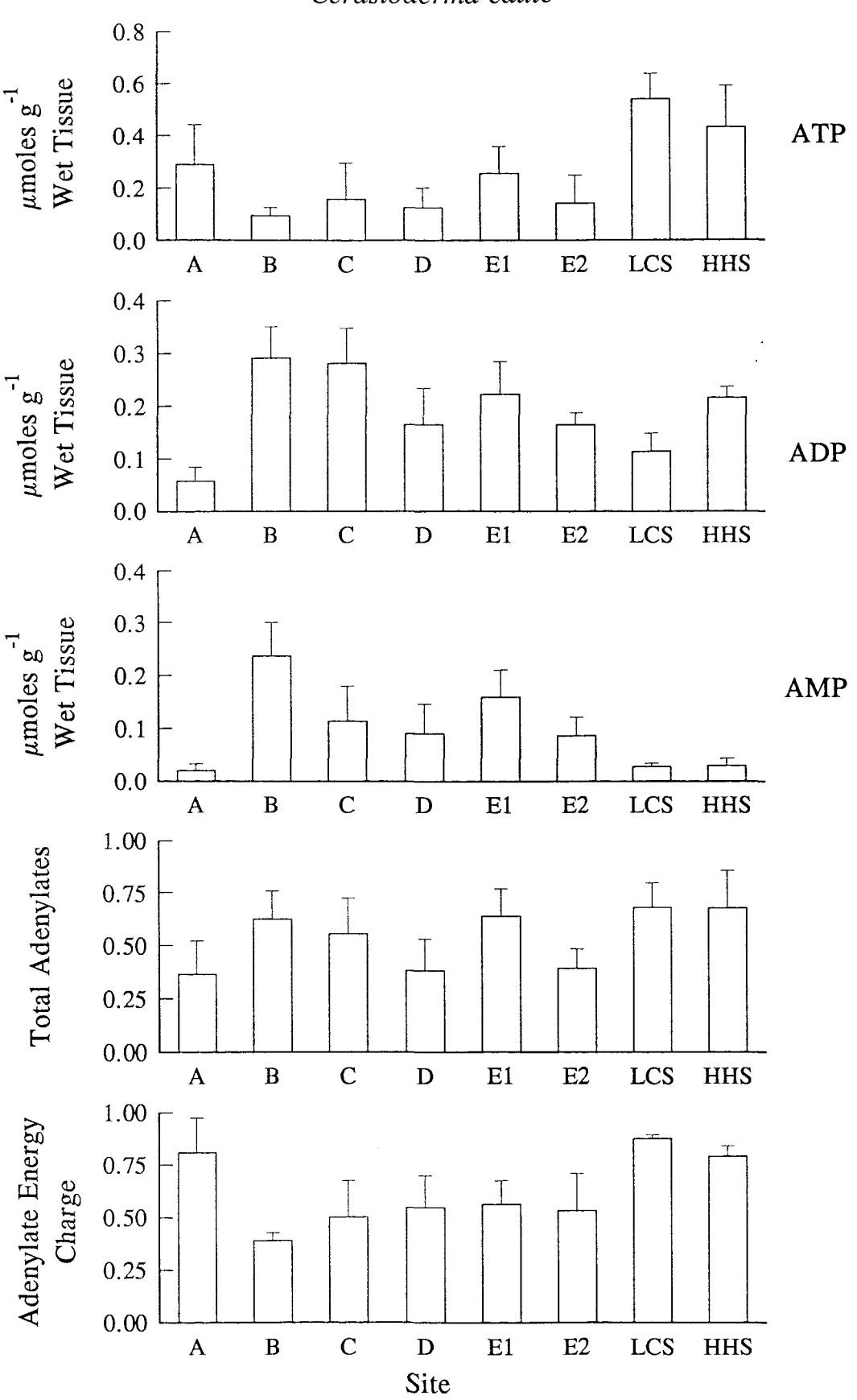


Fig. 30. Mean adenylate results for *C. edule* at the different sites. Error bars indicate standard deviation (shown in one direction only).

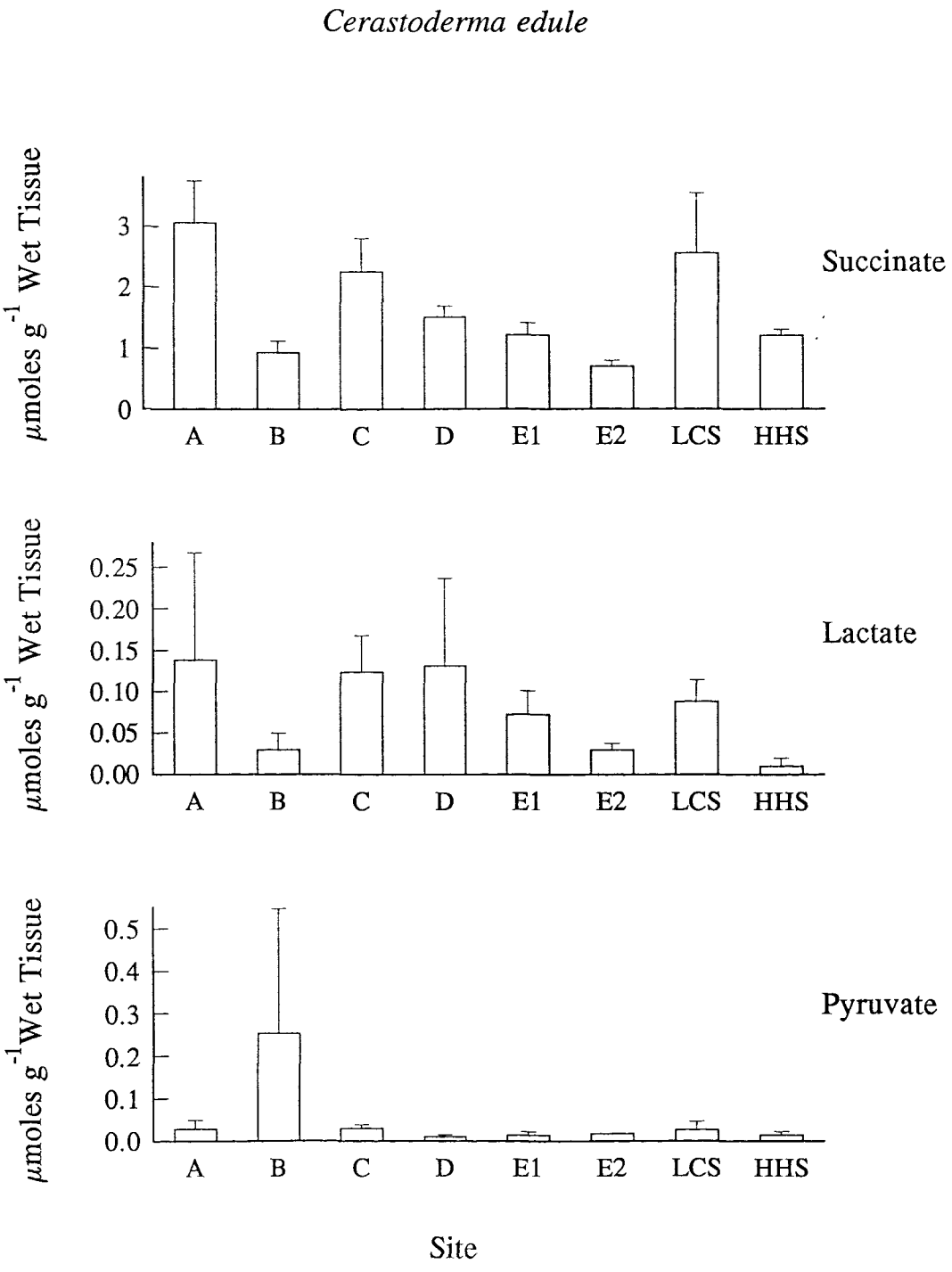


Fig. 31. Mean succinate, lactate and pyruvate results for *C. edule* at the different sites. Error bars indicate standard deviation (shown in one direction only).

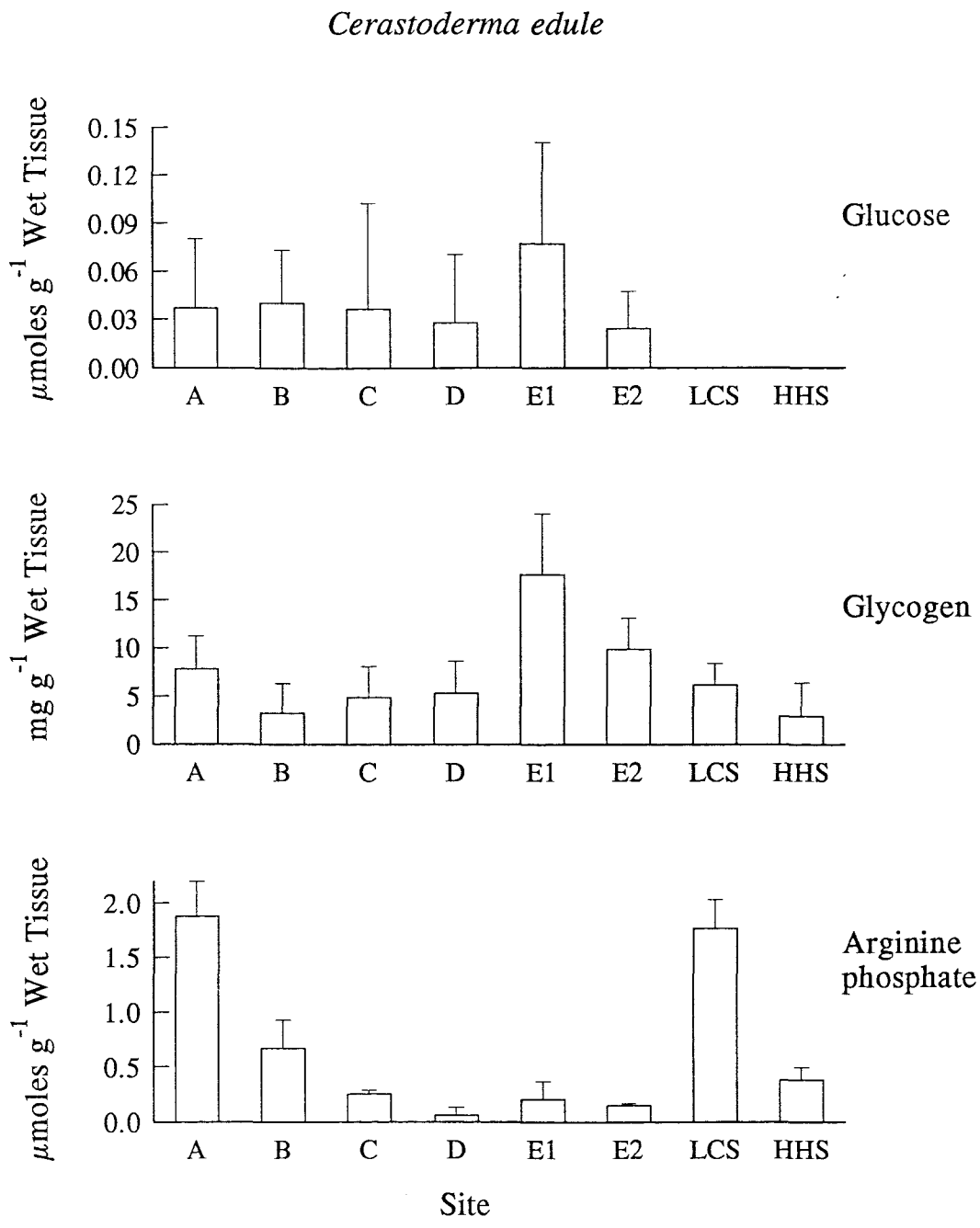


Fig. 32. Mean glucose, glycogen and arginine phosphate results for *C. edule* at the different sites. Error bars indicate standard deviation (shown in one direction only).

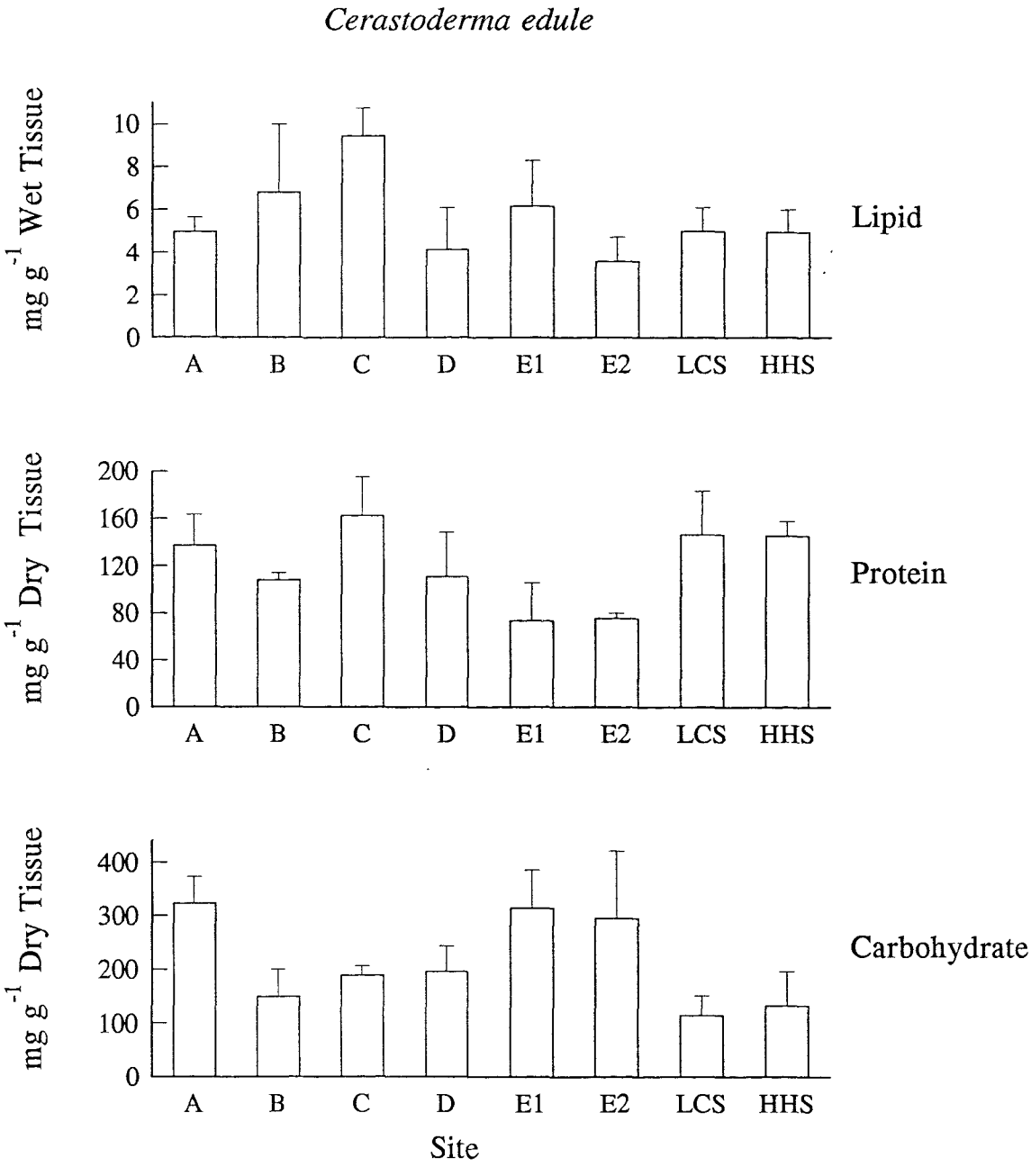


Fig. 33. Mean gross biochemistry results for *C. edule* at the different sites. Error bars indicate standard deviation (shown in one direction only).

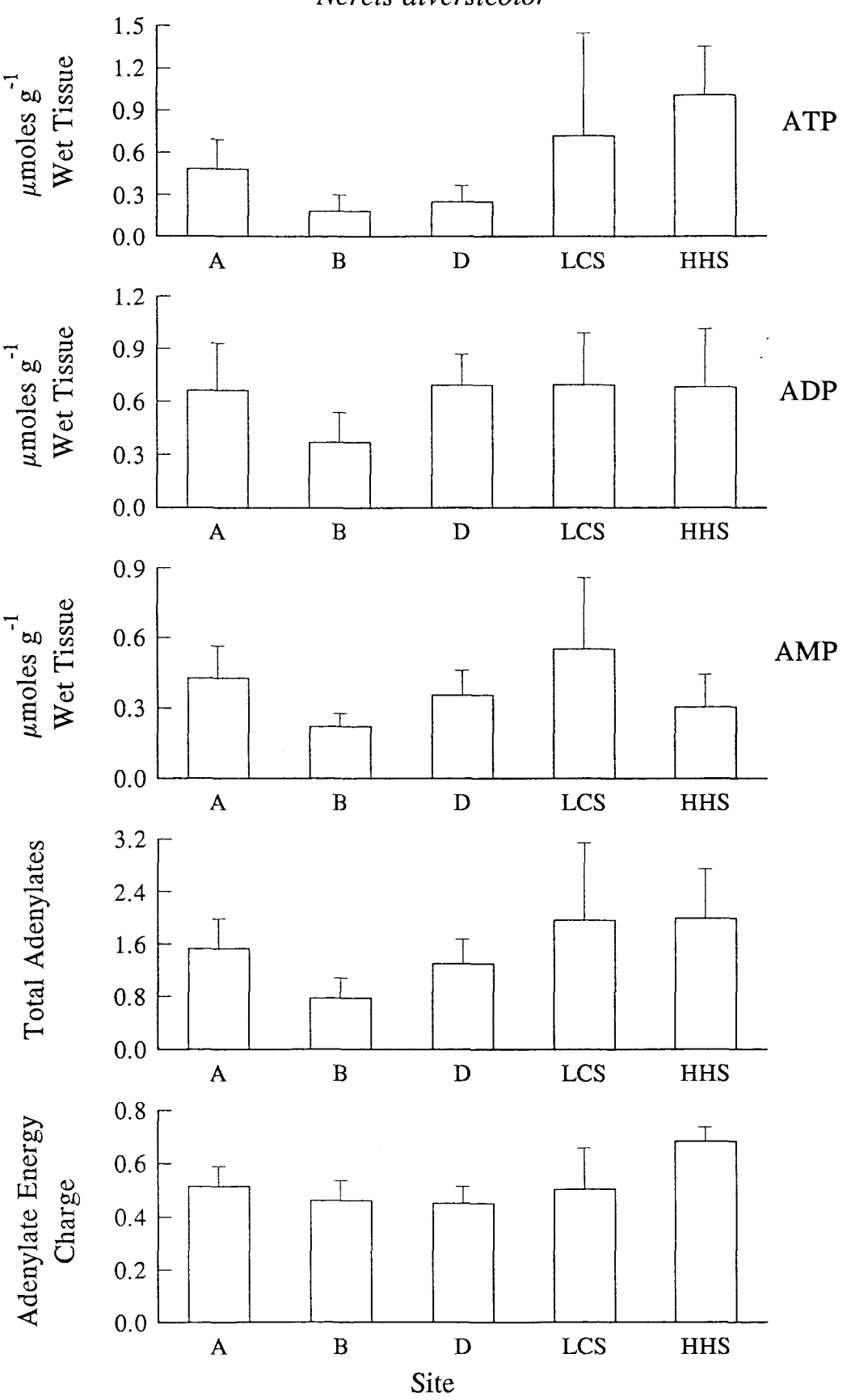


Fig. 34. Mean adenylate results for *N. diversicolor* at the different sites. Error bars indicate standard deviation (shown in one direction only).

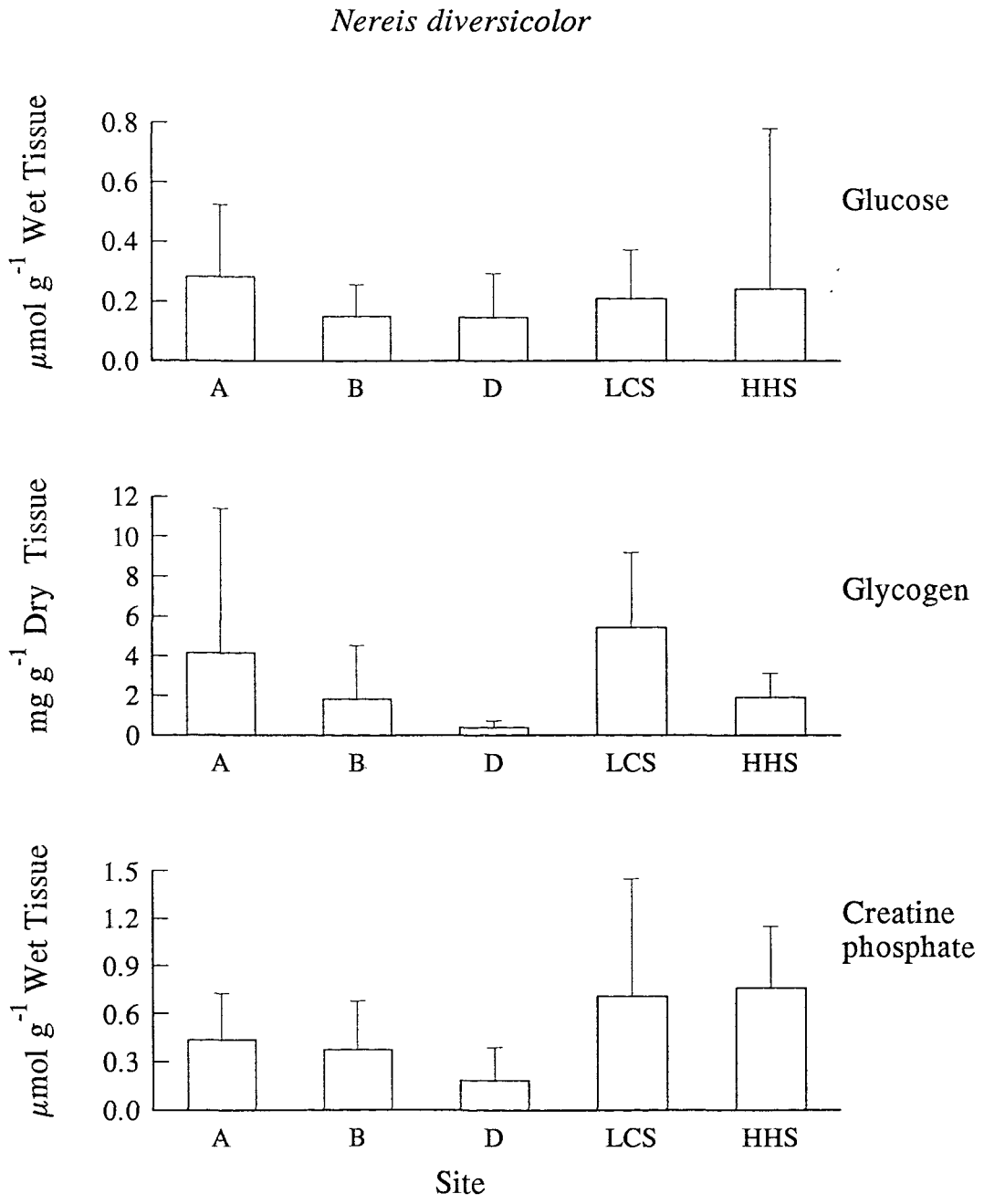


Fig. 35. Mean glucose, glycogen and creatine phosphate results for *N. diversicolor* at the different sites. Error bars indicate standard deviation (shown in one direction only).

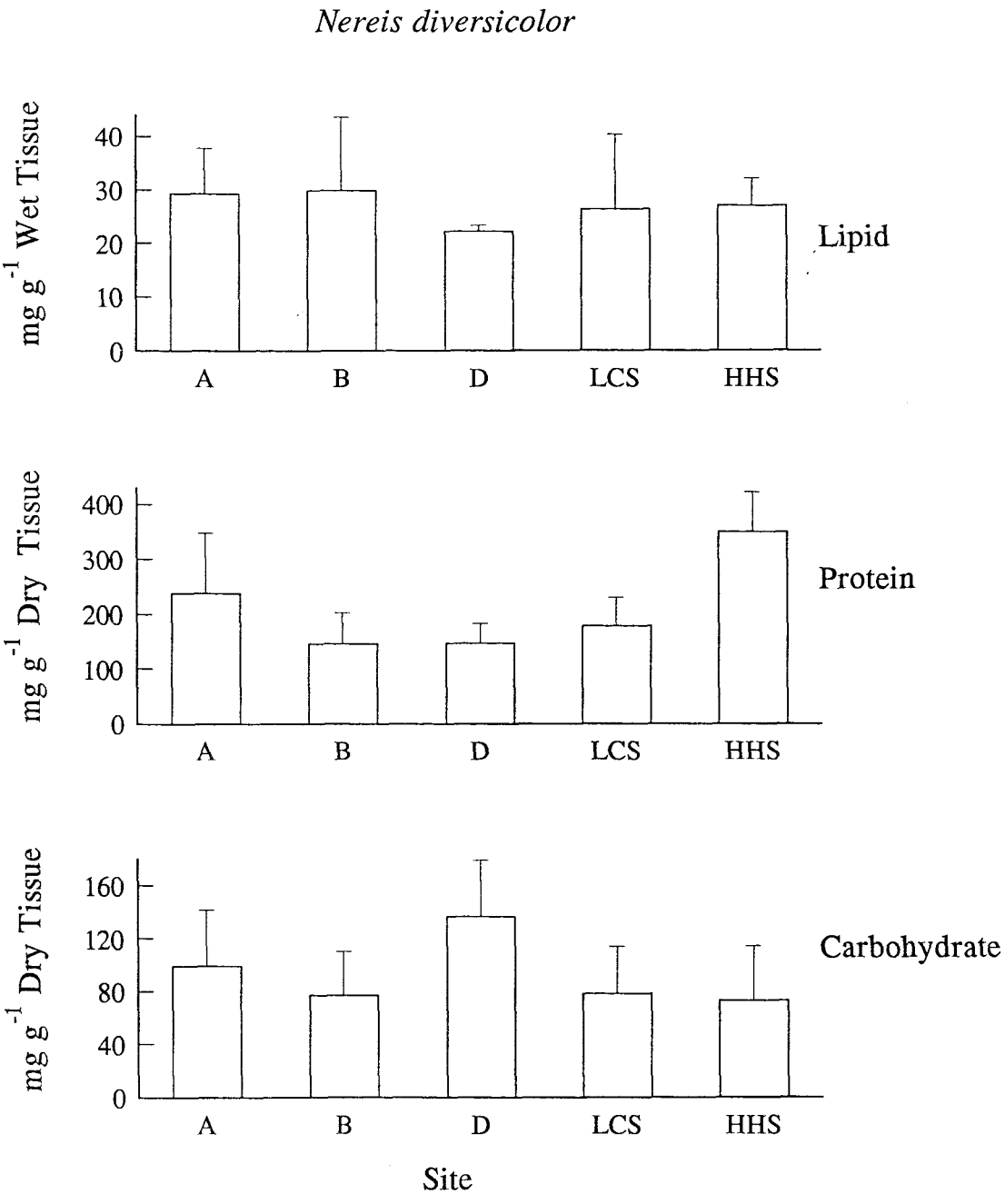


Fig. 36. Mean gross biochemistry results for *N. diversicolor* at the different sites.
Error bars indicate standard deviation (shown in one direction only).

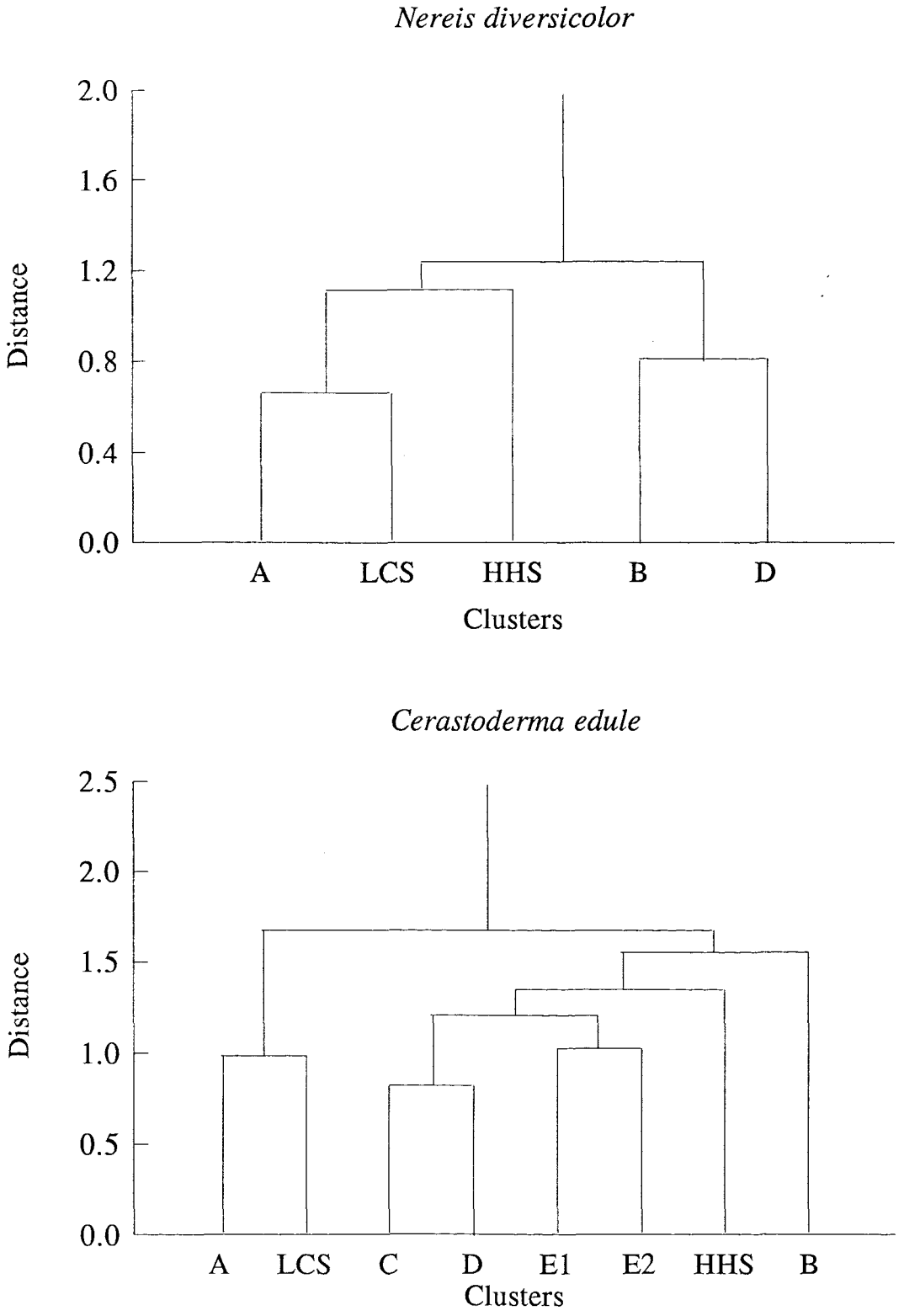


Fig. 37. Cluster diagrams for both key species (Bray-curtis similarity).

Mean values for the three adenylates for the cockle (fig. 30) showed a large inter-site variation although the mean adenylate pools were much closer. LCS and HHS results were more similar to each other than to the other sites, except in AEC where site A cockles were also similar to LCS and HHS cockles (and high), the other sites were similar. The succinate, lactate and pyruvate results (fig. 31) were also very site variable, LCS and HHS cockles showing values within the range of the other sites. Pyruvate levels in cockles at site B were exceptionally high. This compound is the precursor to the Krebs' cycle which is the main metabolic process requiring aerobic conditions, and this site was anoxic, possibly suffering some eutrophication because of its close proximity to the river Maye. The redox layer was close to the surface. Gaping and dying cockles were evident (pers. obs.), thus it seems probable that the high pyruvate levels were a result of some respiratory stress because of sub-surface anoxia.

There was no glucose present in cockles at LCS and HHS (fig. 32) but small amounts were evident in cockles at the other sites. Individual variation was large, however, so site differences were not apparent. Glycogen showed highest levels in E1 cockles, the other sites were similar. Arginine phosphate levels were higher in cockles at LCS and site A.

The gross biochemistry (fig. 33) again revealed that LCS and HHS cockles had similar values, within the range encountered at the other sites, although carbohydrate was slightly lower.

The adenylates in *N. diversicolor* (fig. 34) also varied greatly between sites, LCS and HHS worms had the highest mean adenylate pools. The AEC values were similar at all sites. Individual variations in glucose, glycogen and creatine phosphate (fig. 35) obscured any significant site differences, but worms at site D had very little glycogen. The gross biochemistry results (fig. 36) were similar for all sites.

The cluster diagrams of fig. 37 show the site similarities derived from these metabolic components, with the y-axis (distance) in arbitrary units. Although only a few sites contained *N. diversicolor* the clustering of these sites corresponds well with the type of sediment found at each site. *N. diversicolor* from site A and LCS were the most similar in biochemistry; they were in the same region of the estuary and the sediment at both sites was primarily clean sand. Animals from

sites B and D formed a second cluster and, although the sites were not geographically close, the sediment in both cases was relatively muddy and anoxic. It is also worth noting that these obviously more stressful sites (because of the anoxia) displayed the lowest glycogen and AEC levels in the worms of all the sites. The similar levels of these possible stress indices explains their clustering.

HHS worms were intermediate between the two clusters, and the sediment was also intermediate; muddier than at LCS but not particularly anoxic. Salinity conditions at all sites were similar, and faunal assemblage patterns did not correlate with the clusters.

In the cockle cluster diagram, three pairs of sites were evident. Again, A and LCS were similar in their animal biochemistry as well as close geographically. Unsurprisingly, E1 and E2 clustered together, indicating that in the six months since their removal from site B, the biochemistry of E1 cockles had adapted to the new site and become more similar to the indigenous cockles than to those remaining at site B. Results from cockles at sites C and D also clustered; this was unexpected as the sites bore little resemblance to each other in their sediment characteristics or in faunal composition. Also, site C was marine whilst site D was definitely estuarine. Nor was the modal age of the cockles similar. Those at C were very young, those at D were mature, and several years of age. The two sites were however geographically close. Geographical location within the estuary seems to have had some influence on the cockle biochemistry since the clusters formed were between close sites. HHS and site B did not cluster, and HHS especially was somewhat spatially removed from the other sites.

On the basis of these observations there seems little doubt that site determines the animals' biochemistry. In the case of *N. diversicolor* the important factor would appear to be the sediment. This is not unexpected in an infaunal detritivore and predator and, indeed, the PCA earlier in this chapter revealed the importance of sedimentary properties in determining the polychaetes' biochemistry. For the cockle, the underlying cause is not so apparent. The A/LCS and E1/E2 clusters suggest that type of site has an influence, but the C/D cluster and the lack of clustering at B and HHS suggest that location was more important. The cause is unlikely to have been shore position since site B and HHS were of a similar height to site A and LCS, and would experience similar tidal

immersion/exposure cycles. It may be that it was the water column itself that was having an effect since geographically close regions will have been covered with the same body of water at high tide. Since *C. edule* is a suspension feeder water continually passes over its soft tissues. It is therefore likely that differences in water quality, caused by such factors as river proximity or hydrodynamic conditions, would greatly influence the animals' biochemistry. The PCA also suggested some metabolic effects resulting from water column material. This is highly speculative and ideally further studies should be undertaken but there is much evidence to propose that location was an important metabolic controlling factor.

5.4 Chapter Discussion

Multivariate approaches with this type of data are relatively novel but this form of analysis would seem logical since the metabolites were linked by numerous complex biochemical reactions and an integrated approach was necessary to extract all of the available information. The analyses of the two key species presented here have shown that much more insight into environmental/biochemical interactions can be gained using multivariate methods than would have been possible if each metabolite had been considered separately.

Metabolic responses have been examined in both temporal and spatial situations and in both cases it has been possible to detect the environmental influence exerted by the different sites. Additionally, the differential effects of environmental parameters common to the two species have been indicated. For example the metabolism of *N. diversicolor* would appear to be closely linked to the sediment, whilst that of the cockle appears more dependent on water column factors and tidal cycles. The metabolic adaptability of the cockle in switching between aerobic and anaerobic modes of respiration in response to low oxygen tensions would account for this difference. *N. diversicolor* is known to be remarkably tolerant of low oxygen conditions; of four nereid species studied by Theede *et al.* (1973), *N. diversicolor* exhibited the broadest tolerance range to oxygen deficiency in Kiel Bay (Western Baltic).

An additional component of the spatial investigation was to determine whether LCS and HHS animals lay within the biochemical range displayed

throughout the estuary. In general, the animals at these two sites did not display the extremes of the metabolite levels measured in the estuary.

The cluster diagrams (fig. 37) revealed that the biochemistry of *N. diversicolor* was not especially different at LCS and HHS, at least not as disparate as A/LCS and B/D. This suggests that the PCA, in detecting site differences, was a remarkably sensitive analysis. The PCA for the cockle revealed more definite differences between LCS and HHS animals and the clustering shows these two sites as comparatively distant.

These two studies have also supported the original hypothesis that environmental factors determine metabolic responses and ultimately affect population structure. Although little correlation was found between faunal assemblages or key species densities and sites in the spatial investigation, this was not fully investigated at the time of the study as the biochemical aspect was given greater priority. The more intensively investigated population structure at LCS and HHS can, however, be partially attributed to the biochemical responses to the different environmental conditions. The PCA revealed a lack of natural metabolic cycles in both species at HHS. It is logical to assume that the animals were exerting greater metabolic effort to maintain themselves at this more heterogeneous site. This, combined with the physical and biotic interactions described in chapter 3, led to the differing faunal densities observed at the two sites.

CHAPTER 6

Development of Glycogen as a Biochemical Index

6.1 Introduction

The preceding chapters have described the value of biochemical measurements in detailing the relationship between the organism and its environment. One aim of the study was to verify that such measurements could indicate the "well-being" of the organisms and then to adapt suitable indices for use with preserved biological material. The approach adopted in chapter 5 was to integrate all of the biochemical components to reveal overall metabolic patterns. This procedure was undoubtedly valid for the Baie de Somme study, using fresh, frozen animals, however, many of the metabolites included are not applicable to preserved material. Those more labile components (for example the adenylates) would not give reliable results because of the inevitable time delay between collection and fixation, even if the preservation procedure did not affect these metabolites. In any case, the short-term response components have been shown to contribute little to the observed metabolic cycles. It may still be possible to use a multivariate approach and this will be discussed in chapter 8.

Chapter 2 highlighted the potential of the storage component glycogen as a single, representative biochemical index. The study so far has indicated that it fulfils most of the necessary criteria suggested in chapter 1. Its levels are unaffected by short, sudden perturbations, responding instead to sustained disturbances and its measurement involves a straightforward assay. Given these potentially exploitable characteristics a detailed study was made of the relationship between environmental disturbance and glycogen levels. It was proposed that the assay technique be developed to deal with preserved material.

6.2 Laboratory Experiments

6.2.1 Introduction

The highly dynamic and complex estuarine system of the Baie de Somme was unsuitable for the determination of the effects of specific isolated stressors.

The opportunity arose to utilise controlled laboratory experiments to determine whether or not a continually applied, single stress had an effect on an organism's tissue reserves, resulting in a decline in tissue glycogen levels.

The species used was *Nereis virens*, a ragworm closely related to *N. diversicolor* but commercially available. External stress was imposed by acidification of the seawater in which the animals were kept. Such a stress does not often occur in the natural habitat, the pH range of normal seawater being between 7.6 and 8.4. Freshwater inputs may increase this range to between 6.8 and 9.25, so estuarine animals should be more tolerant, but values lower than this may be expected to be outside the experience of the animal, and adaptation would not be expected.

Two experiments were performed since mechanical failure prevented the first experiment from running for the desired duration. The first ran for ten days, the second for the intended time span of thirty days; both experiments will be discussed. Experimental conditions were identical in both cases, with the exception of the ambient seawater temperature. The experiments were run at different times of the year, thus water temperature varied between 17.2 and 19.5°C in experiment 1 and between 8.0 and 10.1°C in experiment 2. An effect of this was that the worms were more active in the warmer water of the first experiment.

A variety of factors were measured in an attempt to detect the adverse effects of acid seawater exposure including survival, growth factors, glycogen levels and the dissolution of the chaetae.

6.2.2 Methods

The experimental apparatus design was that described by Bamber (1987). The test animals were held on 1 millimetre glass beads in 2 litre aquaria with a continuous flow-through of fully aerated, sand-filtered seawater. The water was acidified by the addition of "Analar" sulphuric acid to give a range of eight pH levels including a control. There were four replicates of each pH channel with five worms per replicate (four worms in the second experiment because the worms were of a larger size).

Prior to the start of the experiment five worms were frozen and five preserved as start controls. During the experiment worms were fed 5% body

weight, per day, after any uneaten food was removed. At the end of each experiment three worms from two of the four pH replicates were removed, blotted dry and frozen individually. The remaining worms were preserved in 100% isopropanol. Records were made of numbers surviving and numbers of surface burrow openings.

The frozen worms were analysed for weight and glycogen content as described in chapter 2. The preserved animals were analysed for width (to the nearest 10 μm) with a micrometer eyepiece and chaetal state, to assess the dissolution of the chaetae.

$$\text{Chaetal state is defined as } \frac{\text{Chaetal width - Body width}}{\text{Body width}}$$

6.2.3 Results and Discussion

Figures 38 and 39 show the results of experiments 1 and 2 respectively. Glycogen levels in both experiments were reduced at the lower pH's, although in experiment 1 (fig. 38) only the lowest pH showed glycogen levels below the start control. The decline in glycogen with falling pH was more noticeable in the longer experiment 2 (fig. 39). Dry weight also showed a decline with falling pH. This variable depended on the initial weight of the worms but since they were of identical age (they came from commercial stocks) initial weights were comparable. The large variation in the individual width measurements, in both experiments, prevented any relationship with pH. Chaetal state also showed no effect from low pH in either experiment. Percentage survival in experiment 1 fell rapidly below pH 6.5 but experiment 2 showed high survival, 100% at the lowest pH. This could either be a result of the larger size of worms in experiment 2, enabling greater tolerance, or that the colder temperature reduced activity, thus lessening the lethal effects of the acidity. Burrows were not evident at all in experiment 2 because of the cold-induced inactivity but in experiment 1 a clear decline in burrow numbers with lowering pH was noted.

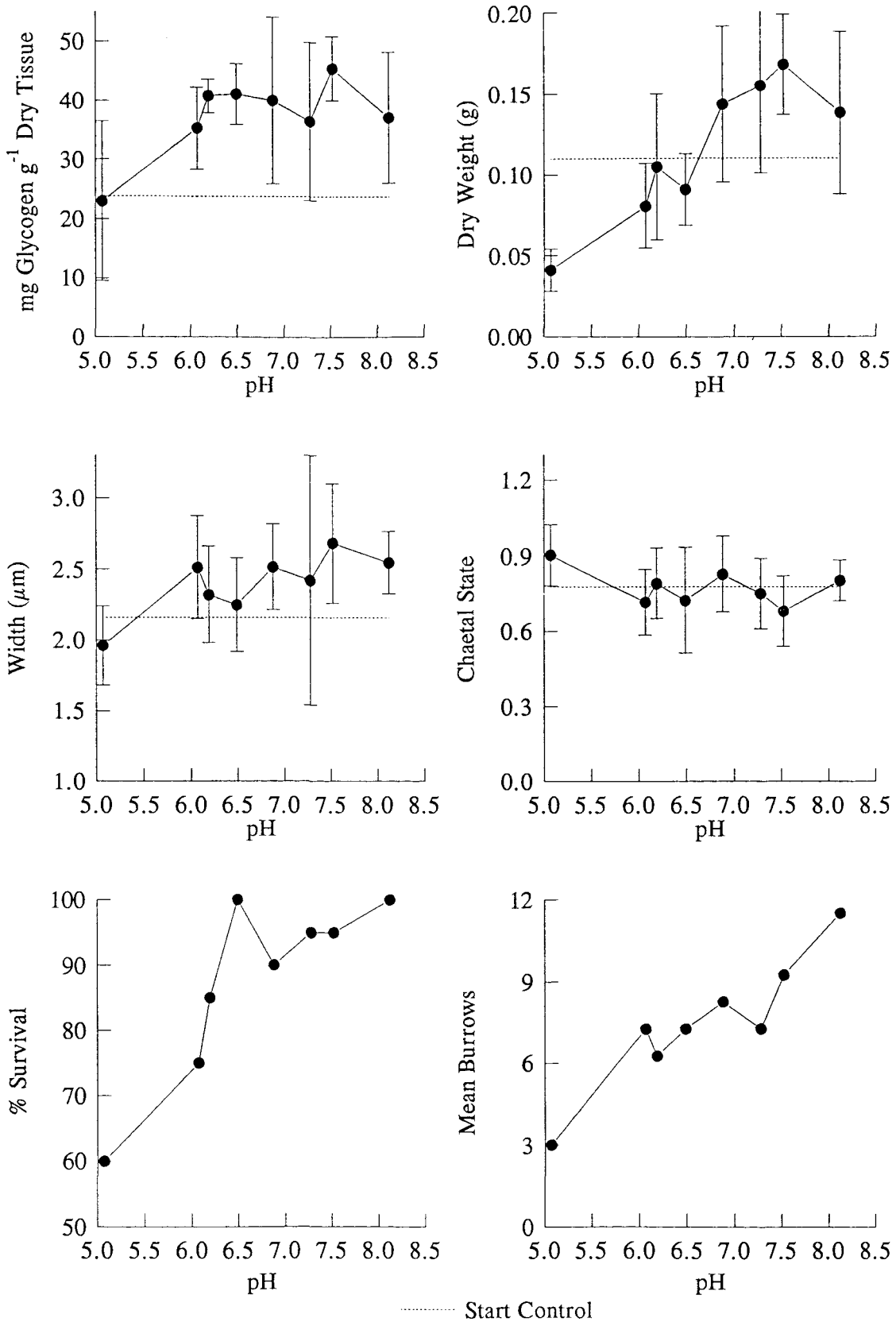


Fig. 38. Results of experiment 1 to detect the effects of acid seawater on *Nereis virens*.

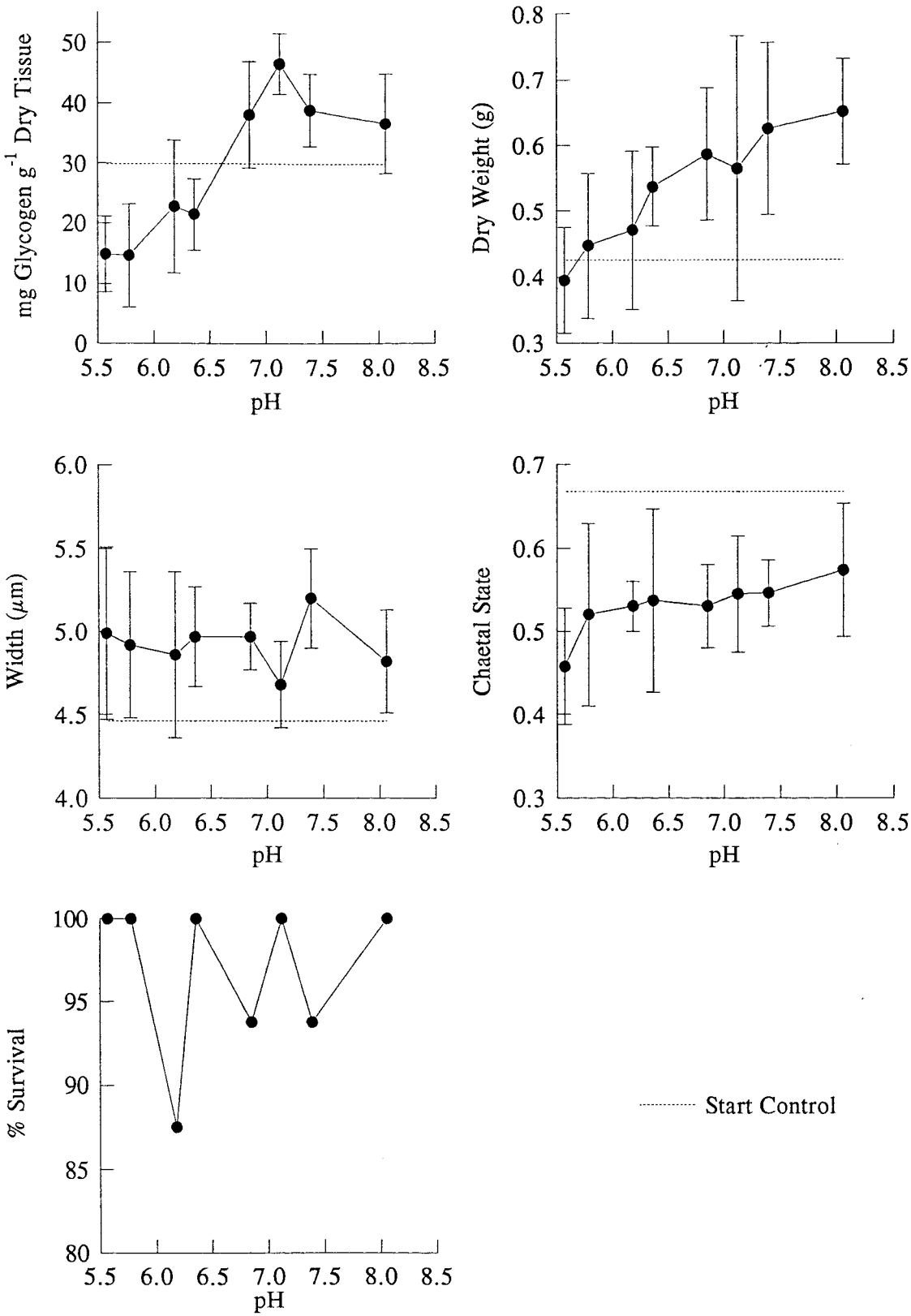


Fig. 39. Results of experiment 2 to detect the effects of acid seawater on *Nereis virens*.

Several of the chosen indices, in addition to glycogen level measurements, have indicated the detrimental, sub-lethal effects of low pH. However, detecting the effects using percentage survival or number of burrows required knowledge of the number of worms and burrows at the start of the experiment. In a field situation such data would be extremely difficult to obtain. Similarly, use of dry weight changes as an index in field applications would require detailed information on the size distribution of the animals in the tested populations, before detrimental effects could be determined. Glycogen level measurements, however, require no such additional information to define stress effects, thus enhancing its efficacy as a stress index. This index has now been proven to be responsive in both field and experimental situations.

In addition, although no mortalities occurred in the pH 5.5 worms in experiment 2, glycogen levels were clearly depressed below pH 6.5. They were also lower than the start control levels, indicating that the worms had been forced to use stored reserves. The detection of reduced glycogen, despite full survival, indicates the sensitivity of this storage product to sub-lethal stress conditions, an essential attribute of a stress index.

6.3 The Measurement of Glycogen in Preserved Material

6.3.1 Introduction

Quantitative examination of formaldehyde-fixed material is normally restricted to histological studies, because of the obvious problems in determining biochemical composition. Proteins are denatured by formaldehyde fixation and any soluble components are dissolved by the storage medium, non-polar media causing leaching of lipids. However, histological methods to detect glycogen do exist. For example Pearse (1980) described histochemical methods that use amylase digestion as part of the procedure for detecting glycogen in chemically preserved, sectioned material. Treatment with ethanol (also a commonly used storage medium) is part of the method for the enzymatic determination of glycogen described by Johnson and Fusaro (1966) and Passoneau *et al.* (1967). These are accepted methods, therefore it would seem possible to make measurements of glycogen in preserved material that should be comparable with levels found in fresh or frozen samples.

The measurement of glycogen in material examined so far in this study has

used an enzymatic method involving amylase digestion. Clearly the presence of any formaldehyde in the sample would denature the enzyme and inhibit the assay, thus its removal was essential. Material preserved in acidic media, such as unbuffered formaldehyde, should not be examined as glycogen would be hydrolysed by the acid to form soluble oligo- and monosaccharides that would be lost in the storage medium so causing an underestimation of glycogen content.

Taking these factors into consideration, the existing enzymatic method was adapted to measure glycogen in preserved specimens. The protocol has been described by Hawkins *et al.* (1992) but will be detailed more fully here.

6.3.2 Adaptation of the Method

It was first necessary to remove all traces of formaldehyde from the tissue to prevent enzyme inhibition. This was achieved by washing the tissue with multiple changes of 70% ethanol, over a period of several days. Clearly, the necessary amount of washing would be dependent on the size and density of the examined tissue. A compromise was achieved between enough tissue for accurate assay and small amounts for efficient washing by using pieces of tissue of approximately 1 gram wet weight. Such tissue required four changes of ethanol with a minimum of twenty-four hours in each solution. Material fixed with formaldehyde but stored in alcohol required only three washes. It was possible to detect the presence of aldehydes and free reducing sugars from glycogen breakdown in the discarded washing ethanol using the Schiff reaction test described by Vogel (1948). Consecutive negative tests implied complete removal of the formaldehyde.

The washed material was then freeze-dried to remove the ethanol and to give dry tissue weights, increasing the accuracy of the assay.

The method described in chapter 2 involved the amylase digestion of glycogen to glucose with subsequent spectrophotometric detection of glucose. In fresh material it was also necessary to measure tissue glucose levels prior to the digestion, to subtract from the end value (glucose plus glycogen) thus giving the true amount of glycogen. Glucose, however, is highly soluble, therefore in the preserved material any glucose not removed by the storage medium should be removed by the washing procedure. This was found to be the case when tested,

thus it was not necessary to measure initial glucose in addition to the end product of the amylase digestion, reducing the assay time.

The assay was further simplified since the stability of glycogen granules meant that extraction with perchloric acid and subsequent centrifugation and neutralization, was unnecessary. The tissue could be placed directly into the incubating digestion solution; the larger pieces of tissue were broken up to increase the available surface area. After digestion the solution was centrifuged prior to the spectrophotometric assay described in chapter 2. In addition to reducing the number of steps in the assay, the accuracy was improved since the dilution caused by the perchloration procedure inevitably introduced slight errors.

Comparison between preserved material and fresh, frozen material was performed to check for the effects of preservation and also of long term storage on glycogen levels. The test between fresh and preserved tissue collected at the same time was performed on liver samples from the deep sea fish *Coryphaenoides rupestris*, collected from two depths (700m and 1350m), from the Rockall trough in January 1990 (stations 74/90/1 and 2). The fish were dissected at the time of collection, liver samples were removed and either frozen or preserved in buffered formaldehyde solution. All specimens examined were female. The oily nature of the fish liver required that the frozen samples be washed with ethanol before analysis since it was found that high lipid levels prevented complete enzymatic hydrolysis of the stored glycogen. With this exception, glycogen analysis proceeded as previously described.

The effects of long term preservation were assessed using tissue from the holothurian *Oneirophanta mutabilis*. Preserved samples collected between 1979 and 1983 during the IOSDL Porcupine Seabight Programme (Rice *et al.*, 1991) were compared with fresh, frozen specimens collected from similar depths in May 1991 during RRS Challenger cruise 79. The tissue examined was the body wall, and, in the event that preferential glycogen storage occurred in certain body wall areas, 1 cm diameter discs were taken from the anterior ventral region of each animal.

6.3.3 Results and Discussion

The following tables give the results of the comparative assays.

Table 10. Comparison of liver glycogen contents of specimens of *Coryphaenoides rupestris*.

Depth (m)	Glycogen mg g^{-1} dry tissue weight			
	Fresh-frozen		Formaldehyde preserved	
700	x = 3.70	s.d. = 2.53	x = 3.18	s.d. = 1.37
1,350	x = 3.64	s.d. = 1.27	x = 3.09	s.d. = 2.90

Table 11. Comparison of body wall glycogen levels of specimens of *Oneirophanta mutabilis*.

Preservation method	Glycogen mg g^{-1} dry tissue weight	
	Mean	Standard deviation
Ethanol (70%)	1.414	0.589
Fresh-frozen	1.482	1.220

A t-test was applied to the results shown in table 10, to determine the differences between the contemporaneous fresh and preserved samples. Values of t were obtained of 0.767 and 0.737 for the 700m and 1350m samples respectively, indicating that the difference between the two treatments was non-significant in both cases (p = 0.45 and 0.47). The results in table 11 were also analysed with a t-test, giving a t value of 0.166, which was again non-significant (p = 0.87).

Thus, with these methodological adaptations, it is possible to measure glycogen in formaldehyde preserved material. The effects of preservation on glycogen levels have been shown to be negligible, and long term storage causes no appreciable loss of glycogen. This last point is particularly important if the index is to be applied to archived and/or time series material. This application will be considered next.

6.4 Baie de Somme Archive

6.4.1 Introduction

The choice of the Baie de Somme as the study site for biochemical validation was partly because of the presumed existence of a time series of archived, preserved specimens collected since 1982. On close examination, however, this time series was not as comprehensive as had been hoped. Nonetheless, it was still examined to further validate the use of glycogen measurements.

Both cockles and *Nereis* had been regularly collected from LCS and some samples were also available from HHS. All samples had been preserved and stored in 4% buffered formaldehyde solution. Population information was also available for the same time period (Desprez, unpublished data), so that correlation between any unusual glycogen levels and population changes could be made.

6.4.2 Methods

Six individuals of each species were removed from each sample. Age was noted from the cockle growth rings, but in the case of *Nereis* only mature two/three year old worms were taken. In the majority of samples only one age cohort of cockles was present, but in the few instances where two cohorts existed, samples from both were taken.

Cockle tissue was removed from the shell and both this and whole *Nereis* individuals were washed in 70% ethanol and freeze-dried according to the protocol described in section 6.3. Determination of glycogen levels also proceeded as described in the previous section.

In order to determine whether or not the measured glycogen levels in the archive samples were unusual, it was first necessary to account for the normal, seasonal fluctuations in glycogen levels. As it has been demonstrated in chapter 5 that both species in the two year (1990-1992) validation study showed no abnormal fluctuations in seasonal cycles, the values from this study were used as a base-line with which to compare the archive results. The results of two years of sampling were combined to give the "expected" glycogen levels for each month. The values obtained from the archive study were compared with the values from the same month from the validation study. LCS and HHS samples were treated separately.

Any significant differences indicated abnormal glycogen levels which were then correlated with the population information.

6.4.3 Results and Discussion

Figures 40 to 43 display the tissue glycogen levels and population densities of both species for the archive samples available. Tables 12 and 13 show the results of the t-test comparing the archive sample glycogen levels with the base-line time series results.

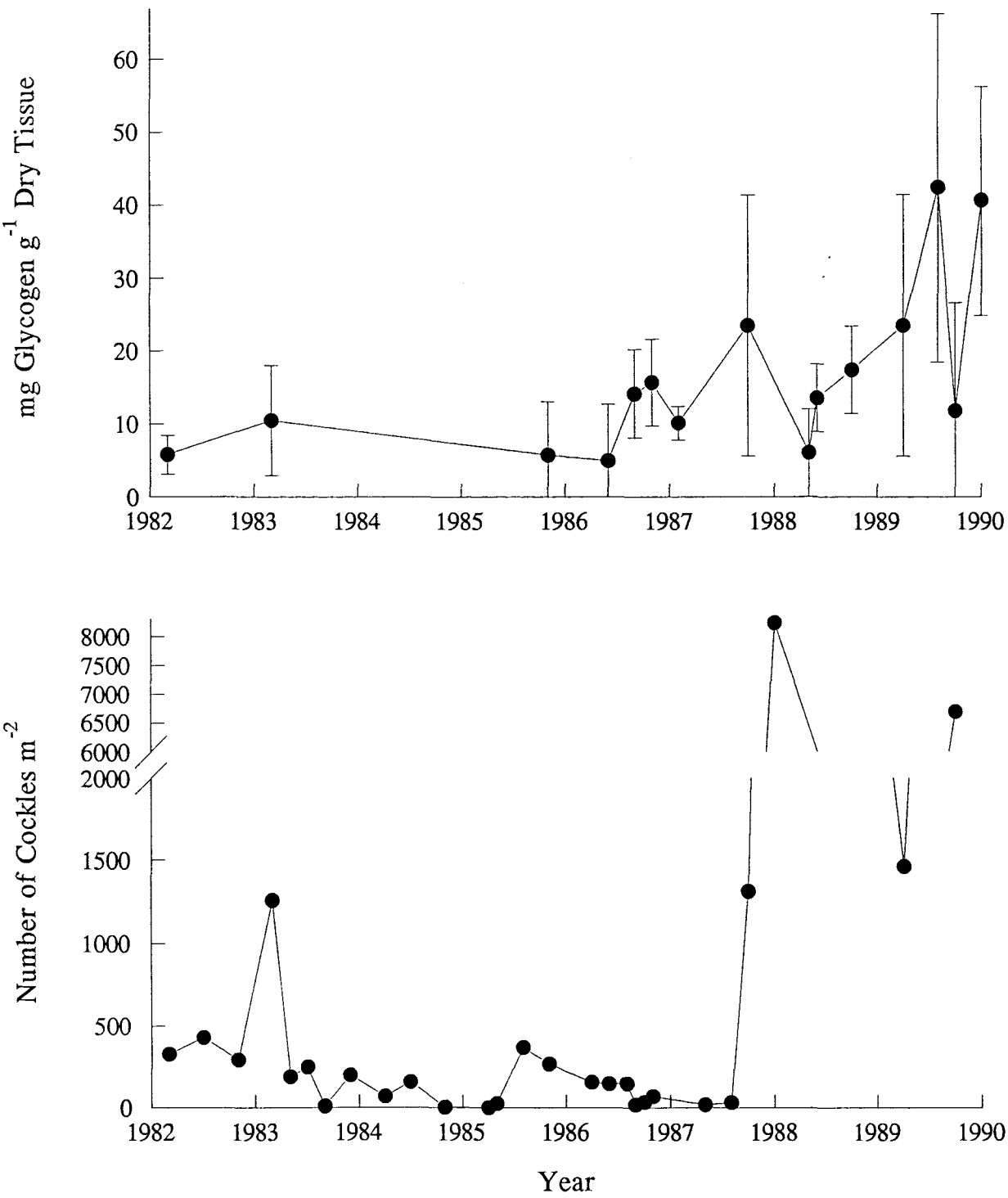


Fig. 40. Mean tissue glycogen results from archive LCS *C. edule*. Error bars indicate standard deviation.

Population data from Desprez (unpublished data).

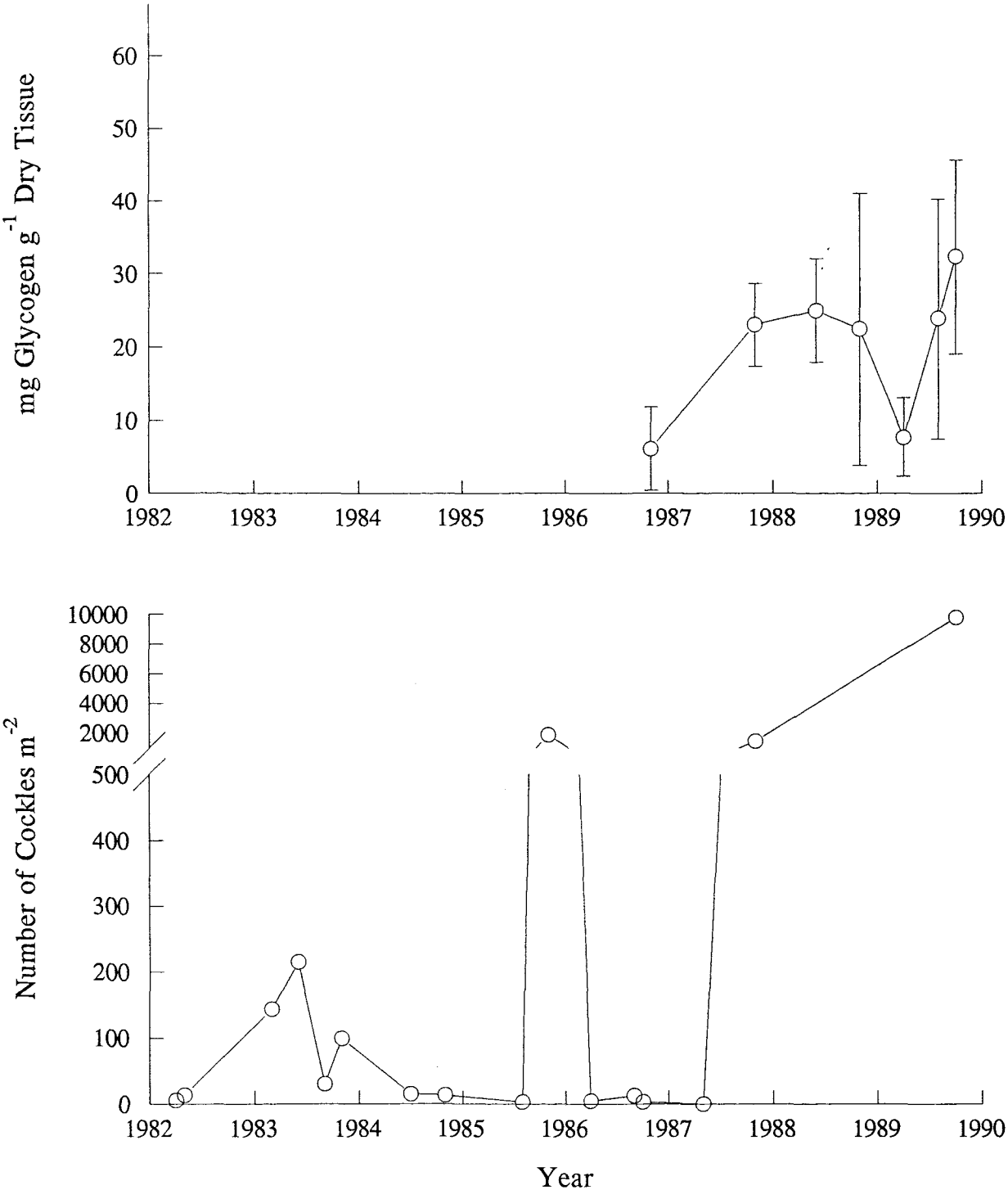


Fig. 41. Mean tissue glycogen results from archive HHS *C. edule*. Error bars indicate standard deviation.

Population data from Desprez (unpublished data).

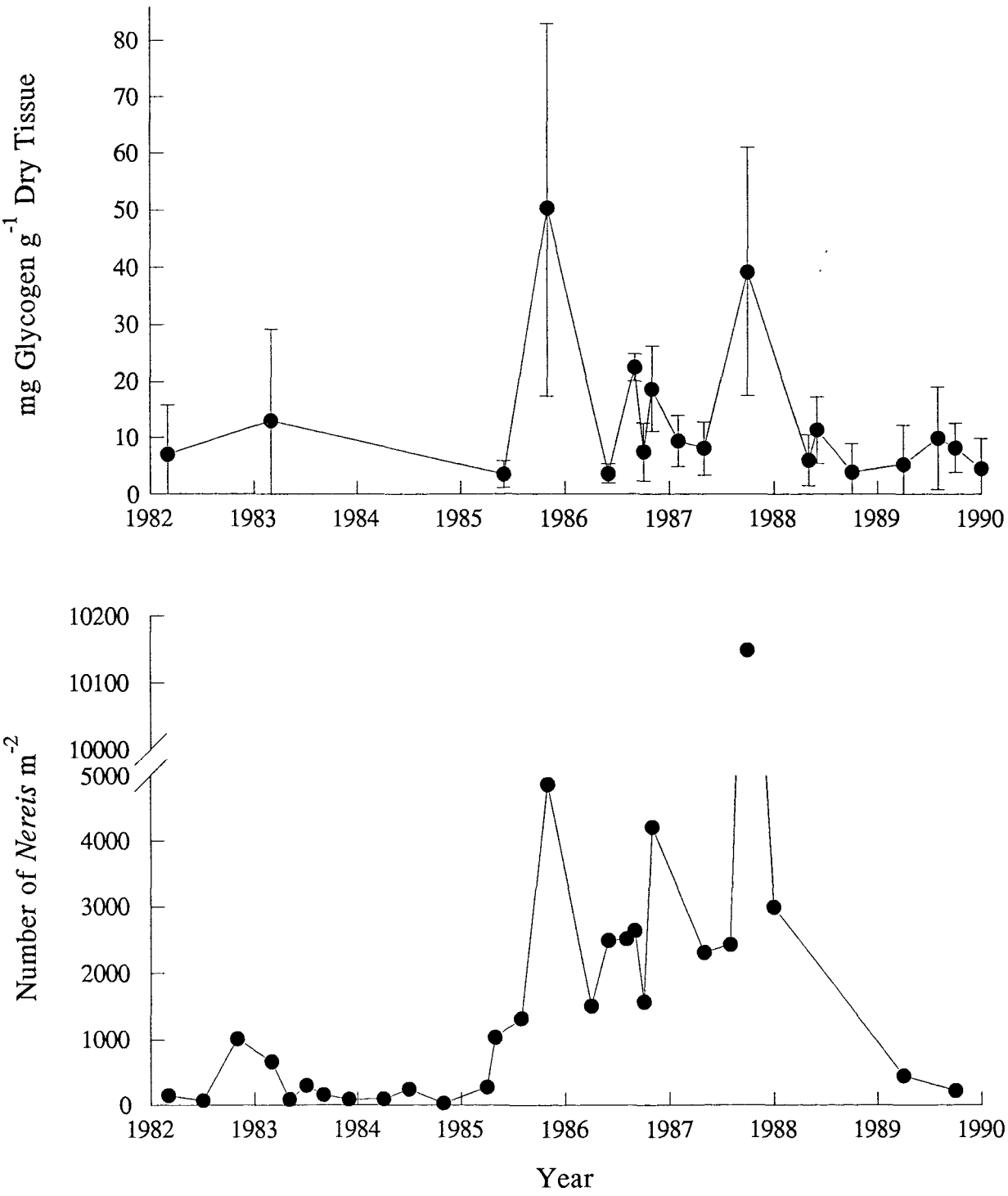


Fig. 42. Mean tissue glycogen results from archive LCS *N. diversicolor*. Error bars indicate standard deviation.

Population data from Desprez (unpublished data).

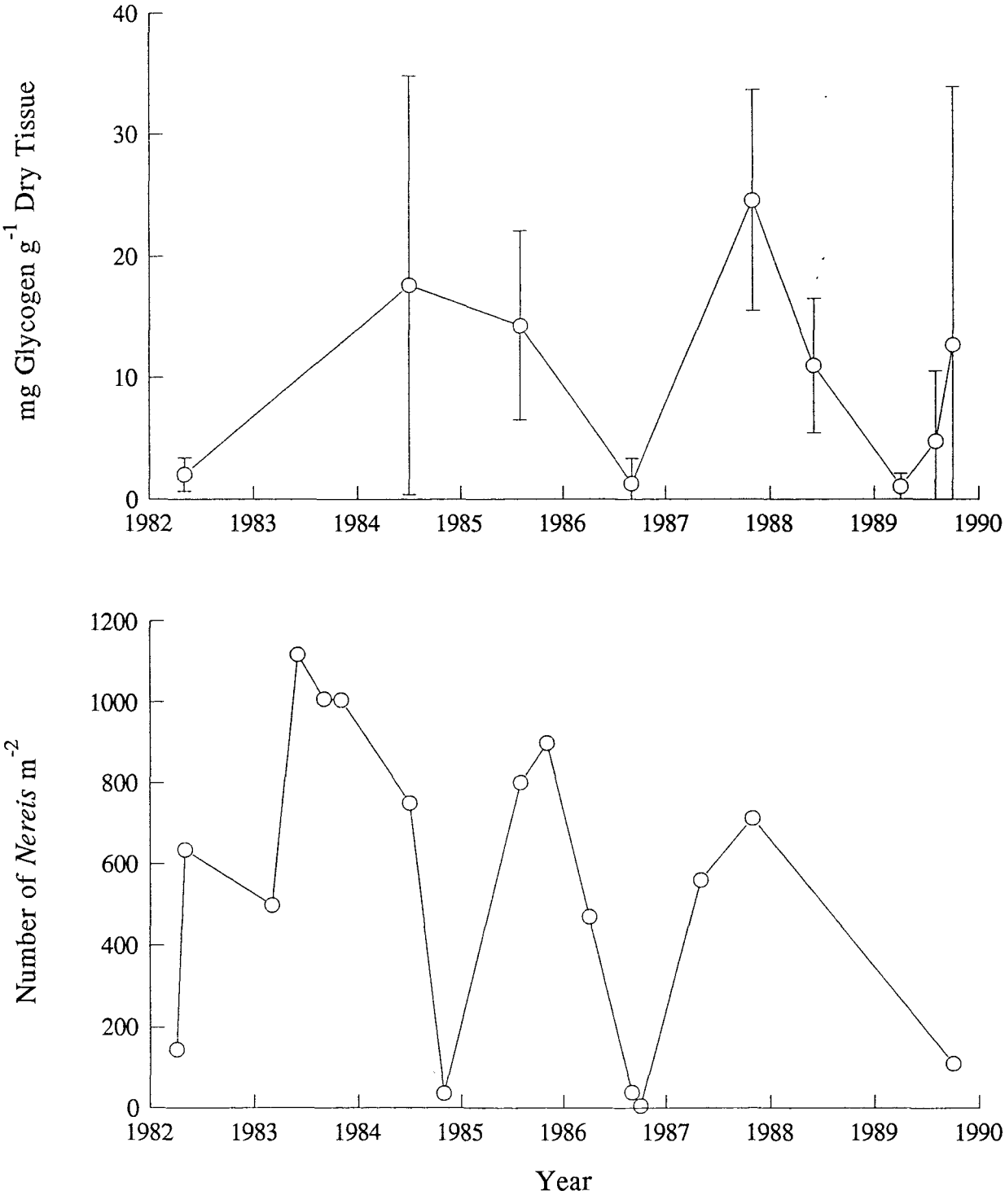


Fig. 43. Mean tissue glycogen results from archive HHS *N. diversicolor*. Error bars indicate standard deviation.

Population data from Desprez (unpublished data).

Table 12. Results of the t-test between monthly time series and archive samples, to detect differences in glycogen levels in *C. edule*.

LCS		HHS	
DATE	SIGNIFICANCE	DATE	SIGNIFICANCE
20-2-82	n.s.		
8-2-83	n.s.		
23-10-85	$p < 0.001$		
9-5-86	$p < 0.001$		
1-8-86	n.s.		
23-10-86	$p < 0.001$		
1-1-87	n.s.		
9-9-87	$p = 0.009$		
7-4-88	n.s.	14-10-86	n.s.
25-5-88	$p < 0.001$	21-10-86	$p = 0.001$
30-9-88	$p = 0.001$	20-10-87	$p = 0.011$
30-3-89	$p = 0.005$	31-5-88	n.s.
7-7-89	$p = 0.006$	30-3-89	$p = 0.032$
19-9-89	$p < 0.001$	8-7-89	$p = 0.014$
4-12-89	n.s.	21-9-89	n.s.

Table 13. Results of the t-test between monthly time series and archive samples, to detect differences in glycogen levels in *N. diversicolor.*

LCS		HHS	
DATE	SIGNIFICANCE	DATE	SIGNIFICANCE
20-2-82	n.s.		
8-2-83	n.s.		
10-5-85	$p = 0.004$		
23-10-85	$p = 0.042^*$		
9-5-86	$p = 0.004$		
1-8-86	n.s.		
25-9-86	$p = 0.022$		
23-10-86	n.s.		
1-1-87	n.s.		
4-4-87	$p = 0.041$	30-4-82	n.s.
9-9-87	n.s.	22-6-84	$p = 0.041$
7-4-88	$p = 0.023$	5-7-85	n.s.
25-5-88	$p < 0.001$	1-8-86	$p = 0.039$
30-9-88	$p = 0.025$	20-10-87	n.s.
30-3-89	n.s.	31-5-88	n.s.
7-7-89	n.s.	30-3-89	n.s.
19-9-89	$p = 0.041$	8-7-89	n.s.
4-12-89	n.s.	21-9-89	n.s.

* Significantly higher.

Several of the archive samples were significantly different from the normal glycogen levels derived from the two year study. In all but one of these cases (that marked *) the glycogen levels were significantly lower than normal, suggesting that the animals were experiencing some stress. It is possible that these differences were a result of interannual reproductive cycle variations. For example, the reproductive cycle may have been temporally shifted, perhaps as a result of meteorological abnormalities, and the apparent significant glycogen differences may not be the result of stress. The time series study allowed the interannual fluctuations from two years to be combined, but this would not have included the potential glycogen cycle shifts evident over an eight year period. Such a comparison was, however, the most comprehensive analysis possible with the limited data available.

Isolated significant differences may be the coincidental result of insufficient data, but prolonged differences are more likely to reflect actual periods of environmental stress. Therefore it would be more appropriate to consider periods when the glycogen levels were consistently different for several samples.

For LCS cockles two such periods are evident in table 12; the latter part of 1985/start of 1986, and the period from May 1988 to September 1989. Examination of the population data shown in fig. 40 reveals that these two periods were coincident with higher cockle densities, particularly the 1988/89 period where numbers exceeded 8,000 per square metre. Data for HHS cockles were more sporadic but the period of March 1989 to July 1989 with low glycogen levels was coincident with high cockle densities of around 10,000 per square metre, shown in fig. 41.

Consistent periods of low glycogen in LCS *N. diversicolor* were difficult to identify (table 13). The period from May 1985 to May 1986, where all differences were significant, also contains one sample with significantly higher glycogen, therefore this period can be assumed not to have been stressful. The period from April 1988 to September 1988 showed consistently low glycogen, but unfortunately no population observations were available for this period. Numbers of *N. diversicolor* were very high (approximately 10,000 per square metre) at the end of 1987, however (fig. 42), and by early 1989 had dropped to only several hundred per square metre. Therefore it can be supposed that 1988 saw declining

Nereis numbers.

There were no significantly different periods evident in the sporadic data for *N. diversicolor* at HHS; however the two isolated samples with low glycogen levels (June 1984 and August 1986) coincided with declining numbers (fig. 43).

These results do suggest some relationship between low glycogen levels and population changes. In the case of the cockle this coincidence occurred with high population densities, but subsequent population declines did occur. During 1986 numbers at LCS fell to only tens per square metre from the 1985 high (when the low glycogen occurred) of several hundred per square metre. There was a more obvious peak in numbers in early 1988 followed by a drop during 1989 from > 8,000 to 1500 per square metre, coincident with significantly low glycogen, although some recovery to numbers of 6500 per square metre was noted by the end of 1989. At HHS the high density period during 1989 was coincident with low glycogen and data from the commencement of the two year study revealed declining cockle numbers to only tens per square metre during 1990. There would thus appear to be a predictive element to the cockle glycogen measurements. Low glycogen levels associated with high population densities indicate a subsequent population decline. However, the samples were too infrequent throughout this time span to be more than speculative about the results.

The results for *Nereis* suggest less of a predictive ability since low glycogen occurred during population decline, rather than before it, but again the data were not sufficient to eliminate the possibility of population prediction.

An additional point is that for both species at both sites the final archive sample, at the end of 1989, showed no significant differences in glycogen levels from the two year study values. This reinforces the proposed suggestion that the intensive two year study covered a period of relative stability, with no environmental (and thus metabolic) abnormalities which commenced towards the latter part of 1989.

6.5 Summary

This chapter has extended and developed the potential of glycogen as a biochemical index. Using controlled laboratory experiments it has been shown to respond sensitively to a single imposed stress. The results of these experiments

also suggested its suitability to field situations, in preference to measurements of growth or survival which require previous knowledge of the studied populations.

The method for glycogen detection has been adapted for the study of preserved material and no significant effects of long term preservation have been found.

Application of this method to a preserved archive of samples revealed the potential to predict future population declines. Although data were not fully adequate to fulfil this central aim of the study, this suggested potential when combined with the irrefutable responses of glycogen noted in the validation study confirm its usefulness as a biochemical index.

Sufficient attributes of glycogen measurements have now been determined to enable it to be applied to preserved deep-sea material, and this will be examined in the next chapter.

CHAPTER 7

Studies on Deep-sea Material

7.1 Introduction

Studies on deep-sea benthic communities have been confined almost exclusively to taxonomic and numerical ecology investigations. Comparisons between communities and inferences on the levels of disturbance have been made from the number of individuals of the species present, for example the studies of Thistle *et al.* (1985) and Grassle and Morse-Porteous (1987). As described in chapter 1, information on the physiological well-being of the organisms should yield more accurate estimates of the community structuring processes than abundance data alone, since biochemical changes are the primary result of environmental change. A valid method for the detection of the physiological state of deep-sea organisms has now been developed (see chapter 6) and preliminary studies of deep-sea material will be described in this chapter.

The application of the glycogen protocol required some methodological definition, for example the type of tissue that should be examined, the lower tissue size limit for reliable detection and the most useful supporting data to be included, such as histological evidence, size and sex measurements. To this end, a range of taxa were investigated to attempt to evaluate the most efficient and informative approach when examining deep-sea material.

The number and variety of taxa that could be examined were constrained by the time available and access to samples; nevertheless several phyla have been studied. The majority were benthic organisms, but a pelagic fish, *Synaphobranchus kaupi*, was included for comparison. Several of the following studies involved the examination and comparison of related species, the remainder were confined to single species studies.

7.2 Examination of *Nephrops norvegicus* and *Nephropsis atlantica*

7.2.1 Introduction

The storage organ in decapod Crustacea is the hepatopancreas, which stores both lipid and carbohydrate. Glycogen forms between 3% and 11% of total carbohydrate present in the hepatopancreas (Gibson and Barker, 1979). Glycogen levels within decapods have been known to vary throughout the year in conjunction with moulting and reproduction cycles (Parvarthy, 1971). During the period of ecdysis, crustaceans do not feed and rapid depletion of hepatopancreatic glycogen reserves has been noted by Williams and Lutz (1975) after only a few days of starvation.

With these observations in mind, preserved samples of *Nephrops norvegicus* and *Nephropsis atlantica* from the Discovery collection were examined. All specimens were collected from the Porcupine Seabight between 1979 and 1983.

7.2.2 Methods

The carapace of each individual was cut along the dorsal mid-line and laterally to allow the hepatopancreas, which lies immediately below the carapace, to be removed. A portion of the tissue was reserved for histological examination and the remainder underwent the washing, freeze-drying and glycogen assay described in chapter 6. This study was undertaken before the completion of the assay protocol and glycogen was measured as glucosyl units, not as a proportion of the tissue weight calculated by comparison with a standard. Comparisons of glycogen levels within this study are valid, but absolute glycogen values, for comparison with other species, are not available.

Histological examinations were performed to determine if the information gained from glycogen measurements could be substantiated or extended. The hepatopancreas samples were dehydrated in an alcohol (ethanol) series from 70% to 100% v/v by successive immersions for several hours, followed by a second immersion in absolute alcohol. The tissue was cleared in "Histoclear" and embedded in paraffin wax prior to sectioning at 5-10 μm. After de-waxing and rehydrating (by reversal of the alcohol series) the sections were treated with the following stains to highlight relevant features. Haemotoxylin with an eosin counter

stain was used to reveal nuclear material and plasma, and the periodic acid-Schiff (PAS) reaction was used to indicate the presence of carbohydrates. The sections were viewed under a microscope and observations noted.

7.2.3 Results and Discussion

Figure 44 shows the hepatopancreatic glycogen changes with depth.

The samples were taken over a depth range, because of bathymetric changes during the course of sampling, therefore the depth that has been plotted was the mid-depth of the range (this protocol has been applied in all subsequent studies). *N. norvegicus* showed increasing glycogen with increasing depth although the number of samples was too small for statistical validity. This species is generally found at depths shallower than 500m, therefore those animals at the optimal depth had lower glycogen levels. *N. atlantica* generally occurs between 1000m and 1500m, and again glycogen levels are lowest at the optimal depth of occurrence, a finding contrary to that expected.

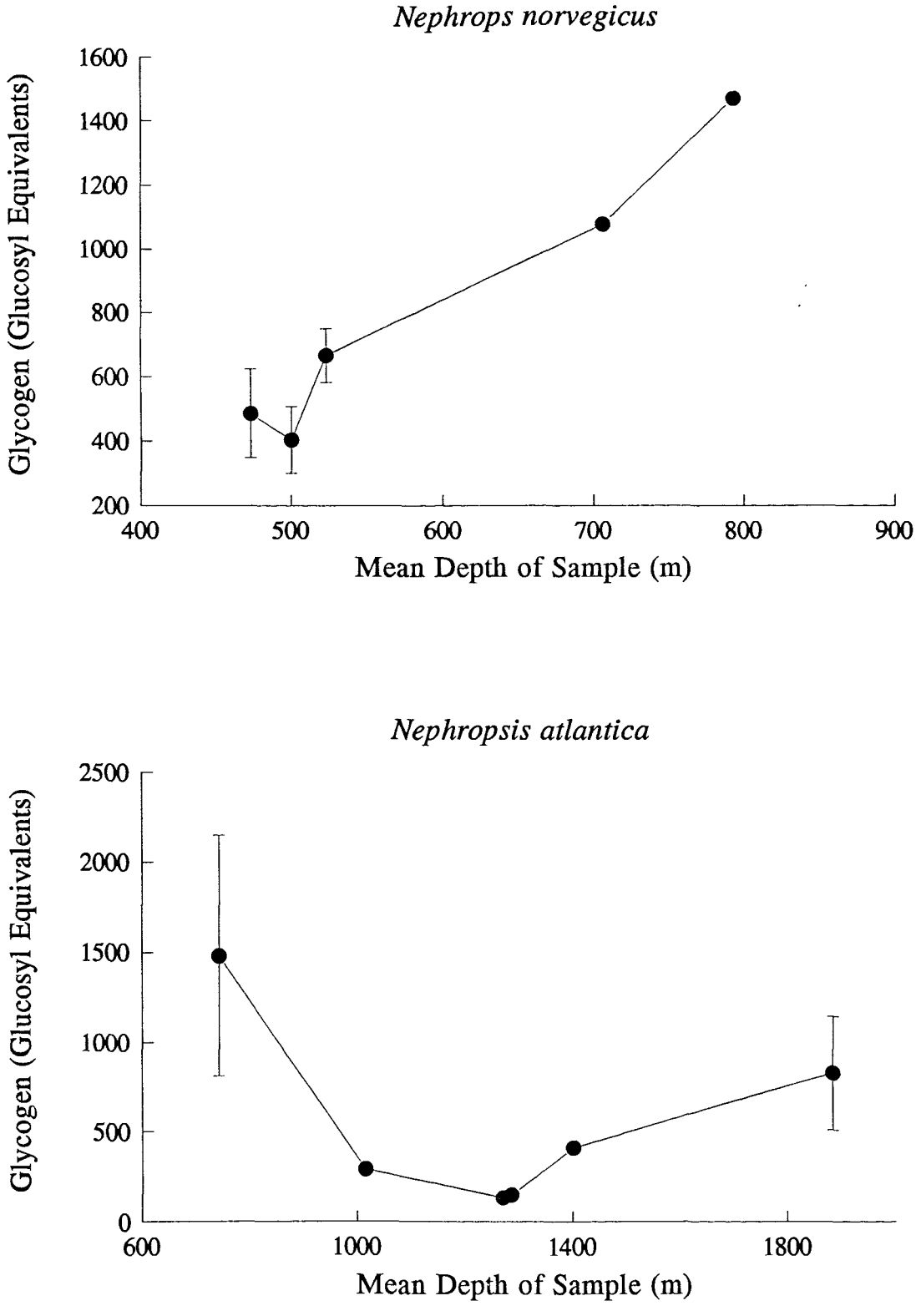


Fig. 44. Mean changes in hepatopancreatic glycogen with depth in the decapods *N. norvegicus* and *N. atlantica*. Error bars indicate standard deviation.

Observations of the histologically prepared hepatopancreas revealed that the tubules of the animals caught within their optimal depth range had large lumena surrounded by epithelial cells with large, presumably lipid containing vacuoles. By contrast, the tubules of those individuals caught at the extremes of the depth range had comparatively narrow, partially occluded lumena surrounded by epithelial cells containing large amounts of PAS-positive granular material, indicating carbohydrates. This pattern was apparent in both species (although very shallow *N. norvegicus* were not studied), despite the differing optimal depths. Although purely qualitative, this histology suggests an hypothesis for the counter-intuitive glycogen results. It can be proposed that organisms at their optimal depths have an efficient metabolism with a rapid turnover of glycogen resulting in lipid storage. The decapod hepatopancreas is known to contain a much higher proportion of lipid than glycogen, 35% of dry tissue in the case of *Homarus americanus* (Gibson and Barker, 1979), compared with a maximum glycogen content in any decapod of 11%, although both storage products depend on the reproduction and moult cycles. The histology revealed large vacuoles and less granular material in the optimal-depth animals suggesting lipid rather than glycogen storage. The large amounts of carbohydrate granules in the tubules of animals from their extremes of distribution indicate the reverse strategy. There is well documented evidence to suggest that many enzyme systems in deep-sea organisms are pressure sensitive (Hochachka *et al.*, 1972; Siebenaller and Somero, 1978). It is thus possible that the glycogen accumulation evident at sub-optimal depths was the result of a reduction of metabolic activity caused by pressure related reductions in the specific activities of key metabolic enzymes.

No allowance has been made for the potential reproductive state of the individuals studied, the samples were too few and covered a wide range of months. Given that variations in storage components with reproductive state have been noted in decapods, it is essential that this be investigated further to validate the above hypothesis.

7.3 Examination of Two Holothurian Species

7.3.1 Introduction

Chemically preserved specimens of two species of holothurian were examined, again from the Discovery collection, collected between 1979 and 1983. The species were *Laetmogone violacea* and *Oneirophanta mutabilis*, both very common in the Porcupine Seabight and Porcupine Abyssal Plain regions but with very different depth distributions. *L. violacea* occurred between 500m and 1500m whilst *O. mutabilis* was found between 2500m and 5000+m (Billett, 1988).

Holothurians do not have a specific storage organ, material being distributed within the body wall; therefore the glycogen content of this tissue was examined. In case of differential storage levels throughout the body wall, tissue samples were removed from the anterior, ventral region of each individual. A cork borer was used to cut 1cm diameter discs of tissue which were then washed and assayed as previously described.

7.3.2 Results and Discussion

Figure 45 shows the changes in body wall glycogen levels with depth for the two species.

It can be seen that in both cases there was a decline in glycogen with increasing depth. In *L. violacea* the correlation was highly significant ($p < 0.001$) but in *O. mutabilis* the correlation was only significant at the $p = 0.052$ level.

The lowest glycogen levels found in *L. violacea* occurred at the maximum depth of occurrence. The sudden drop in glycogen in *O. mutabilis* between 4200m and 4400m coincided with a change in the depth distribution of this species. Below 4200m the animal was twice as abundant as above this depth (Billett, 1988). There would thus appear to be a relationship between glycogen storage and the bathymetric distribution of holothurians.

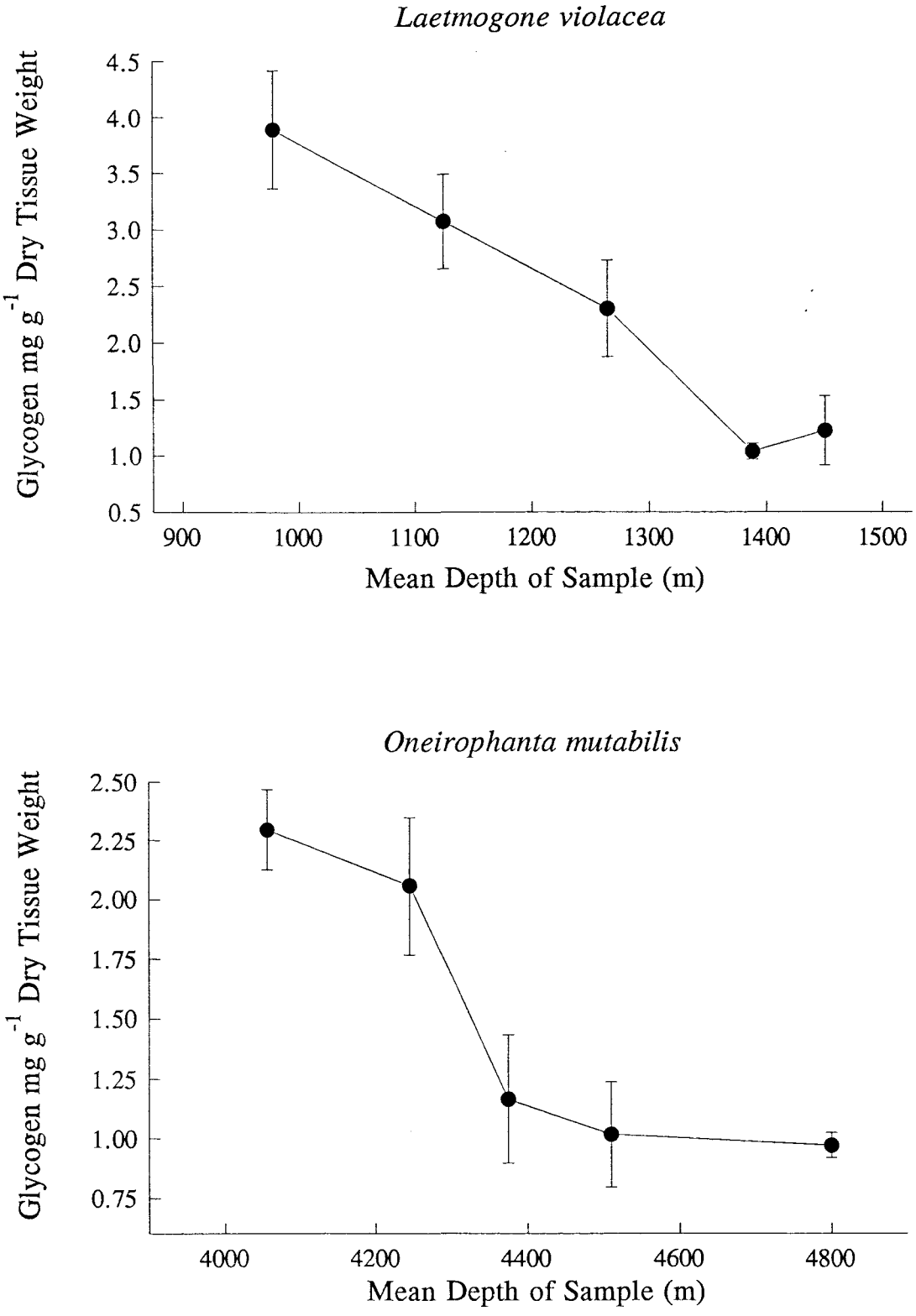


Fig. 45. Mean changes in body wall glycogen with depth in two holothurian species *L. violacea* and *O. mutabilis*. Error bars indicate std. deviation.

L. violacea showed the expected lowest glycogen in the deeper samples. However, specimens from the extreme shallow end of the distribution were not available, so it is not possible to say if these would also have shown lower glycogen. *O. mutabilis* exhibited the lowest glycogen at its most abundant depth of occurrence. Presumably the greater abundance indicates that this was the preferred depth for this species. This being so, it may be that pressure related changes in glycogen metabolism at such great depths caused the unexpected low levels at the preferred depth, consistent with the hypothesis proposed for the decapod results in the previous section. The anatomy of holothurians, with the lack of a specific storage organ, did not allow histological examination to substantiate these findings.

7.4 Examination of Three *Glyphocrangon* Species

7.4.1 Introduction and Method

Three preserved species of the decapod genus *Glyphocrangon* were examined. The species were *G. longirostris*, found between 1500m and 2200m, *G. sculpta*, occurring between 2200m and 3400m and *G. atlantica*, found between 2500m and 4000+m. This depth distribution data was derived from the sampling information of the specimens examined, however they are in agreement with the depth ranges recorded by Holthuis (1971); *G. longirostris* 1280-2500m, *G. sculpta* 1645-3219m and *G. atlantica* 3885-6364m. The species overlap only slightly, so it would appear that one replaced the other as depth increased.

The available material comprehensively covered both depth ranges and the time period from 1976 to 1983. Because of time restrictions, and the preliminary nature of this study, only the depth component was investigated.

The majority of the samples contained only one or two individuals per depth, but where more were present a maximum of six individuals per depth were examined.

The carapace length of each animal was measured with callipers to the nearest millimetre along the dorsal mid-line, from the posterior centre of the carapace to a line running laterally between the posterior points of the eye sockets. The carapace was cut along the mid-line and laterally to enable the hepatopancreas to be removed. The sex of each individual was noted, unless juvenile, and the hepatopancreatic glycogen levels determined as previously described.

7.4.2 Results and Discussion

Figure 46 shows the hepatopancreatic glycogen levels against depth for each of the three species. It became apparent during the course of the assay that there was a relationship between carapace length and amount of hepatopancreatic glycogen. The smaller individuals had proportionately more glycogen than the larger animals. A linear regression between carapace length and glycogen confirmed that this relationship was significant for both *G. longirostris* and *G. sculpta* with r values of 0.618 and 0.627 respectively ($p < 0.001$ in both cases). Such a correlation could obscure any depth/glycogen relationships which were indeed not evident in fig. 46.

The correlation between carapace length and glycogen was then used to calculate the expected hepatopancreatic glycogen for each individual. The difference between the expected value and the actual measured value (the residual, either positive or negative) was then plotted against depth, shown in fig. 47. It was not possible to perform a multiple linear regression since the relationship between glycogen and depth could not be assumed to be linear, as observed in *Nephropsis atlantica* both shallow and deep depth extremes exhibited elevated glycogen levels. The residual plots in fig. 47 however, revealed no detectable relationship between depth and hepatopancreatic glycogen. There was also no evidence that smaller individuals were confined to particular depths.

The absence of a depth/glycogen correlation was not wholly surprising considering the bathymetric distributions. Each of the three species occupied a particular depth range, with almost no overlap between species (an exception was the *G. atlantica* sample from 2400m, however since no other animals were caught until 3800m this could well have been an erroneous sample). It would seem that each species occupied its optimal depth range, being replaced by the next species as depth increased. The observed depth ranges were much narrower than those recorded by Holthuis (1971) suggesting that each species was restricted in its range (perhaps by interspecific competition), and no animals occurred at depths that would be particularly stressful. Thus no unusual glycogen values were obtained.

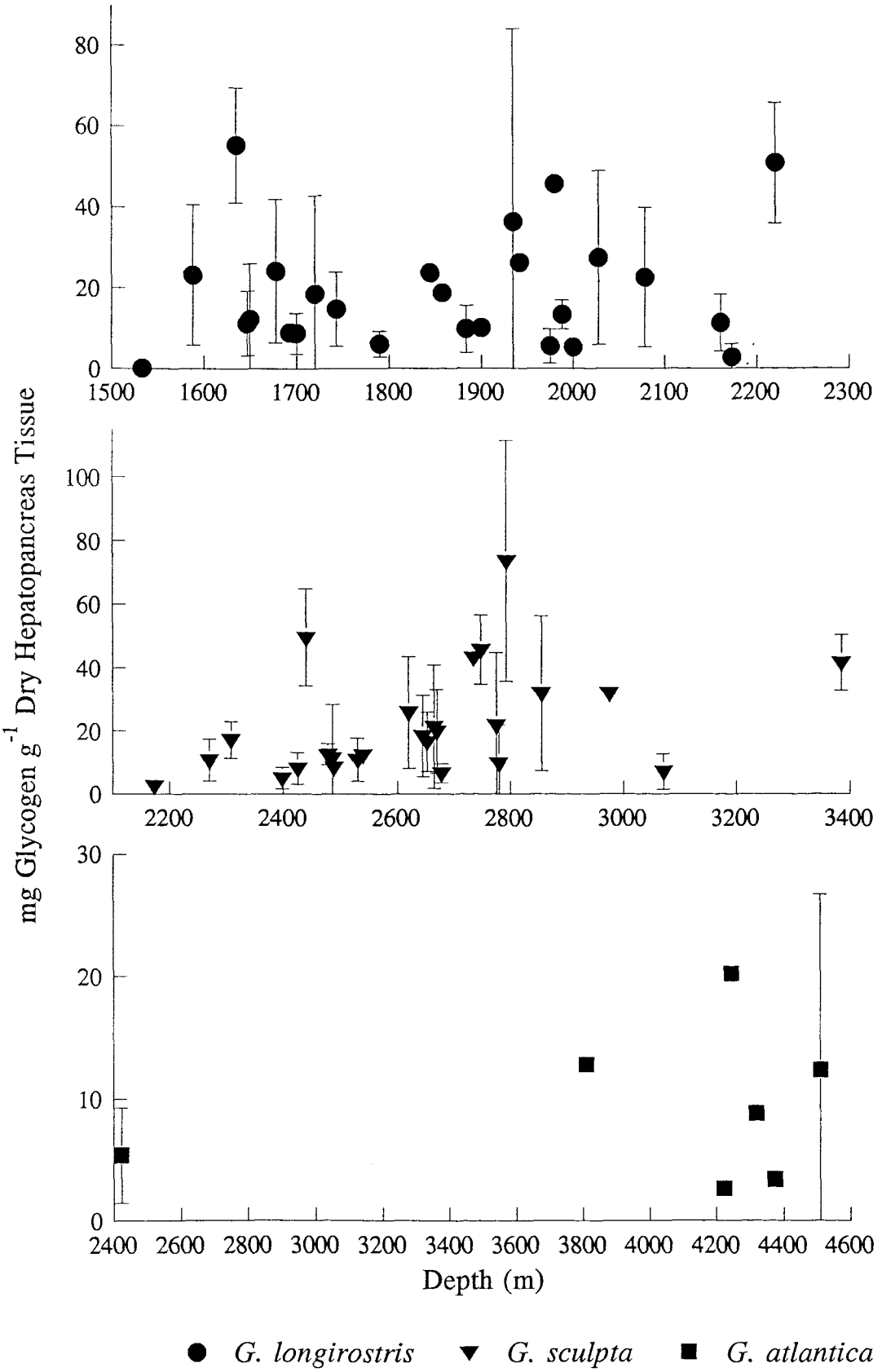


Fig. 46. Mean hepatopanreatic glycogen levels with depth in three *Glyphocrangon* sp. Error bars indicate standard deviation.

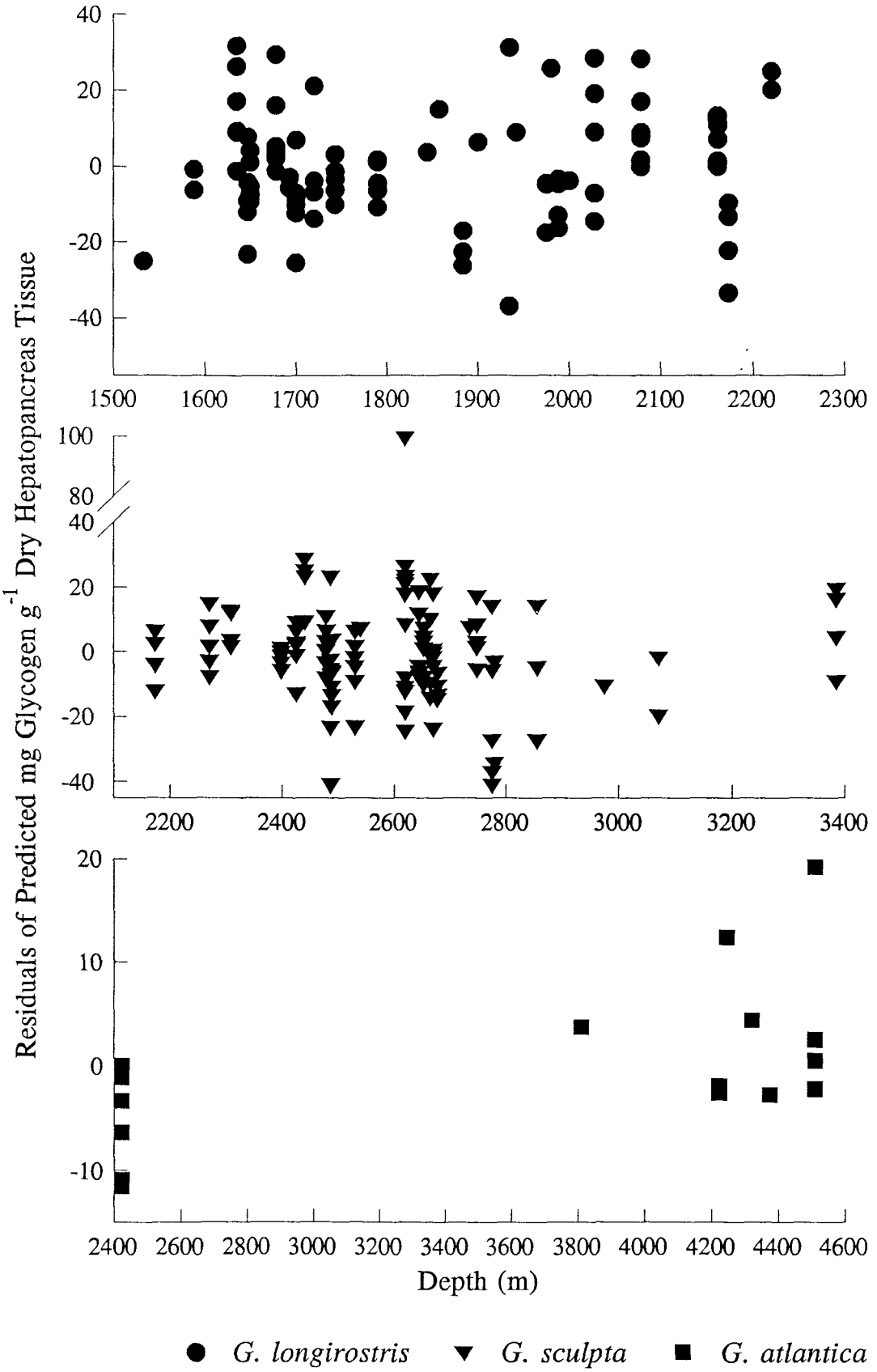


Fig. 47. Plot of residuals of predicted glycogen for each individual *Glyphocrangon* against depth.

Each of the three species showed similar glycogen levels, despite occurring at different depths, which were generally in the range 20 to 40 mg glycogen g^{-1} dry hepatopancreas (2-4%). Such proportions are comparable with those reported for other decapods (Gibson and Barker, 1979). The smaller individuals of *G. longirostris* and *G. sculpta* had values as high as 14%. It may be that *G. atlantica* juveniles also showed higher glycogen, but very few specimens were available for assay, including only one juvenile, consequently no significant size/glycogen correlation could be detected.

No account has yet been taken of the sex or reproductive state of the individuals. The majority of those assayed were female, adult males accounting for 14% in *G. longirostris*, 13% in *G. sculpta* and 44% in *G. atlantica*. It is not certain whether these are the genuine sex ratios or whether behavioural differences meant that females were more likely to be captured by the sampling technique. Excluding males from the analysis made no detectable difference to the depth/glycogen relationship, therefore sex effects were negligible. Reproductive state has not yet been examined, but the reproductive tissue has been retained and will be examined in the future.

7.5 Examination of *Synaphobranchus kaupi*

7.5.1 Introduction and Methods

Synaphobranchus kaupi is a very common pelagic fish of the synaphobranchid eel family. It was included in this series of assays because individuals travel vertically throughout the water column and are not confined to one depth. It would therefore be expected that glycogen levels should not vary with the recorded depth of capture. The lifestyle of *S. kaupi* is very different to that of most benthic macrofauna, where individuals are confined to comparatively narrow depth ranges by their inferior mobility and the more gradual bathymetric changes that occur in the horizontal plane. *S. kaupi* moves rapidly in three dimensions and was therefore included as a comparison with the benthic macrofauna, to define the assay capabilities.

Individual *S. kaupi* were obtained specifically for this assay, thus the animals were dissected at the time of capture and the tissue for assay was frozen since preservation was an unnecessary step. Data from two separate collections

have been combined. The first included animals captured in the Rockall Trough area of the north east Atlantic in February/March 1992 (RRS Challenger cruise 89/92) from depths of 2200m, 1200m and 800m (24 animals per depth). The second assay was performed on animals captured in the same area during August 1992 (RRS Challenger cruise 94/92) from depths of 700m, 1200m, 1300m, 1600m and 1625m (10 individuals per depth). The total length of each individual was determined to the nearest 0.5cm and they were cut ventrally from the operculum to the anal opening so that liver and reproductive tissue could be removed. The liver is the site of glycogen storage in vertebrates and was frozen immediately until the glycogen assay could be performed. The sex of each animal was noted and reproductive tissue preserved for future determination of reproductive state. As the liver was frozen rather than preserved, the washing procedure was unnecessary. Tissue glucose, however, had to be measured as an additional assay for subtraction from the total glucose released by the amylase digestion, as explained in section 6.2.

7.5.2 Results and Discussion

Figure 48 shows the liver glycogen levels at each depth for the two combined assays.

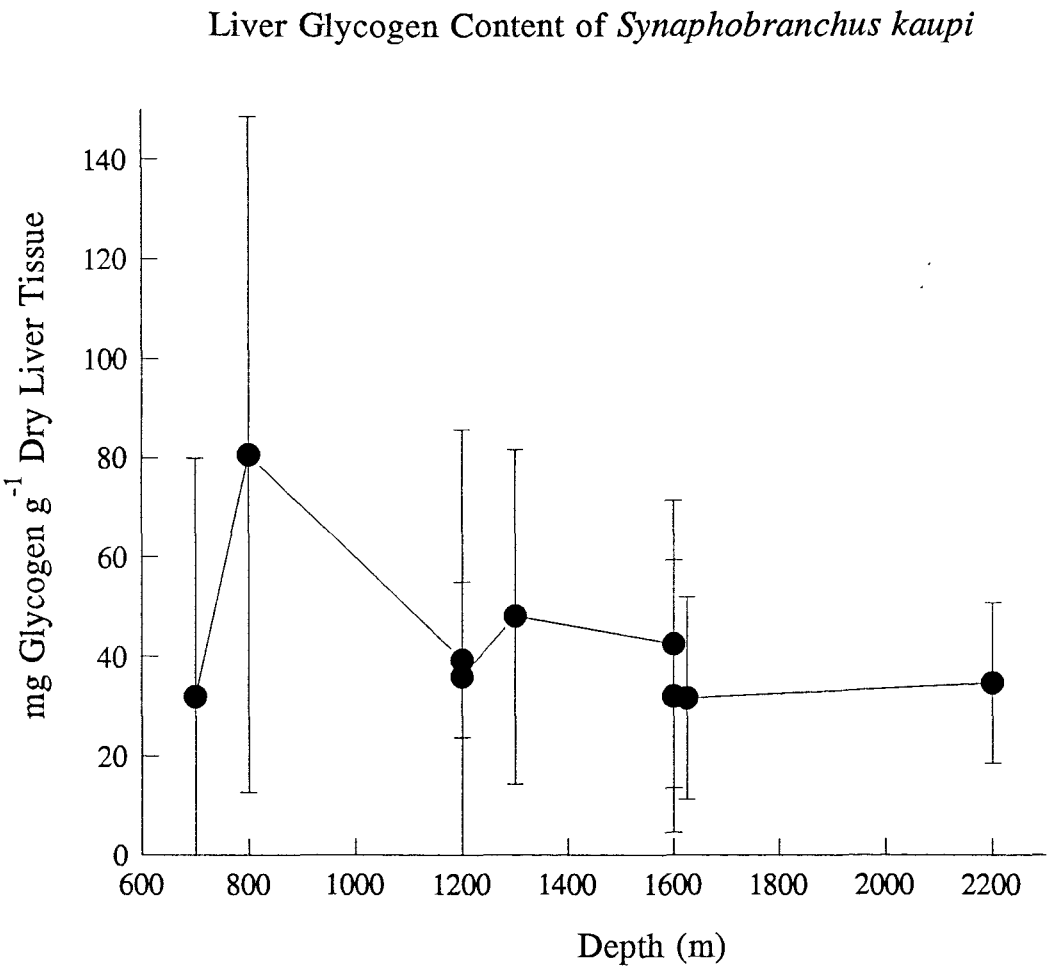


Fig. 48. Mean liver glycogen levels with depth in *Synaphobranchus kaupi*. Error bars indicate standard deviation.

It was noticed during the first assay that the animals from 800m were smaller than those from 1200m and 2200m. A Mann-Witney test was applied and revealed no significant size difference between the 1200m and 2200m samples but the 800m sample was significantly different in size from both of these ($p < 0.001$ in both cases). The occurrence of smaller *S. kaupi* in shallower depths in the Rockall Trough and Porcupine Seabight has also been recorded by Gordon *et al.* (1986). The 1200m and 2200m samples were combined so that the relationship between size and glycogen levels could be investigated (no significant differences in size or glycogen levels existed between the depths). There was a significant linear correlation between length of individual and liver glycogen, smaller animals having proportionately more ($r = 0.385$, $p < 0.007$). The animals at 800m did not show a size/glycogen correlation, but only a narrow range of sizes was present. Despite the relationship between size and liver glycogen, no significant differences in glycogen between the depths existed.

The results of the second assay were included in fig. 48 to extend the range of depths examined. There were again no significant differences between depths either within the assay or between the two assays; seasonal effects were thus negligible.

The hypothesis that *S. kaupi* should not show a depth/glycogen relationship because of its vertical mobility has thus not been disproved.

7.6 Examination of *Stereomastis nana*

7.6.1 Introduction and Method

Preserved specimens of the polychelid decapod *Stereomastis nana* were examined. The samples were again from the Discovery collection, collected in the Porcupine Seabight region between 1978 and 1983. The samples comprehensively covered the depth range of this species between 1500m and 3000m. Other polychelid species existed at different depths in the region but have not yet been examined.

Carapace length was measured as described in the *Glyphocrangon* assay, the animals were sexed, dissected and the hepatopancreas assayed for glycogen content.

7.6.2 Results and Discussion

Figure 49 shows the hepatopancreatic glycogen changes with depth. Individual results have been plotted since many of the samples contained only one or two animals per depth.

There was again a change in the size of animal with depth, the deeper animals tending to be smaller, and this linear correlation ($r = 0.319$) was significant ($p < 0.001$). There was, however, no significant relationship between the size of animal and hepatopancreatic glycogen. The correlation between depth and glycogen shown in fig. 49 proved to be linear ($r = 0.185$) and was significant ($p = 0.028$), glycogen levels decreasing with increasing depth. The hypothesis suggested in section 7.2.3 that low glycogen in decapod hepatopancreas could indicate optimal metabolic conditions may not be true in this case. The low glycogen at depth was not simply the result of the smaller size of the deeper animals since there was no size/glycogen correlation. The smaller size is suggestive of reduced growth which, coupled with the low glycogen, indicates suboptimal conditions.

The bathymetric distribution of this species is not fully known, although the samples used in this assay suggested greatest abundance between 1500m and 2500m. The related species *Polycheles validus* was caught in this area in the same samples between 1800m and 2800m. This distribution overlaps considerably with the deeper *S. nana*, and interspecific interactions may thus have occurred. The lower hepatopancreatic glycogen levels and reduced size of deeper *S. nana* suggest that they were stressed to some degree and it remains to be seen whether the shallower *P. validus* exhibit similar indications.

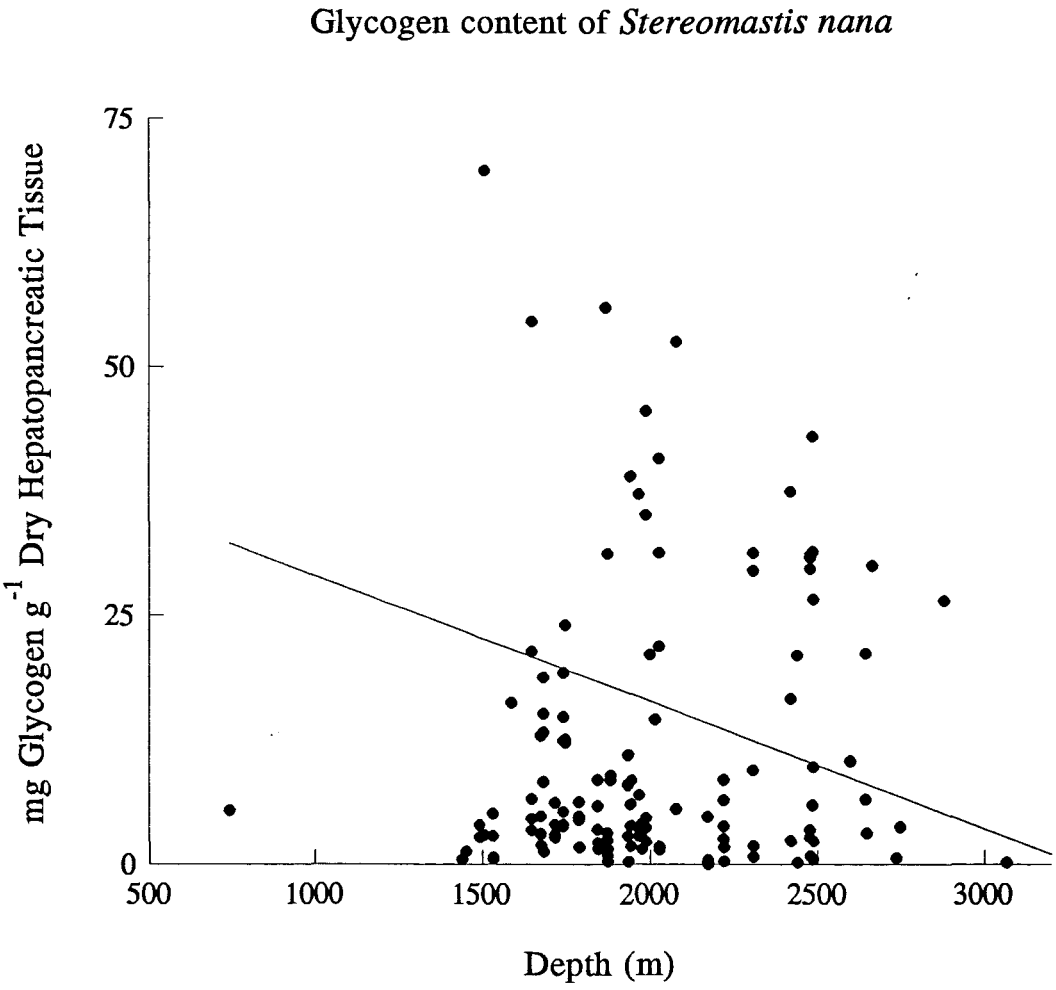


Fig. 49. Hepatopancreatic glycogen levels in each individual *Stereomastis nana* with depth. The correlation between depth and glycogen is indicated by the straight line ($r = 0.185$).

7.7 Examination of *Glycera mimica*

7.7.1 Introduction

The studies to date have dealt with deep-sea benthic macrofauna at the larger end of the size spectrum. The numerically dominant taxa however are often much smaller and whole communities may be composed of organisms such as polychaetes and crustaceans with dimensions smaller than one centimetre. These species are often much less mobile than their larger co-inhabitants and are more susceptible to small scale disturbances, which may even have been generated by the activities of larger macrofauna such as holothurians.

It therefore seemed essential that the method was applied to such organisms, since they may be the most sensitive indicators of environmental disturbance.

The species chosen for investigation was the polychaete *Glycera mimica*, one of the most dominant polychaetes in the Rockall Trough area, with a depth range of at least 400m to 3000m. Preserved specimens from several stations were examined, but the small size of the worms required that the assay procedure be modified.

7.7.2 Methods

The accuracy of the glycogen assay is dependent on accurately determining the weight of the examined tissue. With extremely small samples high accuracy is difficult to obtain using conventional balance apparatus. For this study a Cahn™ microbalance was used, which electromagnetically measures the weight of material only tens of micrograms in mass. Each worm was washed, freeze-dried and weighed. The maximum dry weight of an individual worm was 6.44mg, the minimum was 0.02mg. Between procedures the specimens were kept in a desiccator to avoid re-hydration and the consequent weight gain.

The volume of solutions previously used in the enzymic digestion were clearly too large for this assay since such a dilution of tissue glycogen would prevent accurate determination. The ratio of the component solutions was maintained but the final volume within the incubating vessel was reduced to 105μl (5μl phosphate buffer, 20μl amyloglucosidase and 80μl distilled water, with 10μl of glucose standard replacing part of the water in the standard tube). This volume

allowed the removal of 50μl to a cuvette for the spectrophotometric determination of the glucose. This was the volume removed in the original assay so that this step of the assay remained unaltered.

Each whole individual worm was incubated in a separate tube, and glycogen was determined for the entire worm. Between three and ten individuals were examined per depth.

7.7.3 Results and Discussion

Figure 50 shows the glycogen level changes with depth for whole *G. mimica* and also the abundance of this polychaete, as a percentage of the total polychaete community, at each of the depths (data from Paterson, pers. comm.). There was no significant relationship between size of worm (the size variable was dry weight) and glycogen content at any of the depths. The large variation in glycogen content meant that significant differences were not detectable with a t-test between most of the depths. Exceptions were between the 400m and 1000m samples ($p = 0.004$) and between the 1000m and 1200m samples ($p = 0.011$).

Comparison between the graphs of glycogen content and abundance revealed a degree of agreement. The lowest glycogen, occurring in samples at 1200m, coincided with the lowest percentage abundance. The fauna at this depth had a high diversity (Paterson, pers. comm.) which may explain the low percentage abundance of *G. mimica*. However, this polychaete is capable of high dominance in favourable conditions so the low glycogen may be indicative of environmental unfavourability at this site. The pressure-related hypothesis suggesting low glycogen in optimal situations (section 7.2.3) is unlikely to be true in this case as 1200m is well within the wide depth distribution of *G. mimica*. Although high diversity in deep-sea benthos generally indicates favourable environmental conditions, this may not be specifically applicable in this case to *G. mimica*.

If a prediction had to be made on the relative abundances of *G. mimica* at each depth using only the data from the glycogen graph of fig. 50, it would bear close resemblance to the actual abundances observed. This is particularly notable when the low numbers of individuals examined and their small size are taken into consideration. Such a "model" is speculative only and perhaps the most significant

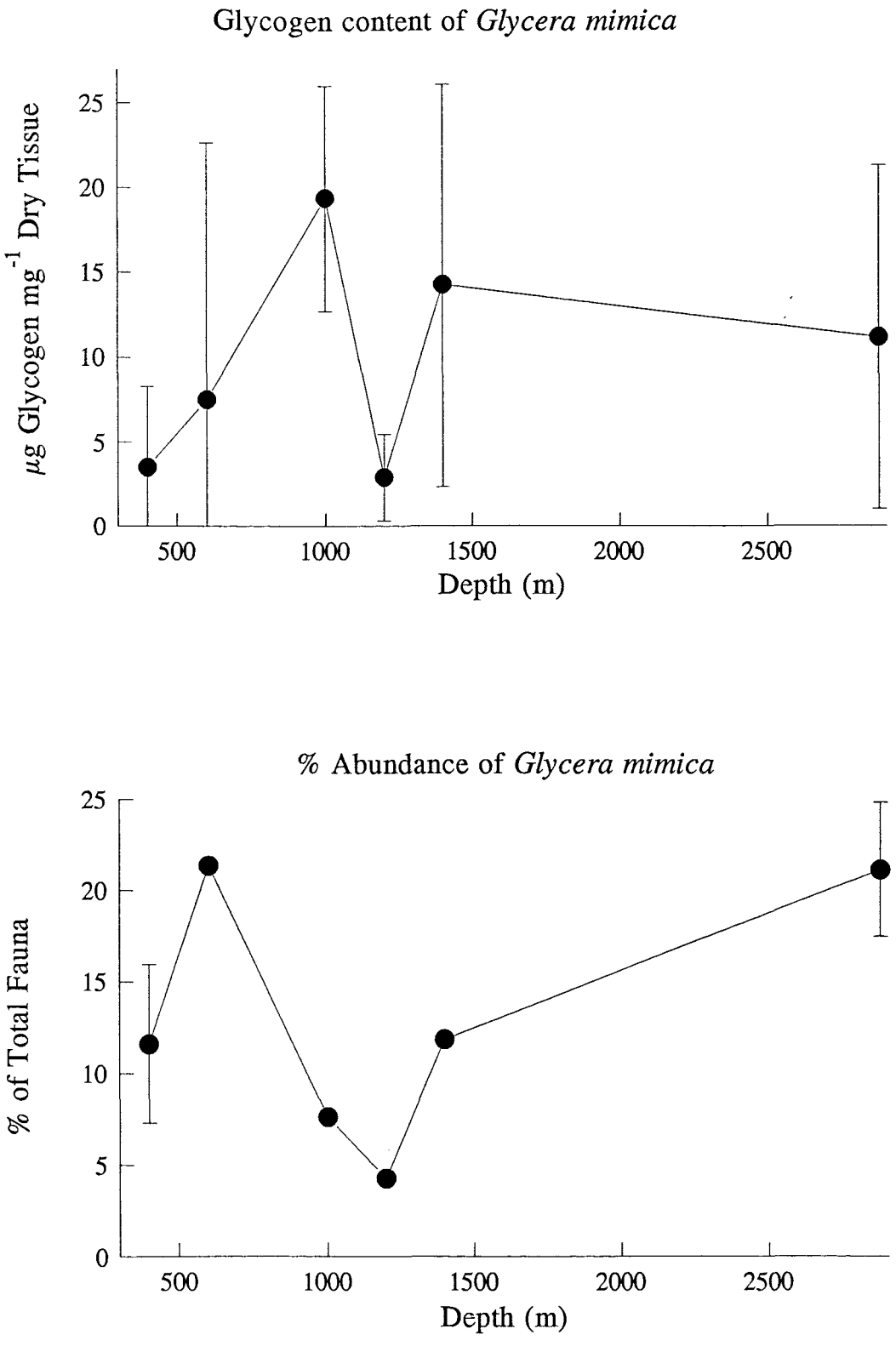


Fig. 50. Mean glycogen and relative abundance changes with depth of *G. mimica*. Error bars indicate standard deviation.

component of this assay was that glycogen could be determined in very small (less than one centimetre in length) individual worms to give ecologically sensible results.

7.8 Chapter Discussion

This chapter has described the preliminary application of the glycogen assay to preserved, archived deep-sea material. The intention was to examine as wide a range of taxa as time permitted, albeit somewhat superficially, in preference to detailed investigations on only one or two taxa to define the extent of applicability of the method. The range of taxa examined covered four phyla, Crustacea, Chordata, Echinodermata and Annelida and the Crustacea examined included three separate assays on closely related decapod species, allowing some comment on the possible generalizations that could be made.

These assays have highlighted the necessity for sufficiently numerous specimens to detect size/glycogen and size/depth relationships so that they can be accounted for in the analysis. Such relationships would appear to be remarkably specific, for example the *Glyphocrangon* specimens showed significantly increased glycogen in smaller individuals, whilst the polychelid decapod *Stereomastis nana* showed no such size/glycogen relationship but exhibited a size/depth correlation. No evidence was found of depth related glycogen changes in any of the *Glyphocrangon* species, *S. nana* showed a linear decline in glycogen with depth, and *Nephropsis atlantica* displayed a nonlinear effect of depth on glycogen levels. Generalizations may thus be possible between species of the same genus (all of the *Glyphocrangon* species showed the same correlations), but assumptions about more distantly related species should not be made.

These studies have generally concentrated on the detection of depth effects, the hypothesis being that animals outside their optimal depth should suffer a degree of stress. Factors such as competition or areas of environmental instability could potentially create depth related glycogen changes as well as changes evident in the horizontal plane. Examples can be found in these assays. The *Glyphocrangon* species showed no pattern in glycogen levels with depth, but each species occupied a distinct depth band with little overlap. *S. nana* did display a depth related decline in glycogen which may have been influenced by its overlap

with a closely related species. *G. mimica* exhibited reduced glycogen at a depth where its relative abundance was known to be low. *Synaphobranchus kaupi*, which moves vertically through the water column, showed no relationship between depth of capture and glycogen. The effects of pressure when existing outside the optimal depth range cannot be ignored. As discussed earlier in this chapter (section 7.2) pressure related changes in enzyme systems of deep sea organisms are known to exist. Such effects presumably define the extreme possible depth distribution of a given species, but may well be evident inside this range should an animal be forced to exploit a region outside its optimal depth range. This may explain the depth related glycogen changes observed in the decapods *N. norvegicus*, *N. atlantica* and *S. nana* and the holthurians *L. violacea* and *O. mutabilis*.

Such reasoning may be an oversimplification, since there are a multitude of unrecorded factors that could have influenced each separate study. The fact remains however, that these studies have provided examples of the ecological information that can potentially be obtained from previously biochemically unexaminable material.

These studies have also enabled the methodological approach to be defined. The limitations and applicability of the method will be discussed in the final chapter.

CHAPTER 8

Summary and Conclusions

The ultimate aim of this study was to develop a biochemical method that could detect the effects of environmental disturbance in preserved deep sea benthic macrofauna. The taxonomic abundance studies currently employed allow the definition of historical population changes, i.e. what has happened, but there is a need for a predictive capability so that future studies can be more suitably defined. Disturbance in the deep-sea will inevitably increase, primarily from anthropogenic sources, therefore monitoring studies will become crucial. The ability to detect detrimental effects before they alter the community structure would seem essential. If such a procedure could be simple and cost-effective it would indubitably be more useful. The ability to analyse preserved material is the most cost-effective option since the expense of obtaining the material has already occurred and the monitoring component could be "back-dated" to the beginning of the preserved archive. This procedure would extract information on past, present and future populations, an unsurpassable ability.

The validation of this proposed aim was initially carried out on readily-accessible littoral sites in the Baie de Somme. It was unforeseen however that the strong hydrodynamic activity in the region would prevent the isolation of specific disturbances, and population changes in the monitored species were minimal. Comparisons between the two sites did however reveal that the biochemical indices were sensitive enough to detect site differences in the relative levels of environmental disturbance. Integration of all of the biochemical measurements and using multivariate statistical analyses revealed the probable environmental factors that structured the observed biochemical patterns (chapter 5). The Baie de Somme exercise demonstrated the undoubtable validity of biochemical techniques in isolating the underlying processes that create community structure.

Once this had been established it was necessary to develop the biochemical techniques for application to preserved, archived material. Although the integration of all of the biochemical components had been informative, the majority of them

were unsuitable for further development, as has been explained in section 6.1. An index for deep-sea animals required a metabolite that would be unaffected by the stresses of collection but which would have responded to sustained environmental disturbances in the animals' habitat. Glycogen was the most suitable representative metabolite of those examined.

Laboratory experiments revealed that tissue glycogen levels were responsive to a single imposed stress, and sensitive to sublethal stress levels.

The assay method applied to fresh, frozen material from the Baie de Somme study was then modified, justification given by the accepted histological techniques of Pearse (1980) for glycogen determination in preserved, sectioned material. The preservation procedure and long term storage had no noticeable effect on tissue glycogen levels.

An archive of preserved material from the Baie de Somme covering the decade prior to the present study was then used to test the preserved glycogen methodology. Unfortunately the time series was too temporally dispersed to allow precise correlation with population data. The results were suggestive, however, of a predictive capability in the glycogen assay, tissue glycogen levels were seen to fall prior to population declines. Despite the insufficiencies of the archive data set it was apparent that the technique could be applied to archive material.

The Baie de Somme validation exercise also revealed the applicability of biochemical studies to ecological investigations of shallow water environments. Intensive examination of representative species of a particular habitat, such as *C. edule* and *N. diversicolor* in the Baie de Somme, would contribute to environmental monitoring. The biochemical measurements have been shown to be extremely sensitive to environmental disturbance, with implications for pollutant monitoring studies.

Preliminary application of the technique to deep-sea specimens has been described in chapter 7. The assays were designed to provide methodological definition and suggestions as to the subsequent studies that should be undertaken.

It was proposed in chapters 5 and 6 that a multivariate approach, combining different biochemical indices sensitive to different time-scales of disturbance, would be the most informative way to compare samples that had experienced differing degrees of disturbance. An identical approach could not be

performed on preserved material, but it may be possible to make supportive measurements of other stress-affected components. Histology would figure prominently in such measurements. The brief histological investigation of the *Nephrops* and *Nephropsis* samples (section 7.2) revealed structural differences in the hepatopancreatic tubules and variations in the ratios of storage components. Quantitative assessment of such observations could be developed, for example direct measurements of cell or tubule dimensions. Measurements have been made on the digestive gland of *Mercenaria mercenaria* exposed to hydrocarbons (Tripp and Fries, 1987) revealing detectable changes in tubule dimensions.

Stored lipids are removed from tissues by the preservation procedures, however the vacuoles in which they were stored remain visible in histologically prepared material. A quantitative measurement of lipid storage could be gained from the vacuolar percentage composition of the cell. A ratio between this value and glycogen measurements could indicate changes in the ratio of stored components. The qualitative observations on the *Nephrops* and *Nephropsis* material suggested correlations between the stored lipid:glycogen ratio, tubule morphology and depth distribution.

Assessment of reproductive state would also be a useful contributory measurement, either to help explain particular glycogen levels or as a separate indicator of environmental disturbance, since reproductive output may be affected. Correlations between glycogen measurements and reproductive state could assist in the determination of the seasonal storage cycles of certain species. This has implications for extending the existing knowledge on the seasonal breeding patterns of some echinoderm species which have attracted a great deal of recent interest (Gage and Tyler, 1991).

Determination of the sex of examined individuals should be carried out. No differences were found between the sexes in any of the studies described in chapter 7 (where sex was noted) but it cannot be assumed that this would be true for all species. Indeed, many species show sexually dimorphic cycles so that glycogen levels might be expected to differ seasonally between the sexes.

Morphological measurements should also be made. There was some evidence in the *Stereomastis nana* study (section 7.6) that the deeper dwelling, glycogen poor individuals were reduced in size, suggesting lower growth

efficiency. In both the *Glyphocrangon* (section 7.4) and *Synaphobranchus kaupi* (section 7.5) studies, significant correlations between size and glycogen content were noted. Size measurements should be included if only so that such relationships can be accounted for in the analysis. Each of the above supportive measurements are insufficient in themselves to indicate disturbance effects, but taken as a whole they may provide irrefutable evidence of detrimental conditions.

When attempting to isolate the effects of disturbance it is important to have as few variables as possible. Seasonal cycles and depth, for example, may both influence tissue glycogen levels; therefore one or the other variable should be kept constant, or a sufficiently large data set examined so that their effects can be accounted for. If the material is collected specifically for this assay then such a step is easy. The *S. kaupi* study, for example, used individuals collected on only two occasions, therefore the seasonal changes (had any existed) were simple to observe and account for.

Any measurements on biological material incur the problem of individual variation. Sufficient numbers must be examined to provide statistical validity, and paucity of specimens is probably the largest potential problem with deep-sea studies. Sampling difficulties and the low numbers of individuals of some species in the deep-sea reduce the availability of specimens. The extraction of small infaunal species of polychaete and crustacea relies on time-consuming sieving and sorting procedures. The ability to use preserved animals does allow all available samples to be analysed as one data set, thus maximising the available information.

Variation between individuals may be particularly large in certain taxa. The rapid decline in the hepatopancreatic glycogen of starved decapods, mentioned in section 7.2, is further complicated. There is an increase to well above normal levels once feeding starts and this is maintained for a few days before another sudden drop (Williams and Lutz, 1975). Such natural phenomena could confuse disturbance signals in glycogen levels, particularly in less known species where these phenomena are as yet unrecorded.

The *Glycera mimica* study (section 7.7) demonstrated that the lower size limit of the assay capability was as small as could be accurately weighed. Glycogen levels in individual polychaetes can be determined, although the weighing procedure may be time-consuming. It would be possible to combine

individuals and assay several worms as one sample, to increase the volume of material, if sufficient numbers of individuals have been collected. The mean glycogen level with depth has usually been plotted, so combining individuals in this way would not reduce the available information as long as sufficiently large numbers existed for replication.

Preliminary deep-sea studies have now been performed, the next step therefore should logically be an intensive study of one particular set of samples. Ideally this should incorporate investigations of several different taxa, from the small, numerous polychaetes to the larger echinoderms and crustaceans in a region where disturbance is known to have occurred so that the susceptible organisms can be identified. The investigation should include as many of the supportive measurements suggested previously as possible.

A data set as comprehensive as this, covering depth, time and numerous taxa may be difficult to obtain but it would be the ultimate validation exercise. Collaboration between institutions with comparable material may provide such data.

Our present knowledge of deep-sea communities and structuring processes is so slight that any method which can elicit information from animals that have already been captured and preserved can only be of great value.

APPENDIX 1

Composition of Reagents Used in Biochemical Procedures

(i) Lipid

Chloroform-methanol solvent; 2 pts chloroform: 1 pt methanol

KCl; 0.05 M

(ii) Carbohydrate

Phenol; 5% w/v

Sulphuric acid reagent; 5g L^{-1} Hydrazine sulphate in conc. H_2SO_4

(iii) Protein

NaOH; 0.1 M

Reagent mixture, $CuSO_4$; 1% w/v

NaK tartrate; 2% w/v

Na_2CO_3; 20g L^{-1}

Folin and Ciocalteau Reagent; 1 pt reagent: 1pt dist. H_2O

(diluted immediately before use)

Stock standard solution of Bovine serum albumen fraction V; 100 mg protein

in 100 cm^{-3} 0.9% w/v KCl

Note: Unless stated otherwise, all reagents for the following enzymatic procedures stored at 2-4°C.

(iv) Adenylates

ATP buffer; 71.25 cm^{-3} 0.05 M triethanolamine hydrochloride (TEA)

(stock 0.5 M,adjusted to pH 7.6 with NaOH and diluted to 0.05 M before use)

0.75 cm^{-3} 0.5 M $MgCl_2.6H_2O$

0.75 cm^{-3} 20 mM NADP+ (aqueous solution)

(stored at -20°C and added just before use)

G6PDH; 0.6 mg cm^{-3} (from yeast, grade II)

5 mg cm^{-3} suspension in 3.2 M $(NH_4)_2SO_4$

Glucose; 0.4 M D-glucose (stored at -20°C)

HK; 2.0 mg cm^{-3} (from yeast, grade II)

10 mg cm^{-3} suspension in 3.2 M $(NH_4)_2SO_4$

ADP-AMP buffer; 65 cm^{-3} TEA (as ATP buffer)

0.88 cm^{-3} 0.5 M $MgSO_4.7H_2O$

1.88 cm^{-3} 2 M KCl

0.125 cm^{-3} 10% w/v solution (saturated)

Ethylenediaminetetracetic acid (EDTA)

0.75 cm^{-3} 10 mM NADH-Na_2 (diluted with 5% w/v $NaHCO_3$)

1 cm^{-3} 40 mM phosphoenolpyruvate, monosodium salt

(PEP) (aqueous sol.)

0.25 cm^{-3} $ATPNa_2H_2$ (aqueous sol.)

NOTE: the latter 3 solutions stored in one vial at -20°C

and added just prior to use.

LDH; 1 mg cm^{-3} (from rabbit muscle)

10 mg cm^{-3} suspension in 3.2 M $(NH_4)_2SO_4$

PK; 2 mg cm^{-3} (from rabbit muscle)

10 mg cm^{-3} suspension in 3.2 M $(NH_4)_2SO_4$

MK; 1.25 mg cm^{-3} (from rabbit muscle)

5 mg cm^{-3} suspension in 3.2 M $(NH_4)_2SO_4$

(v) Glucose and Glycogen

ATP; 0.1 M $ATPNa_2H_2$ (aqueous sol.)(stored at -20°C)

Phosphate buffer; 0.2 M $NaH_2PO_4.2H_2O$ + 0.1 M $Na_2HPO_4.2H_2O$

(adjusted to pH 7 by addition of one or the other solutions)

Amyloglucosidase; (1000 E.U cm^{-3}), 1 mg made up in solution containing

2.3 cm^{-3} 0.1 M citric acid + 2.7 cm^{-3} 0.1 M (tri)sodium citrate (pH 4.8)

Glycogen standard; 1 mg cm^{-3} oyster glycogen (aqueous sol.)

(stored at -20°C)

(vi) Arginine- and Creatine phosphate

ODH; (from scallops) 100 E.U cm^{-3} in 50:50 glycerol:H_2O

HCl; 0.5 M

NaOH; 1 M

ADP; 63 mM ADP-Na_2 (aqueous sol.) (stored at -20°C)

CP; 5 mg cm^{-3} (aqueous solution)

(vii) Succinate

Succinate buffer; 1 cm^{-3} glycylglycine buffer;
2.4 g glycylglycine + 0.6 g $MgSO_4.7H_2O$ in 50 cm^{-3} H_2O,
adjusted to pH 8.4 with 2 M NaOH and made up to 60 cm^{-3} with H_2O
0.1 cm^{-3} 9 mM β-NADH (23 mg NADH-Na_2 + 30 mg $NaHCO_3$ in 3 cm^{-3} H_2O)
0.1 cm^{-3} coenzyme mixture (30 mg coenzyme A +
30 mg inosine 5'-triphosphate + 30 mg PEP in 3 cm^{-3} H_2O)

NOTE: These solutions stored in one vial at -20°C

SK; (from pig heart) 10 E.U cm^{-3} suspension in 3.2 M $(NH_4)_2SO_4$

(viii) Lactate

Lactate buffer; 0.67 cm^{-3} Glutamate buffer; 20 cm^{-3} neutral glutamate sol.
(56.75 g L-(+)- glutamic acid in 380 cm^{-3} 1 M NaOH,
made up to 500 cm^{-3} with H_2O) + 9.4 cm^{-3} 1 M NaOH
0.1 cm^{-3} 24 mM β-NAD (aqueous sol.)

GPT; (from pig heart) as supplied suspension

(ix) Pyruvate

Pyruvate buffer; 3.9 cm^{-3} ADP-AMP buffer (before addition of NADH, PEP and ATP)
0.12 cm^{-3} NADH (10 mg NADH in 2 cm^{-3} 5% $NaHCO_3$)

APPENDIX 2

Results of Sediment Particle Size Analysis

Table 14. LCS surface 1 cm layer.

ϕ	% Each Fraction per Month								
	9-90	12-90	3-91	6-91	9-91	12-91	3-92	6-92	9-92
0+	0.076	0.018	0.103	0.114	0.060	0.068	0.172	1.417	2.109
0.5	0.060	0.073	0.036	0.122	0.160	0.031	0.088	0.076	0.188
1.0	0.252	0.075	0.060	0.191	0.264	0.076	0.103	0.163	0.228
1.5	0.423	0.354	0.081	0.308	0.663	0.259	0.224	0.196	0.255
2.0	3.160	6.958	2.096	4.406	5.262	8.234	4.181	2.911	4.856
2.5	25.71	40.54	31.84	26.40	31.16	45.64	33.26	23.70	29.08
3.0	56.70	46.34	54.94	48.86	51.17	39.99	51.65	50.28	44.03
3.5	9.259	3.672	5.680	11.63	7.891	3.221	7.202	14.09	12.10
4.0	1.430	0.805	1.419	3.031	1.764	0.483	1.349	3.106	2.309
4.5+	2.923	1.169	3.746	4.939	1.611	2.002	1.771	4.065	4.848

Table 15. LCS surface 10 cm layer.

ϕ	% Each Fraction per Month								
	9-90	12-90	3-91	6-91	9-91	12-91	3-92	6-92	9-92
0+	0.057		0.109	0.973	1.554	0.539	1.733	0.405	1.172
0.5	0.007		0.029	0.072	0.038	0.059	0.069	0.036	0.116
1.0	0.014		0.058	0.102	0.079	0.087	0.079	0.065	0.123
1.5	0.080		0.148	0.226	0.239	0.207	0.155	0.116	0.259
2.0	2.384		2.754	4.439	4.385	4.922	2.931	1.979	4.999
2.5	22.34		29.71	27.29	31.08	33.15	26.08	16.53	32.83
3.0	60.20		54.75	51.14	45.51	44.07	50.52	45.08	43.82
3.5	10.15		6.388	9.224	10.79	8.515	12.08	20.55	9.753
4.0	1.597		1.612	2.410	2.303	2.673	2.782	6.594	2.694
4.5+	3.174		4.438	4.133	4.016	5.781	3.575	8.645	4.238

Table 16. HHS surface 1 cm layer.

ϕ	% Each Fraction per Month								
	9-90	12-90	3-91	6-91	9-91	12-91	3-92	6-92	9-92
0+	2.781	0.529	0.056		0.005	0.000	0.004	0.061	11.71
0.5	0.063	0.118	0.039		0.004	0.014	0.011	0.023	1.969
1.0	0.052	0.111	0.076		0.022	0.013	0.022	0.023	1.023
1.5	0.092	0.109	0.158		0.072	0.028	0.071	0.033	0.472
2.0	1.856	1.558	2.924		1.330	0.501	1.303	0.468	0.894
2.5	39.67	33.95	30.53		32.83	17.80	26.06	10.53	2.925
3.0	51.81	58.58	54.91		56.27	69.92	51.81	56.91	16.10
3.5	1.675	3.753	8.873		6.459	8.590	10.65	22.23	20.65
4.0	0.251	0.562	1.680		1.256	1.824	4.313	5.370	12.14
4.5+	1.752	0.730	0.759		1.759	1.314	5.754	4.354	32.12

Table 17. HHS surface 10 cm layer.

ϕ	% Each Fraction per Month								
	9-90	12-90	3-91	6-91	9-91	12-91	3-92	6-92	9-92
0+	0.627	4.265	0.042		2.285	0.066	0.059	0.730	0.473
0.5	0.078	0.035	0.004		0.031	0.037	0.015	0.065	0.044
1.0	0.102	0.048	0.015		0.035	0.053	0.067	0.088	0.074
1.5	0.188	0.083	0.037		0.076	0.094	0.118	0.247	0.100
2.0	2.072	0.999	0.748		0.905	0.780	1.208	0.801	0.709
2.5	32.58	20.74	26.80		21.87	12.28	22.14	10.50	10.18
3.0	57.60	49.62	58.24		60.68	55.44	54.89	54.58	57.13
3.5	3.767	11.73	7.783		8.750	19.30	12.22	20.77	20.01
4.0	0.608	3.438	2.223		2.150	5.816	3.620	5.238	5.443
4.5+	2.375	9.041	4.111		3.225	6.136	5.662	6.977	5.835

APPENDIX 3

Results of Principal Components Analysis

Table 18. Principal Component values for *C. edule* at LCS.

Date	Principal Component			
	1	2	3	4
12-90	-1.9322	-0.3530	-0.3211	0.3657
1-91	-1.3173	1.8807	-1.9505	-1.0832
2-91	-1.1423	0.6749	-2.3772	0.4985
3-91	-1.2894	0.0084	-1.8007	-0.0844
4-91	-2.6990	-0.1620	0.5472	-1.4532
5-91	-1.6507	-2.6379	1.2413	1.9425
6-91	0.0405	-4.0722	0.2404	-0.9626
8-91	-1.9299	-0.5917	-0.2432	-0.1015
9-91	0.1587	0.9342	3.2697	0.0100
11-91	0.7622	2.2771	1.8550	-1.0526
1-92	4.4654	-1.8836	0.0661	-1.5413
2-92	2.7815	1.2757	-0.8412	1.5686
4-92	3.1995	-0.2236	-1.7725	0.4141
6-92	0.4677	0.6742	1.1108	1.9267
9-92	0.0853	2.1988	0.9760	-0.4474

Table 19. Principal Component values for *C. edule* at HHS.

Date	Principal Component			
	1	2	3	4
12-90	-3.1449	1.1347	0.4405	-0.0540
1-91	-0.1333	-1.2536	-0.8874	-0.0803
2-91	-0.0667	-2.7479	-0.4612	-0.2180
3-91	-1.6823	-0.9663	-1.9536	0.9695
5-91	-3.5131	1.4439	2.4098	-0.0028
7-91	0.5146	-1.4397	-0.4902	-1.0449
8-91	-3.2785	-0.5226	0.4628	-0.1323
9-91	2.2826	-3.6205	2.7396	0.1568
10-91	2.1684	-1.6007	0.4135	3.1179
1-92	1.7431	-0.3281	0.0630	-1.5598
3-92	1.3842	0.3880	-1.2720	0.09297
5-92	0.8861	0.3667	-0.0034	0.1686
7-92	2.9046	3.0014	0.8735	-1.0910
9-92	-0.0649	0.0639	-2.3349	-0.3224

Table 20. Principal Component values for *N. diversicolor* at LCS.

Date	Principal Component			
	1	2	3	4
1-91	2.7603	2.3988	-1.1185	-0.1547
2-91	-2.8411	-1.8153	-0.4338	-0.7225
3-91	-2.1069	-0.5581	-0.2301	-2.0913
4-91	1.5235	0.1396	-0.9258	-0.1124
5-91	3.9224	-1.9697	1.0721	-1.8140
6-91	2.2693	-3.0780	0.4393	1.2075
8-91	-0.2022	-1.1822	-0.6793	1.2534
9-91	-2.2671	-0.1061	-0.5743	0.0150
10-91	-0.3072	-1.1987	0.3428	-0.1398
11-91	0.5336	1.3969	-0.1088	1.0169
12-91	0.01104	2.8310	0.4969	-0.9628
2-92	-1.6902	0.6831	3.2854	1.1325
4-92	0.2553	2.0853	0.7140	-0.3242
6-92	-0.1276	0.1951	-1.2017	1.4395
8-92	0.04725	0.4831	-0.2218	0.4731
9-92	-1.7804	-0.3048	-0.8565	-0.2163

Table 21. Principal Component values for *N. diversicolor* at HHS.

Date	Principal Component			
	1	2	3	4
1-91	4.5137	1.1265	-0.4760	-0.6384
2-91	-1.3248	-1.1162	-1.1189	-0.7676
3-91	-0.5568	-2.2064	-2.0938	0.9115
5-91	1.6518	-3.4906	1.7206	1.0375
7-91	1.4332	0.0067	-0.0296	-1.0742
10-91	-1.2419	0.3295	1.4033	-0.3024
12-91	-1.6558	-0.4603	-0.1672	-0.5595
1-92	-0.8676	1.5623	2.0721	0.9936
3-92	-1.2926	0.1798	-0.3878	-0.7200
5-92	-0.7726	1.4255	0.4876	-0.7899
9-92	0.1136	2.6433	-1.4104	1.9095

Table 22. Eigenvectors of metabolites for significantly correlated principal components in *C. edule* at LCS.

Metabolite	Principal Component			
	1	2	3	4
Protein	+0.3886	-0.0800	+0.0768	+0.2623
Carbohydrate	-0.1549	-0.1455	+0.2095	+0.6469
Lipid	-0.1544	+0.0600	-0.4566	-0.1056
ATP	+0.2136	+0.3920	-0.0577	+0.4207
ADP	+0.3392	-0.1870	-0.3565	+0.0595
AMP	+0.2394	-0.4561	-0.0900	-0.0035
Total Adens.	+0.3962	-0.0210	-0.2397	+0.3009
AEC	-0.0473	+0.5332	+0.1300	+0.0603
Arg. Phos.	+0.3000	+0.2880	+0.2818	-0.2269
Glucose	-0.0794	-0.3686	+0.1950	-0.0655
Glycogen	+0.0670	-0.0849	+0.5254	+0.1514
Succinate	+0.3789	+0.0235	+0.2507	-0.3611
Lactate	+0.4180	+0.0101	+0.0180	-0.1321
Pyruvate	+0.0696	+0.2464	-0.2685	+0.0164

Table 23. Eigenvectors of metabolites for significantly correlated principal components in *C. edule* at HHS.

Metabolite	Principal Component			
	1	2	3	4
Protein			+0.2975	+0.0588
Carbohydrate			+0.4697	-0.2612
Lipid			+0.1457	+0.1222
ATP			-0.0959	-0.1904
ADP			-0.1898	-0.0431
AMP			+0.3808	-0.0117
Total Adens.			-0.0278	-0.1474
AEC			-0.0702	-0.1615
Arg. Phos.			+0.0940	+0.1371
Glucose			+0.4052	+0.0100
Glycogen			+0.4786	-0.1483
Succinate			+0.1384	+0.2338
Lactate			+0.1622	-0.3412
Pyruvate			+0.1619	+0.7844

Table 24. Eigenvectors of metabolites for significantly correlated principal components in *N. diversicolor* at LCS.

Metabolite	Principal Component			
	1	2	3	4
Protein		+0.2042	+0.4763	+0.3228
Carbohydrate		-0.0139	-0.2456	+0.4607
Lipid		+0.0055	+0.1667	-0.4777
ATP		+0.451	+0.0257	-0.3048
ADP		+0.4372	-0.2875	+0.2189
AMP		-0.2923	+0.0824	+0.1053
Total Adens.		+0.2175	-0.0911	+0.0659
AEC		+0.5464	+0.0291	-0.1462
Glucose		-0.3252	+0.1948	-0.2583
Glycogen		-0.1212	-0.2229	+0.3196
Cr. Phos.		+0.1089	+0.7058	+0.3300

Table 25. Eigenvectors of metabolites for significantly correlated principal components in *N. diversicolor* at HHS.

Metabolite	Principal Component			
	1	2	3	4
Protein		+0.4262		
Carbohydrate		+0.1549		
Lipid		-0.2307		
ATP		+0.3732		
ADP		+0.2922		
AMP		-0.0789		
Total Adens.		+0.2206		
AEC		+0.4960		
Glucose		-0.3805		
Glycogen		-0.1170		
Cr. Phos.		+0.2389		

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