New Biological Methods for Assessing Episodic Pollution

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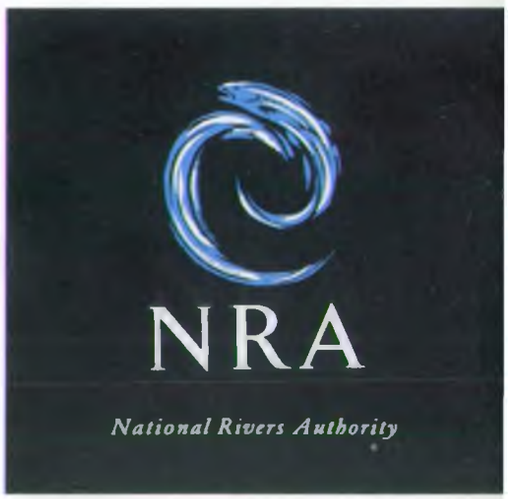

NEW BIOLOGICAL METHODS FOR ASSESSING THE EFFECTS OF POLLUTION

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NEW BIOLOGICAL METHODS FOR ASSESSING THE EFFECTS OF POLLUTION

I Johnson, M Crane, L Maltby, A C Garrood, M Beverley and B D Roddie

EXECUTIVE SUMMARY

Bioassays can provide sensitive and cost-effective methods of monitoring aquatic pollution, particularly where traditional chemically-based assessments are not adequate. This report describes work currently being carried out in four main areas with a view to developing reliable and applicable bioassays for fresh, estuarine and marine waters.

The development of in situ bioassays for fresh water quality assessment is based on pollutant-induced effects on the physiological scope for growth (SFG) index in Gammarus pulex and the biochemical index of glutathione-s-transferase (GST) induction in Sphaerium corneum. Feeding rate has been identified as the important component of the scope for growth assay, and in field deployments this parameter identified differences in water quality above and below discharges. Induction of the enzyme GST, which has a key role in the detoxification of pollutants, has been measured in response to physical stress and pollutant (organochlorine compounds and zinc) exposure. In addition, these approaches appear to have potential to indicate prior exposure to episodic events.

The studies on enhancing the applicability of the marine oyster embryo toxicity test through the cryopreservation of oyster gametes and developing toxicity tests for estuarine pollution using copepod species are currently in the developmental stages. However, as with the freshwater approaches, these bioassays should ultimately provide the National River Authority with robust, sensitive and reliable bioassays which can be routinely applied to assessing fresh, estuarine and marine pollution.

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GENERAL INTRODUCTION

Fresh, estuarine and marine waters are subjected to a wide range of chronic and episodic polluting discharges. Traditionally, discharges have been monitored and regulated on the basis of effluent chemistry. However, biological methods providing toxicity-based assessments have become increasingly widely used and sophisticated in recent years (Cairns and Pratt 1987; OECD 1984 and 1986). Regulatory authorities are finding that a purely chemistry based approach to pollution control does not prevent damage to the aquatic environment. The use of *in situ* bioassays has a number of advantages over traditional chemical methods (Hunt 1989; Wall and Hammer 1989). These include a greater direct relevance to the protection of aquatic communities, independence of cost or technical limitations on the analysis of effluents and the ability to measure directly the toxicological effects of effluents of complex and varying composition. The response of a test organism to a complex effluent reflects the combined toxic effects of all components (whether these are synergistic, additive, independent or antagonistic), which allows regulators to apply consent conditions or whole effluents, without the need to consent each of the components independently. Mackay *et al* (1989) have concluded that bioassays can provide a sensitive and cost-effective method of monitoring and controlling effluent discharges, though additional work is needed on their development and standardisation before they are routinely applied in field situations.

This report describes work currently being carried out in four main areas, with a view to developing reliable and applicable bioassays for fresh, estuarine and marine waters. The experimental approaches under investigation include:

a) an *in situ* physiological bioassay for river quality assessment based on pollutant-induced effects on 'scope for growth' (SFG) of *Gammarus pulex*. A validation study of this established technique in field and laboratory situations is being conducted to assess likely sources of

variation in the feeding and respiration rates, which are needed to calculate SFG;

b) an *in situ* biochemical bioassay for monitoring freshwater pollution based on induction of the enzyme glutathione-s-transferase (GST) in *Sphaerium corneum* during pollution exposure. This ubiquitous enzyme system has a major role in the detoxification process of toxicants and the development of a reliable, sensitive and reproducible test has been investigated;

c) studies on the culture of the estuarine copepod *Eurytemora affinis* and marine zooplankton *Acartia tonsa* as candidate species for the routine toxicity testing of estuarine waters. At present the toxicity of discharges to the estuarine environment is generally estimated using non-estuarine organisms in non-estuarine media, where the effects of important modifying factors, such as conservative major ions (Na^+, Cl^-) and other non-conservative components (CO_3^{2-}, HCO_3^-) are not considered. Consequently there is a need to identify appropriate native euryhaline estuarine species for assessing pollution;

d) development of the oyster (*Crasssostrea gigas*) embryo test to expand its applicability for assessing the impact of estuarine and marine pollution. This rapid and sensitive test is widely applied in monitoring the toxicity of water and sediments, and the cryopreservation of the gametes would widen access to the test on a routine basis. The maintenance of frozen gametes will remove potential problems associated with obtaining stocks at certain times of the year, even though there are nov increasing number of dealers able to supply conditioned oysters throughout the year. Furthermore given batches of preserved gametes to be used in tests can be initially characterised in terms of fertilisation success and sensitivity to pollutants.

The bioassays are at varying stages of development, with the feeding rate and GST studies being considerably more advanced than the

copepod and oyster gamete investigations. However all these approaches have the common ultimate aim of providing the National Rivers Authority with robust, sensitive and reliable bioassays which can routinely be applied to monitoring water quality in fresh, estuarine and marine waters.

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SECTION A - A REPORT ON A SERIES OF FIELD TRIALS TO TEST THE *GAMMARUS PULEX* SCOPE FOR GROWTH TECHNIQUE

Mark Crane and Lorraine Maltby

SUMMARY

There is a need for a rapid, simple, sensitive and cost-effective *in situ* bioassay for use by biologists when monitoring the effects of continuous and episodic discharges on receiving water quality. Previous laboratory investigations have shown the potential of 'Scope for Growth' (SFG) and feeding tests with the freshwater amphipod *Gammarus pulex* in fulfilling such a rôle.

This report forms part of a three year programme to develop new biological procedures for assessing the impact of episodic pollution on water and sediment quality. It describes progress in the validation of *G. pulex* SFG and feeding tests in both the field and laboratory situations. Five field sites, representing a geographically and compositionally diverse range of test environments, were selected. The sites received discharges from, respectively, a domestic sewage treatment works, a sewage treatment works processing metallic waste from local industry in addition to domestic sewage, a quarry, a pulp mill and a fish farm.

The main conclusion of the study is that *Gammarus* feeding rate can provide a sensitive indication of changes in water quality between stations upstream and downstream of several different types of effluent discharges. Although there was considerable variation within treatment groups (mean coefficient of variation 55-113%) this was not significantly higher than in many other 'relevant' bioassays. A number of possible ways for reducing this variability are suggested including the development of an artificial, and hence more controllable, food material.

As part of this exercise both inter-population and inter-laboratory variation was investigated. This was considered necessary in order to assess the transferability of the technique between laboratories and its dependence upon the source of the test animals. Obviously it would be impractical for all laboratories to obtain their animals from the same source. It is therefore important to assess how much variation in response there would be between animals from different sites.

During this study two populations of G. pulex were compared, one from Derbyshire and the other from Oxfordshire. Differences in response between these two populations were observed, although these were probably due to the fact that the Oxfordshire animals came from a stream that was contaminated with heavy metals. The sensitivity of this technique is therefore dependent on the source of the test animals used and more work is needed to investigate the extent of this variation. This could be achieved by measuring the response of animals from several 'clean' sites, covering a range of geographical areas, to a number of toxicants.

The second source of variation investigated was inter-laboratory. For some of the trials, the two laboratories (WRc and Sheffield University) measured different levels of response. However, the reasons for this are unclear. Although such variability is unsatisfactory and efforts should be made to reduce it, it did not affect the interpretation of the results. None of the 5 trials resulted in a significant station by laboratory interaction when the response of animals deployed upstream and downstream from a discharge was compared.

At the end of each field trial, the feeding rate of a sample of the test animals was measured for a further 6 days under standard laboratory conditions. The objective of this exercise was to determine whether or not the differences in feeding rate observed in the field persisted in the laboratory. No persistence of field effects were detected in any of the trials. This could either be because reduction in feeding rate is a behavioural response and not a physiological one, or because the loss of accumulated toxicants is rapid. The length of time that effects persist

has implications for the usefulness of the technique in assessing episodic pollution. Further investigations into contaminant accumulation and loss in G. pulex and the effects that these have on feeding rates are therefore of major importance for the development of this test.

Feeding rate is only one component of the SFG assay, the others being the determination of respiration rate and SFG itself. The measurement of respiration rate and subsequent calculation of SFG were not found to be cost-effective or particularly useful and it is recommended that feeding rate alone be used as an ecologically relevant, relative measure of water quality in field and laboratory tests.

SECTION A1 - INTRODUCTION

This report forms part of a three year programme to develop new bioassays for assessing the impact of episodic pollution on water and sediment quality and is a continuation and amplification of the work by Maltby and Naylor (Sheffield University) for WRc Contract No CS 4323 RX. In that contract laboratory techniques were developed to assess the 'Scope for Growth' of Gammarus pulex under both laboratory and field conditions with the aim of eventually employing the technique as a method for assessing episodic pollution.

The term 'Scope for Growth' (SFG) refers to the amount of energy available to an individual for growth and reproduction after its metabolic needs have been met (Equation 1; Warren and Davis 1967).

$$P = C - F - U - R \qquad \text{Equation 1}$$

where P = Scope for growth
C = Energy consumed
F = Energy lost as faeces
U = Energy excreted
R = Energy metabolised

Maltby and Naylor (1989) showed that a number of parameters could significantly reduce the SFG of G. pulex, including low concentrations of dichloroaniline, zinc and ammonia. In three field tests, they found a significant depression in SFG at sites downstream from effluent discharges.

To investigate further the utility of the G. pulex SFG technique in detecting toxicants in the field, five sites were selected for trials with the technique in the spring, summer and autumn of 1989. The seven main objectives were:

1. to investigate the response of G. pulex SFG to different stressors in the field;

2. to investigate the underlying variation in the SFG of stressed and unstressed G. pulex in the field;

3. to compare differences in the sub-lethal stress response in the field of G. pulex from two different populations;

4. to compare differences in measurement between the laboratories involved in the exercise, ie WRc and Sheffield University;

5. to compare the SFG of G. pulex in the field with the invertebrate community at each site;

6. to compare the SFG of G. pulex in the field with the measurement of ambient concentrations of toxicants;

7. to investigate the persistence of the effect of field stressors on the feeding of G. pulex.

These objectives were chosen to provide a solid foundation for a decision to recommend or reject all or part of the G. pulex SFG technique as a routine in situ bioassay for water industry biologists. Continuous discharges were chosen because it was necessary to test the technique under relatively constant conditions before investigating the effect of pollution episodes. The analysis of within-treatment variation was important for determining the optimum sample size, statistical sensitivity and temporal and geographical variability of the technique. The inter-population and inter-laboratory comparisons provided information on the optimum choice of test animals and the transferability of the technique. The investigation into the persistence of any field effects examined the potential of the test for the monitoring of episodic pollution.

SECTION A2 - STUDY SITES

The five field sites were selected to represent a geographically and compositionally diverse range of environments and discharges. The two main selection criteria were the presence of a continuous point effluent discharge and the proximity of a further clean reference site in the same catchment. Table A2.1 outlines the characteristics of the five sites.

Table A2.1 - Field sites used for testing the G. pulex SFG technique

Trial No	Field site	National Grid reference	Composition of effluent discharged	Date of G. pulex SFG field trial (1989)
1	Colne Water, East Lancashire (Reference station Wycoller Beck)	SD86703930 (SD93403920)	Domestic sewage with metallic industrial waste	9 - 15 May
2	River Lea, Essex (Reference station River Ash)	TL12101800 (TL37751333)	Domestic sewage	30 May - 5 June
3	West Okement, North Devon	SX56659308	Quarry effluent	26 July - 1 Aug
4	River Erme, South Devon	SX63605660	Pulp mill effluent	16 - 22 August
5	River Avon, Hampshire	SU17802300	Fish farm effluent	15 - 21 Sept

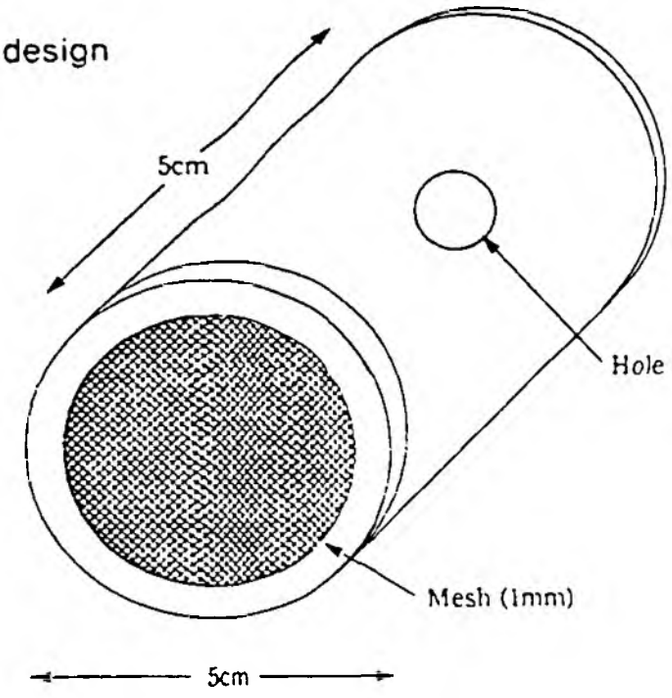
SECTION A3 - METHODS

A3.1 FIELD DEPLOYMENT

For the first four trials one hundred and sixty-eight adult male G. pulex obtained from Crags Stream, Derbyshire (NGR 49707450) and the

same number of animals obtained from Haseley Brook, Oxfordshire (NGR SU62409930) were placed, one each in individual field cages with a known amount of food material (Figure A3.1). The Crags Stream population will subsequently be referred to as 'Population 1' and the Haseley Brook population as 'Population 2'.

Figure A3.1 Field cage design



For the fifth trial, that on the Hampshire Avon, 100 animals from Crags Stream and 100 from a laboratory culture maintained at WRc Medmenham ('Population 3') were used. The chemistry of the water from which the wild populations were taken is shown in Table A3.1. Both wild populations of animals were collected from the field at least one week before each trial and kept in standard conditions (aerated 20 litre tanks; temperature: 15 °C; photoperiod 12L: 12D with 30 minutes artificial dawn and dusk; food: inoculated alder leaves). On each trial every animal received four 17 mm diameter discs, of known dry weight, cut from alder leaves (Alnus glutinosa). The leaf discs were inoculated with the fungus Cladosporium to increase their palatability (Maltby and Naylor 1989).

Table A3.1 - Chemical composition (mg/l) of water from which wild *Gammarus* populations obtained

Parameter	Crags Stream (Population 1)	Haseley Brook (Population 2)	
Alkalinity (as $CaCO_3$)	80	250	
TOC (as C)	0.86	6.6	
Ammonia (as N)	<0.04	<0.04	
Nitrate (as N)	25.5	13.2	
Nitrite (as N)	<0.5	0.07	
SS	5.4	14.2	
Chloride	62.1	57.0	
Sulphate as S	153.0	75.3	
SRP (as P)	-	2.36	
Calcium	114.0	94.0	
Magnesium	57.1	6.71	
Sodium	26.1	69.3	
Potassium	2.3	14.0	
Copper	<0.004	<0.004	
Zinc	0.018	0.02	
Chromium	0.001	0.003	
Nickel	<0.01	<0.008	
Cadmium	<0.004	<0.004	
Lead	<0.05	<0.05	
Manganese	0.011	0.028	
Iron	0.063	0.465	1.384 (0.214)+
Aluminium	0.07 *	0.26 *	0.616 (0.175)+

TOC = Total Organic Carbon
SS = Suspended Solids
SRP = Soluble Reactive Phosphorus
\+ = Mean body burdens (SE) of *Gammarus* sampled from Haseley Brook
* = Values exceeding actual or proposed environmental quality standard

For all but the Hampshire Avon trial a random number table was used to assign 56 field cages containing animals from Population 1 and 56 from Population 2 to a series of six holding baskets, each of which held 22 field cages. Ten field cages without animals were also randomly assigned to each set of six baskets to act as controls for leaf disc weight loss or gain in the field. This random assignment of field cages was repeated twice more to produce three sets of six holding baskets for

deployment at three stations (see Figure A3.2). On the Colne and Lea trials Station 1 was a 'clean' reference site in the same catchment as Stations 2 and 3, which were located upstream and downstream respectively from the discharge point. On the West Okement and Erme trials Station 1 was a 'clean' upstream site located just above the initial discharge point and Stations 2 and 3 were located downstream from the initial and secondary discharges respectively. Figure A3.3 illustrates the relative positions of stations and discharges in each of the five field trials.

For the Hampshire Avon trial a random number table was used to assign 50 field cages containing animals from Population 1 and 50 from Population 3 to a series of five holding baskets. Five leaf control cages were also assigned to each set of five baskets. This assignment of cages was repeated to produce two sets of five holding baskets for deployment at two stations, upstream and downstream from the fish farm.

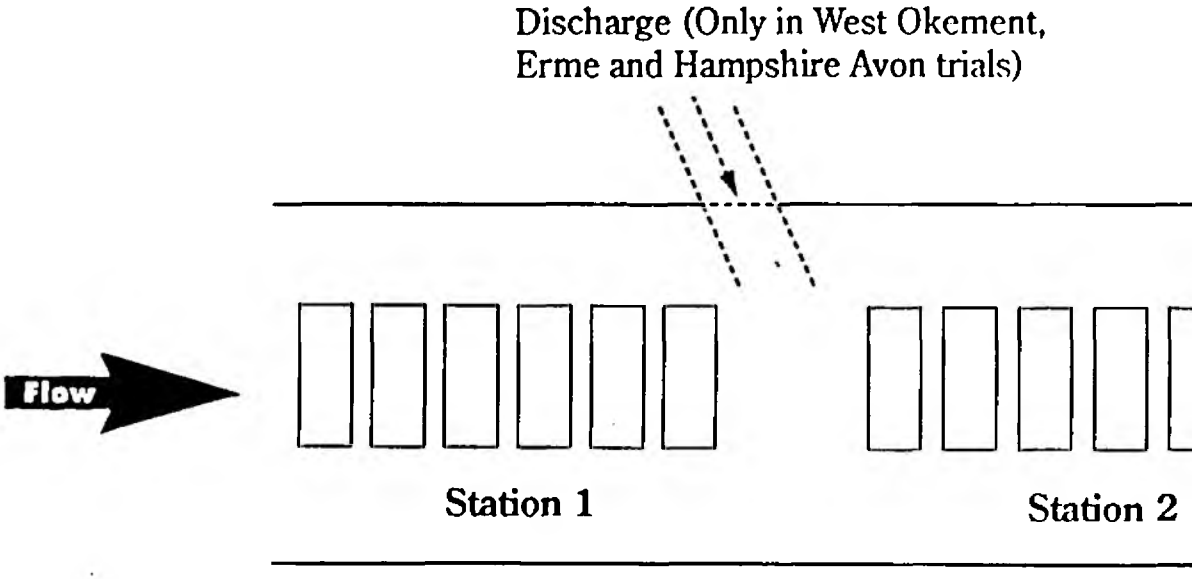
When the field cages had been distributed the holding baskets were secured to the substratum so as to allow water to pass through the bore of the field cages. The following physical and chemical measurements were then taken at all stations:

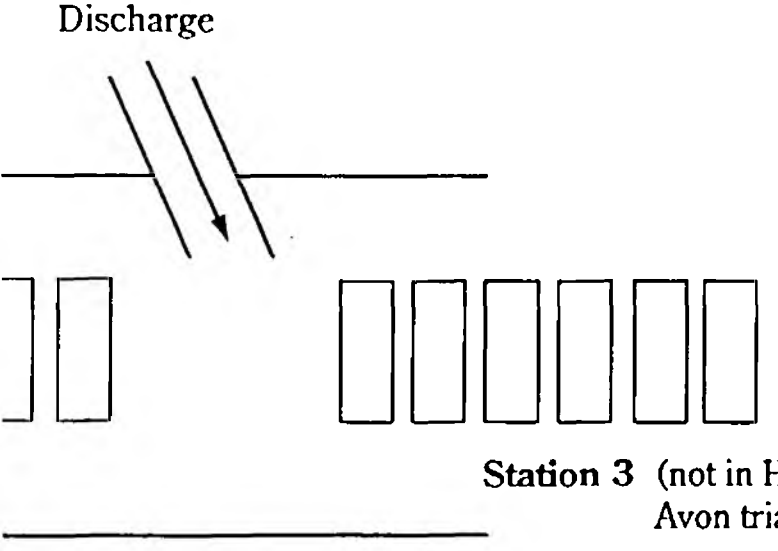
i) Channel width
ii) Channel depth
iii) Flow rate
iv) Substratum size
v) Temperature
vi) pH
vii) Conductivity

One set of water samples per station was also taken for laboratory analysis of the following determinands:

i) Metals: Na, Mg, K, Ca, Al, Mn, Fe, Ni, Cu, Zn, Cd, Cr, Pb
ii) Nitrate

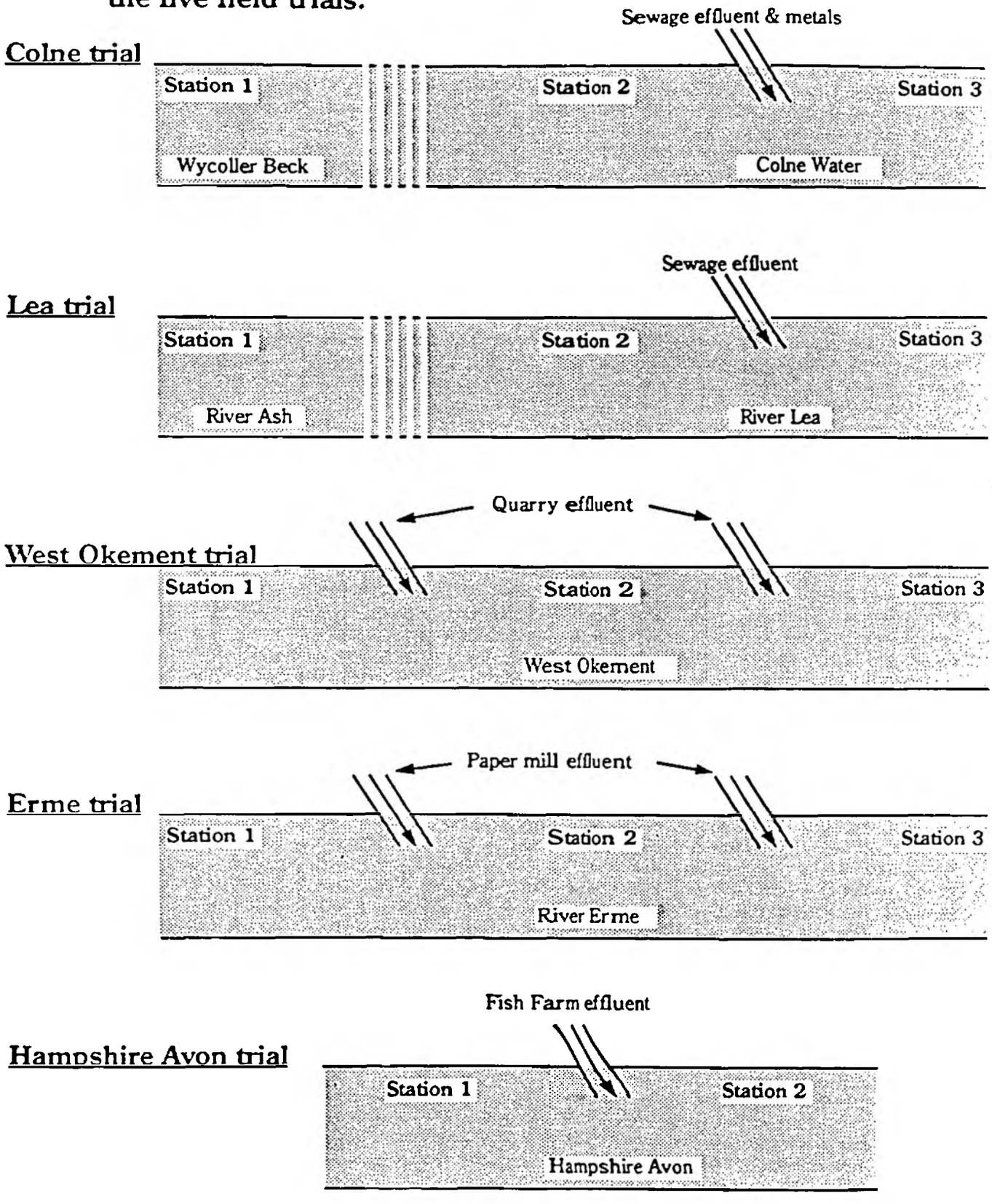
Figure A3.2. Diagram of holding basket positions





Station 3 (not in Hampshire Avon trial)

Figure A3.3. Relative positions of stations and discharges in the five field trials.



iii) Nitrite
iv) Total organic carbon
v) Total ammonia
vi) Soluble reactive phosphorus
vii) Sulphate
viii) Chloride
ix) Alkalinity
x) Suspended solids

Phenol and organochlorine concentrations were also determined for samples taken from the River Erme because the literature suggested that these groups of chemicals may be present in pulp mill effluent. The results from the water samples were compared with actual or proposed Environmental Quality Standards (EQS) in order to assess the relative water quality at the stations used in each trial (Table A3.2).

Three-minute kick-samples with a standard 0.5 mm net were taken at each station. Invertebrates caught by this method were sorted, counted and identified to family or species level.

The field cages were recovered after six days, when a further set of water samples were taken for analysis. The retrieved cages were divided equally by station and population between Sheffield ('Laboratory 1') and WRc ('Laboratory 2') using a random number table and returned to the respective laboratories in large plastic tubs containing water from the field.

Table A3.2 - Accepted and proposed Environmental Quality Standards for the protection of freshwater life in the UK

Parameter	Maximum level	Reference
pH	6.5 - 8.5	Wolff et al 1985
Ammonia (as N)	0.78	Seager et al 1986
Nitrate (as N)	400.0	US EPA 1986
Nitrite (as N)	0.4 - 0.9	EIFAC 1984
SS	25.0	Gardiner and Mance 1984
Chloride	100.0	McKee and Wolf 1963
Sulphate	Not toxic in water	McKee and Wolf 1963
Calcium	555.0	Ellis 1937
Magnesium	100.0	Solbé and Zabel 1988
Sodium	100.0	McKee and Wolf 1963
Potassium	50.0	Jones 1939
Copper	0.001 - 0.028 *	Mance et al 1984
Zinc	0.008 - 0.1 *	Mance and Yates 1984
Chromium	0.005 - 0.05 *	CEC 1986
Nickel	0.008 - 0.2 *	Mance and Yates 1984
Cadmium	0.005	CEC 1983
Lead	0.004 - 0.06 *	Brown et al 1984
Manganese	0.002 - 0.16 *	Solbé and Zabel 1988
Iron	2.0	Campbell 1985
Aluminium	0.05	Solbé and Zabel 1988

Results reported in mg/l
* Lower value = maximum at low alkalinity
  Higher value = maximum at high alkalinity

Upon return to the laboratory, the food material remaining in the field cages was removed, rinsed in clean water and its weight determined after drying in an oven at 60 °C for 2 days. Surviving animals were selected randomly either for respirometry, for investigation of persistence of effect, or for immediate death and determination of dry weight.

A3.2 RESPIROMETRY ON FIELD DEPLOYED *GAMMARUS PULEX*

The sub-sample of *G. pulex* selected for respirometry by each laboratory comprised 8 animals from each population deployed at each station, giving a total of 48 animals. For the Hampshire Avon trial up to 15 animals were selected from each population at each station. A

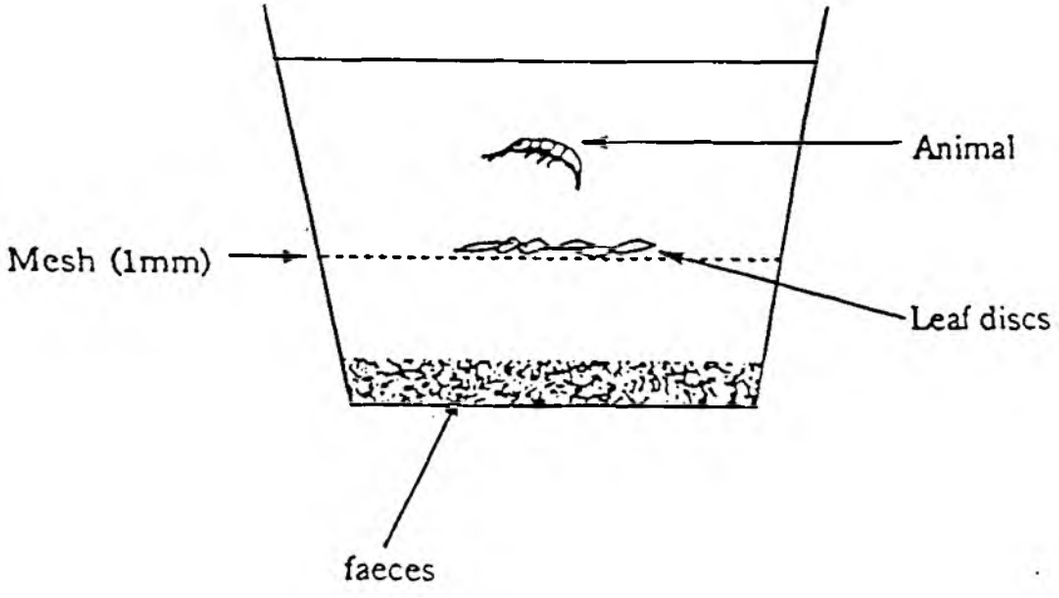
flow-through system was used with a Strathkelvin 781b oxygen meter and Radiometer Copenhagen E5046-0 electrode in a 15 °C constant temperature room.

Animals were acclimatised to the system for at least 3 hours prior to measurement of their oxygen uptake. Respiration rate was determined by comparing the dissolved oxygen content of water leaving respirometry chambers with that leaving blank chambers. Animals were respired in water taken from the field station at which they were deployed. Two or three sets of readings were taken for each animal. After the mean respiration rate of each animal had been determined, they were killed and their dry weight measured.

A3.3 MEASUREMENT OF THE PERSISTENCE OF ANY FIELD EFFECTS ON THE FEEDING RATE OF GAMMARUS PULEX

Only Laboratory 2 (WRc) undertook this part of the exercise. Fifteen animals from each population deployed at each station were placed in 250 ml chambers containing clean groundwater and four alder leaf discs of known dry weight prepared in the manner described earlier (Figure A3.4). The chambers containing the G. pulex and 10 extra chambers containing only leaf discs, to act as leaf weight gain or loss controls, were distributed randomly on a flat, uniformly lit surface in a 15 °C constant temperature room. After six days, the remaining leaf material and animals were removed from the chambers and their dry weight determined. The faeces produced over the six day period were filtered from the chamber and their dry weight determined.

Figure A3.4 Laboratory feeding chamber design



A3.4 CALCULATION OF FEEDING RATE, RESPIRATION RATE AND SFG

The feeding rate of Gammarus in the field or in the laboratory persistence of effect experiments was calculated from Equation 2.

$$C = \frac{J_1((D_1 * L) - D_2)}{W * N}$$

Equation 2

Where C = feeding rate (J/mg animal dry weight/day)

J_1 = energy content of leaf discs (21.552 J/mg) (Maltby and Naylor 1989)

D_1 = dry weight of food material initially supplied (mg)

D_2 = dry weight of food remaining after N days (mg)

W = dry weight of Gammarus (mg)

L = leaf weight change correction factor
(using leaf discs from blank cages or blank feeding chambers)

$$= \frac{\Sigma \text{ (final control leaf wt/initial control leaf wt)}}{S}$$

N = Number of days of experiment

S = Total number of control leaf discs

The amount of energy lost in faeces in the field was calculated from Equation 3

$$F = 0.74C - 0.303 \qquad \text{Equation 3}$$

Where F = energy in faeces (J/mg animal dry weight/day)

C = energy consumed (from Equation 2)

This regression equation was derived from work by Maltby and Naylor (1989) and was used because the direct determination of faecal mass is not possible in the field.

The amount of energy used in respiration was calculated from Equation 4

$$R = \frac{J_2(O_1 - O_2)}{T * W} \qquad \text{Equation 4}$$

Where R = Respiration rate (J/mg animal dry weight/day)

J_2 = Constant (20.432)

O_1 = Mean dissolved oxygen entering respirometry chamber (torr)

O_2 = Mean dissolved oxygen leaving respirometry chamber (torr)

T = Mean flow rate of water through respirometry chamber
(time (sec) taken to fill 250 µl sampling syringe)

W = Dry weight of Gammarus (mg)

The derivation of Equation 4 is given in Appendix 1.

The rate at which an animal assimilated food (A) was calculated from the results of the above equations by subtracting F from C. The SFG was found by subtracting R from A.

A3.5 STATISTICAL ANALYSIS OF FEEDING RATE, RESPIRATION RATE, SFG AND PERSISTENCE OF EFFECT

The feeding rate, respiration rate and SFG results calculated from the equations in Section 3.4 were analysed using a three-factor fixed-effects model analysis of variance (ANOVA). The three factors, or main effects, analysed were:

a) Station: 3 levels (Station 1, Station 2 and Station 3)
b) Population: 2 levels (Population 1 and Population 2 or Population 3)
c) Laboratory: 2 levels (Laboratory 1 and Laboratory 2)

First-order interactions (between two factors) and second-order interactions (between three factors) were also investigated with this ANOVA. If higher order interactions were found then these were considered to be more important than lower order interactions or main effects. For example, if significant station by population by laboratory, and station by population interactions were found then the former was considered important and the latter irrelevant in the interpretation of results. If a significant station by laboratory interaction and a significant station main effect were found, again the higher order interaction (station by laboratory) was considered important.

The reason for this decision can be illustrated with reference to an hypothetical trial in which a significant station by population interaction and a significant station main effect occurs because one population changes in its response with respect to station and one does not. If such is the case then it is meaningless to refer to the significant station main effect, because the populations do not respond similarly at each station such as all the measurements made by one laboratory on one population.

After completing an ANOVA, the power of the test was calculated. The power of a statistical test is, "... the probability of rejecting the null hypothesis when it is in fact false and should be rejected" (Zar 1984). For example, the null hypothesis may be that there is no difference in the feeding rate of Gammarus deployed upstream and downstream from an effluent discharge. If an ANOVA is performed with a power of 80%, there is a one in five chance of not rejecting the null hypothesis when it is in fact false and there really is an upstream, downstream difference in feeding rate. A failure of this kind is called a Type II error. There are two ways of reducing the probability of committing a Type II error. One can either increase the sample size or reduce the significance level of the test, thereby increasing the probability of committing a Type I error. It has been suggested that in environmental impact assessment studies, the latter course should be adopted (Swartz 1984). In this study, however, we have maintained the traditional approach and ascribed significance only to those differences that have a less than 5% probability of chance occurrence.

If significant main effects or interactions at $P<0.05$ were observed in the ANOVA then a Tukey multiple comparison test was used to discover where the significant differences lay. If this Tukey test failed to discover significant differences between treatment means then Scheffés procedure was used to compare biologically realistic sets of treatment groups, such as all the measurements made by one laboratory on one population. Analyses were carried out using either the GENSTAT or MINITAB statistical packages. Manual operations were performed according to Zar (1984) and Sokal and Rohlf (1981). In the analysis of the results from the persistence of effects experiments the factor 'laboratory' was omitted because the experiment was only performed by Laboratory 2 (WRc).

Because the first two trials, in the Colne and Lea catchments, involved the use of reference stations remote from the remaining two stations the latter were also analysed separately. This was done to provide a better indication of upstream, downstream differences in the response of feeding rate, respiration rate and SFG.

The response of Gammarus deployed in different baskets at the same station was analysed to see if they ate a similar amount. A one-way ANOVA was used to look at each population at each station. This analysis was performed in order to assess the effect of basket position on feeding rate, a factor of importance in the future design of deployment methodologies.

A3.6 MEASUREMENT OF TISSUE RESIDUES OF HEAVY METALS IN GAMMARUS AND LEAF DISCS

Pooled samples of Gammarus from both populations and leaf discs deployed in the Colne and West Okement trials were analysed for chromium, copper, zinc, lead, cadmium and nickel. Gammarus and leaf discs from the West Okement were analysed additionally for levels of aluminium, iron and manganese. These two sites were selected for detailed metal analysis because they both received metallic wastes. Ten Gammarus and ten leaf discs selected at random from each station were dried at 60 °C for 48 h and then bulked and weighed. The samples were digested in aqua regia and the metal concentrations determined using an inductively coupled plasma atomic emission spectrometer.

In addition to the above, analysis on individual animals from the West Okement trial was also performed in order to determine variation in body burdens between individuals and populations. Ten individuals from both populations deployed at each station were dried at 60 °C for 48 h. They were then weighed and digested in aqua regia. Nickel, chromium, iron, manganese and aluminium concentrations were determined using a Perkin-Elmer M2100 atomic absorption spectrophotometer linked to an electrothermal furnace. Zinc and iron were determined using a Perkin Elmer M2100 atomic absorption spectrophotometer with an air-acetylene flame.

A3.6.1 Statistical analysis of Gammarus tissue residues of heavy metals

For the West Okement trial, sufficient data were generated from the individual analyses to allow for statistical testing. For each metal a two-factor ANOVA was performed on Gammarus tissue residue level against population and station. Correlation coefficients were also calculated to investigate the relationship between tissue residue levels and feeding rate (Zar 1984).

A3.7 COMPARISON OF DATA FROM DIFFERENT TRIALS

In assessing the utility of the Gammarus SFG technique it was considered important to investigate the following factors common to all of the five field deployments.

A3.7.1 Measurement of food quality

A colorimetric assay was performed in order to assess the variability of fungal inoculation between laboratories and to determine whether the fungal biomass of the food material altered during field deployment. Ten leaf discs prepared by each laboratory from every station on all the trials were analysed.

The estimation of fungal biomass was obtained using a technique modified from that developed by Ride and Drysdale (1972). Dried leaf discs were ground in 70% acetone using a homogeniser. The slurry was then centrifuged in glass tubes at 4000 rev/min for 10 minutes before being washed with distilled water. Any chitin present was deacetylated to form chitosan, a polymer of glucosamine, by autoclaving for 1 h in 3 ml of 21.4M KOH. Eight ml of 75% ethanol was added to the cooled tubes which were then chilled at -20 °C for 15 minutes. Celite suspension (1 g Celite mixed with 20 ml of 75% ethanol) was layered on top of the alkali solution and the tubes centrifuged as before. The resulting pellet was washed once with 40% ethanol and twice with distilled water and then suspended in 1.5 ml of distilled water.

An addition of 1.5 ml of 5% weight by volume $NaNO_3$ and 1.5 ml of 5% $KHSO_4$ was made to each tube. This solution was mixed using a vortex mixer for 15 minutes to deaminate and depolymerise the chitosan. The tubes were then centrifuged at 4000 rev/min for 2 minutes. A 1.5 ml aliquot of supernatant was taken from each sample for the colorimetric assay and 0.5 ml of 12.5% $NH_4SO_3NH_2$ was added to each. The contents of the tubes were mixed and 0.5 ml of 3-methyl-2-benzothiazolinone hydrazone hydrochloride was added to the tubes before heating them to 100 °C for 3 minutes. After cooling to room temperature 0.5 ml of 0.5% $FeCl_3$ was added to each tube and the mixture was allowed to stand for 30 minutes before its absorbance at 650 nm was measured with a Pye Unicam spectrophotometer (Model SP6-550).

Standard curves were prepared from known concentrations of glucosamine and known weights of Cladosporium. Sterile leaves were also analysed in order to correct for background variation.

A3.7.2 Variation in response at 'clean' stations

The feeding rates of the Gammarus deployed at the clean site, Station 1, in each trial were compared, in order to test whether feeding rate was constant under unstressed conditions.

A3.7.3 Variation in weight change of field-deployed control leaves

The weight loss or gain of the control leaf discs used to take account of leaching or depositional processes in the field were compared between stations and laboratories in order to assess variability and the effect that this may have had upon the results.

A3.7.4 The relationship between feeding rate and production of faeces

This was considered important because in the field SFG technique developed by Maltby and Naylor (1990), the energy lost in faeces is estimated by a regression equation (Equation 3) and not by direct observation. Results from the persistence of effect experiments were

used to assess the repeatability of the Maltby and Naylor equation by plotting the energy consumed against the faeces produced by each population across all the trials.

A3.7.5 Optimum sample size

Coefficients of variation (CVs) were calculated from the measurements recorded by each laboratory for each population at every station. The normal range of the CVs was then used to produce a chart showing the sample size (ie the number of Gammarus) required at each station in order to detect a given difference in feeding rates.

SECTION A4 - RESULTS

A4.1 COLNE CATCHMENT

A4.1.1 Chemical and biological sampling

a) Physico-chemical characteristics

Analyses of samples from the three stations are presented in Table A4.1. Both deployment and retrieval samples from Stations 1 and 2 contained levels of chromium above the EQS. Levels of aluminium were also considerably higher than the EQS at Stations 2 and 3 and slightly higher at Station 1. Flow rates varied between stations with a higher rate at Station 3 during both measurement periods. Station 3 also had a higher conductivity than the other two stations, suggesting that there was a higher concentration of ionic compounds in the water at this station. The temperature of the water at Station 1 was slightly lower than at the other two stations during the period of deployment. To summarise, the quality of water deteriorated from Stations 1 to 3, with Station 3 displaying a considerably poorer quality than the other two.

Table A4.1 - Physico-chemical characteristics of Colne deployment stations

Parameter	Deployment (9/5/89) Station			Retrieval (15/5/89) Station		
	1	2	3	1	2	3
Width (m)	3.6	7.5	5.5			
Depth (cm)	21.0	23.0	29.0			
Flow rate (m/s)	0.21	0.3	0.39	0.37	0.27	0.41
Temperature (min-max °C)				6-13	7-16	12-14
Conductivity (µmhos)	155	245	400	145	250	370
pH	8.8	9.2	9.1	7.6	9.3	8.6
Alkalinity (as $CaCO_3$)	61	66	94	61	77	83
TOC (as C)	2.2	3.1	18.7	2.1	2.9	9.6
Ammonia (as N)	<0.1	<0.1	0.59	<0.1	<0.1	0.48
Nitrate (as N)	0.18	0.78	8.0	0.16	0.76	6.37
Nitrite (as N)	<0.01	0.03	0.11	<0.01	0.03	0.1
SS	<0.5	<0.5	2.8	2.0	4.0	11.0
Chloride	13.0	24.5	58.2	11.8	24.4	43.6
Sulphate	16.7	44.0	64.3	16.0	43.2	53.5
SRP (as P)	<0.003	0.008	2.492	<0.003	0.05	2.150
Calcium	27.1	36.4	36.9	27.1	39.2	39.1
Magnesium	3.81	6.58	6.68	3.89	6.65	6.70
Sodium	7.69	13.9	79.6	7.80	14.8	40.6
Potassium	0.806	2.29	9.95	0.913	2.3	6.37
Copper	<0.004	<0.004	0.007	<0.004	<0.004	0.005
Zinc	<0.003	0.004	0.054	<0.003	<0.003	0.022
Chromium	<0.005	0.051 *	0.039 *	<0.005	0.052 *	0.047 *
Nickel	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008
Cadmium	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004
Lead	<0.05	<0.05	0.224	<0.05	<0.05	<0.05
Manganese	0.01	0.042	0.081	0.01	0.048	0.053
Iron	0.09	0.519	0.739	0.078	0.508	0.589
Aluminium	0.056 *	0.123 *	0.308 *	<0.04	0.096 *	0.154 *

TOC = Total Organic Carbon
SS = Suspended Solids
SRP = Soluble Reactive Phosphorus
All results reported in mg/l unless otherwise stated. Values exceeding actual or proposed EQS levels are asterisked.

Table A4.2 - Invertebrates found in kick samples from three stations in the Colne catchment

Taxon	Station 1	Station 2	Station 3
ANNELIDA			
Oligochaeta			
Lumbriculus variegatus	*	*	*
Stylodrilus heringianus		*	
Tubificidae		*	*
Naidae		*	*
MOLLUSCA			
Gastropoda			
Ancylus fluviatilis		*	
ARTHROPODA			
Isopoda			
Asellus aquaticus			*
Plecoptera			
Isoperla grammatica	*		
Leuctra hippopus	*		
Chloroperla torrentium	*		
Ephemeroptera			
Baetis rhodani			*
Ecdyonurus torrentis	*	*	
Rhithrogena semicolorata	*		
Paraleptophlebia submarginata	*		
Trichoptera			
Hydropsyche angustipennis	*		
Rhyacophila dorsalis	*		
Polycentropus kingi	*		
Limnephilidae	*		*
Diptera			
Dicranota sp.	*		
Pedicia sp.	*		
Ceratopogonidae	*		*
Tanypodinae			*
Chironominae			*
Coleoptera			
Limnius volckmari	*		
Potamonectes griseostriatus	*		
Oreodytes septentrionalis	*	*	
Hydracarina			
Lebertia sp.	*		*
Hygrobates sp	*	*	*
BMWP# Score	94	22	17

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b) Invertebrate sampling

The results of kick-samples taken at the three stations on the date of deployment are presented in Table A4.2. The community found at Station 1, Wycoller Beck, contained considerably more species than the communities found at Stations 2 and 3. G. pulex was not present at any station, although the conditions at Station 1 did not appear to be unfavourable.

A4.1.2 Gammarus pulex bioassays

a) Field mortality

The percentage mortality of animals in each population at each station is given in Table A4.3. Mortality was low for both populations, although higher in Population 1 than in Population 2 at Stations 2 and 3.

Table A4.3 - Percentage field mortality of Gammarus deployed in the Colne catchment

Station	Percentage Mortality	
	Population 1	Population 2
1	0	2
2	8	0
3	10	2

b) Feeding rate in the field

i) Three-station analysis

The mean feeding rate of the two populations as measured by both laboratories during the Colne deployment is shown in

Figure A4.1. The results from the ANOVA performed on these data are given in Table A4.4.

The significant station by population interaction shown in Table A4.4 was due to Population 2 eating significantly less at Station 3 than at Station 2 (Tukey Multiple Comparison $q=4.198$, $P<0.05$). The significant station by laboratory interaction was due to Laboratory 2 measuring a significant decrease in feeding rate between Stations 2 and 3 ($q=4.357$, $P<0.05$). No difference was found in the feeding rate between baskets for either population within any of the three stations (ANOVA, highest F for any population at any station = 1.77, $P>0.05$). Thus the positioning of the baskets within stations did not significantly affect the response of the test animals.

Table A4.4 - Results of ANOVA performed on the feeding rate of Gammarus in the Colne trial - three station analysis

Source of variation	df	Sum of squares	Mean square	F	P	Significance level
Station	2	16.497	8.248	4.61	0.011	*
Population	1	1.423	1.423	0.80	0.373	
Laboratory	1	0.771	0.771	0.43	0.512	
Station.Population	2	12.048	6.024	3.37	0.036	*
Station.Laboratory	2	11.333	5.666	3.17	0.044	*
Population.Laboratory	1	5.807	5.807	3.25	0.073	
Station.Pop.Lab	2	7.280	3.640	2.04	0.133	
Residual	201	359.271	1.787			
Total	212	414.398				

* = significant ($P < 0.05$)

ii) Two-station analysis

When the results are analysed only from Stations 2 and 3, upstream and downstream from the discharge, a station main

effect is apparent. Gammarus from both populations deployed at Station 3 ate less than those deployed at Station 2 (Table A4.5). A population by laboratory interaction is also evident due to Laboratory 2 measuring significant differences between the two populations ($q=4.095$, $P<0.025$), a result of little biological significance.

Table A4.5 - Results of ANOVA performed on the feeding rate of Gammarus in the Colne trial - two station analysis

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	1	14.730	14.730	6.27	0.013	*
Population	1	7.339	7.339	3.12	0.079	
Laboratory	1	0.294	0.294	0.13	0.724	
Station.Population	1	3.149	3.149	1.34	0.249	
Station.Laboratory	1	6.276	6.276	2.67	0.104	
Population.Laboratory	1	11.221	11.221	4.78	0.031	*
Station. Pop.Laboratory	1	1.248	1.248	0.53	0.467	
Residual	137	321.872	2.349			
Total	144	366.125				

* = significant ($P < 0.05$)

c) Respiration rate of Gammarus returned from the field

i) Three-station analysis

There were no significant differences in the measurement of Gammarus respiration rate between any station, population or laboratory (Figure A4.2, Table A4.6). However, the power of this test was very low, due to a lov sample size and high variability between replicates. This meant that there was a >60% chance of committing a Type II error and falsely accepting that there was no difference in the response of animals between treatment groups (Zar 1984).

Table A4.6 - Results of ANOVA performed on the respiration rate of Gammarus from the Colne trial - three station analysis

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	2	0.04956	0.02478	0.65	0.524	
Population	1	0.03215	0.03215	0.84	0.361	
Laboratory	1	0.01040	0.01040	0.27	0.603	
Station.Population	2	0.05713	0.02856	0.75	0.475	
Station.Laboratory	2	0.05788	0.02894	0.76	0.471	
Population.Laboratory	1	0.07961	0.07961	2.09	0.152	
Station.Pop.Lab	2	0.03745	0.01872	0.49	0.613	
Residual	81	3.08262	0.03806			
Total	92	3.40682				

ii) Two-station analysis

There is no material difference between the results of a two-station and three-station analysis (Table A4.7). No factors differed significantly in either case.

Table A4.7 - Results of ANOVA performed as the respiration rate of Gammarus from the Colne trial - two station analysis

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	1	0.04028	0.04028	1.04	0.313	
Population	1	0.00020	0.00020	0.01	0.943	
Laboratory	1	0.00402	0.00402	0.10	0.749	
Station.Population	1	0.00684	0.00684	0.18	0.676	
Station.Laboratory	1	0.05670	0.05670	1.46	0.232	
Population.Laboratory	1	0.01333	0.01333	0.34	0.560	
Station.Pop.Laboratory	1	0.00041	0.00041	0.01	0.918	
Residual	53	2.05595	0.03879			
Total	60	2.17773				

d) Scope for growth of Gammarus deployed in the field

i) Three-station analysis

There was a significant difference in the SFG measured between stations (Figure A4.3, Table A4.8). Animals from both populations at Stations 1 and 3 had a lower SFG than those at Station 2 ($q=4.279$ and 3.469, $P<0.025$ and <0.05 respectively). There was also a significant difference in the measurements made by each laboratory, with Laboratory 2 consistently estimating a higher value than Laboratory 1. Unlike the results for feeding rate presented above, no station by population interaction could be detected for SFG. This may have been due to the low power of this test leading to a >80% chance of committing a Type II error. It is thus quite likely that such an interaction did exist but was not detected by the ANOVA.

Table A4.8 - Results of ANOVA performed on the SFG of Gammarus from the Colne trial - three station analysis

Source of variation	df	Sum of squares	Mean square	F	P	Significance level
Station	2	1.3019	0.6509	4.62	0.013	*
Population	1	0.0642	0.0642	0.46	0.501	
Laboratory	1	0.7757	0.7757	5.51	0.021	*
Station.Population	2	0.3211	0.1605	1.14	0.325	
Station.Laboratory	2	0.1574	0.0787	0.56	0.574	
Population.Laboratory	1	0.0775	0.0775	0.55	0.460	
Station.Pop.Lab	2	0.1230	0.0615	0.44	0.648	
Residual	81	11.4004	0.1407			
Total	92	14.2213				

* = significant ($P < 0.05$)

ii) Two-station analysis

Both populations of Gammarus differed significantly in their SFG, with animals deployed upstream from the discharge showing a higher SFG than those deployed downstream (Table A4.9).

Table A4.9 - Results of ANOVA performed on the SFG of Gammarus from the Colne trial - two station analysis

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	1	0.7501	0.7501	4.52	0.038	*
Population	1	0.0140	0.0140	0.08	0.772	
Laboratory	1	0.3531	0.3531	2.13	0.150	
Station.Population	1	0.0093	0.0093	0.06	0.814	
Station.Laboratory	1	0.1137	0.1137	0.69	0.411	
Population.Laboratory	1	0.0499	0.0499	0.30	0.586	
Station.Pop.Labortory	1	0.1230	0.1230	0.74	0.393	
Residual	53	8.7883	0.1658			
Total	60	10.2014				

* = significant ($P < 0.05$)

e) Persistence of field effects on feeding rate of Gammarus

Due to an accident it was not possible to measure the persistence of field effects in more than 3 animals from Station 3, too small a sample size for statistical analysis. Analysis of the two remaining stations revealed no significant differences in feeding rate for either population between Stations 1 and 2, results that agree with that obtained from the field (Figure A4.4, Table A4.10). There was, however, a significant difference in feeding rate between the two populations, with Population 2 consuming more than Population 1. Both populations fed at a considerably higher rate in the laboratory than in the field.

Table A4.10 - Results of ANOVA performed on the feeding rate of Gammarus during the persistence of effect experiment following the Colne trial

Source of variation	df	Sum of squares	Mean square	F	P	Significance level
Station	2	0.591	0.295	0.06	0.943	
Population	1	52.811	52.811	10.45	0.002	**
Station.Population	2	9.264	4.632	0.92	0.405	
Residual	62	313.347	5.054			
Total	67	376.014				

** = very significant (P <0.01)

A4.1.3 Metal loadings in Gammarus and leaf discs

The concentrations of metals found in Gammarus body tissue, and the leaf discs used to feed them, after deployment at the three stations are given in Table A4.11. Chromium levels were high in leaf and animal material from Stations 2 and 3. Lead levels were higher in leaf discs from Stations 2 and 3 but only in animals from Station 3. Nickel levels were raised in leaf material at Stations 2 and 3 but not in animals from any station.

Table A4.11 - Concentration of metals in leaf and Gammarus tissue from the Colne trial

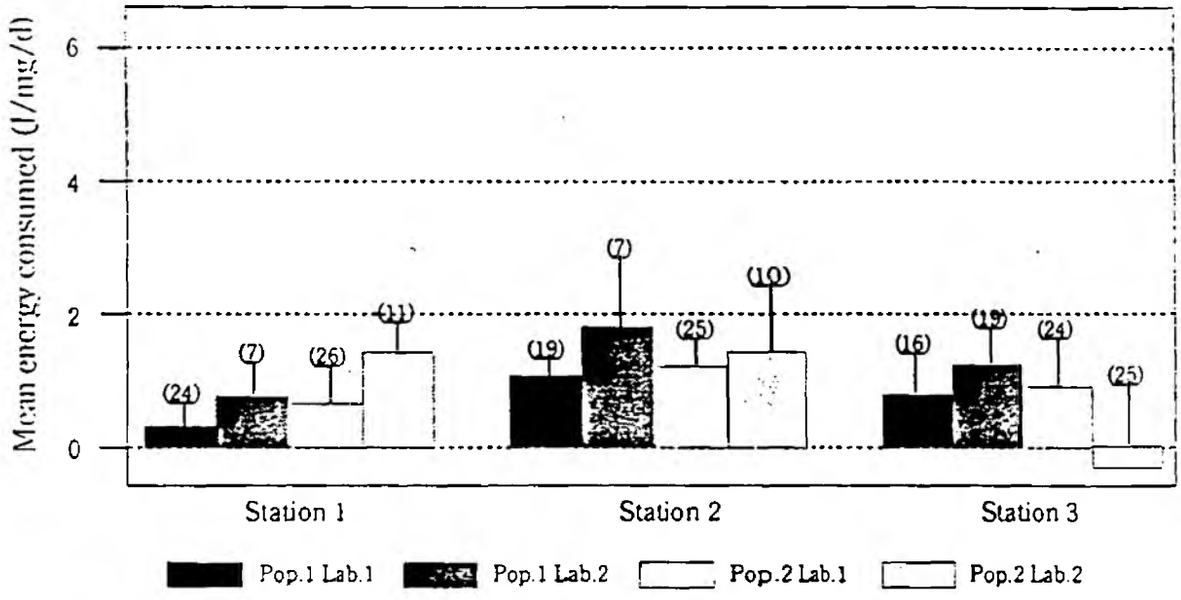
Determinand	Station 1		Station 2		Station 3	
	Gammarus	Discs	Gammarus	Discs	Gammarus	Discs
Chromium	0.0071	0.0073	0.0191	0.209	0.0162	0.267
Copper	0.0513	0.0823	0.0543	0.0504	0.0415	0.0661
Zinc	0.0683	0.199	0.0750	0.219	0.0679	0.223
Lead	<0.002	0.0177	<0.002	0.062	0.0068	0.162
Cadmium	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002
Nickel	<0.003	0.0173	<0.003	0.0594	<0.003	0.038

All results reported in mg/g

A4.1.4 Summary of main results from the Colne catchment

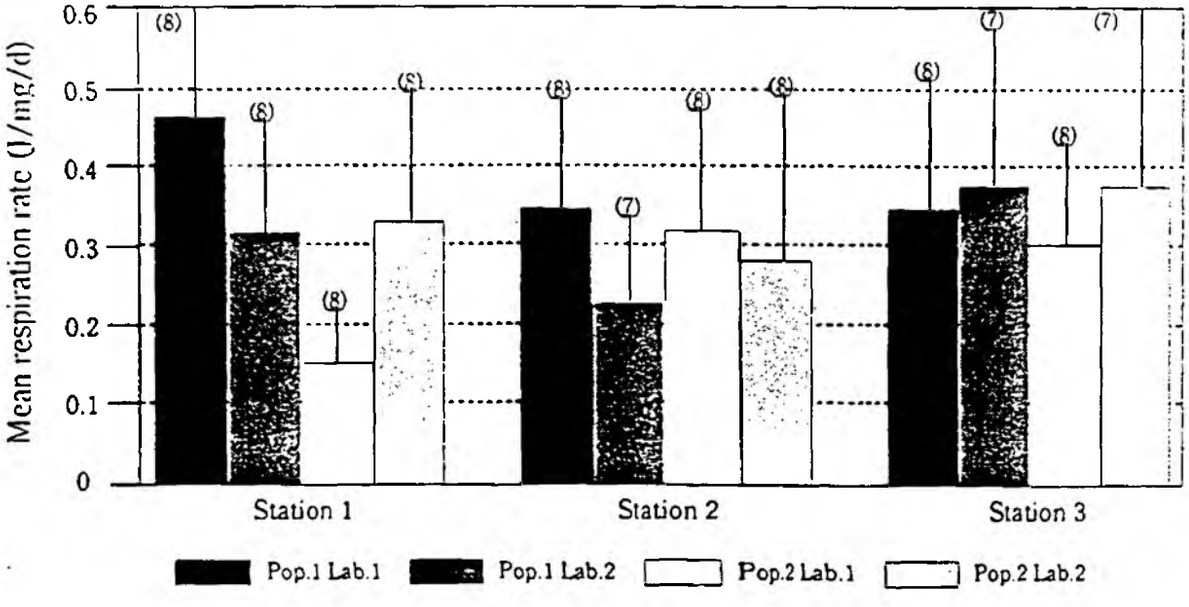
Chemical and biological sampling suggested a marked decline in water quality between Stations 1 and 2, and a further slight decline between Stations 2 and 3. Mortality during field deployment was highest in Gammarus from Population 1 at Stations 2 and 3. The interpretation of the results depended, to a certain extent, upon whether Station 1, the reference site, was included in the analysis. Given the remoteness of Station 1 from the other two stations there is an arguable case for its exclusion. When the upstream and downstream stations alone were analysed feeding rate was shown to differ significantly between stations with upstream animals feeding more than those deployed downstream. A similar trend was apparent in the estimation of SFG. Levels of lead were highest in leaf discs and animals from Station 3, suggesting a possible cause for the downstream reduction in feeding rate and SFG. No difference between stations could be detected when respiration rate and the persistence of field effects were analysed, although the latter were compromised by an accident. These results suggest that water quality deteriorated between Stations 2 and 3, upstream and downstream respectively from the sewage treatment works. They also show that the use of a remote reference station may not be the most appropriate basis for the comparison of polluted with unpolluted sites.

Figure A4.1 Energy consumed by *Gammarus pulex* at three stations in the Colne catchment.



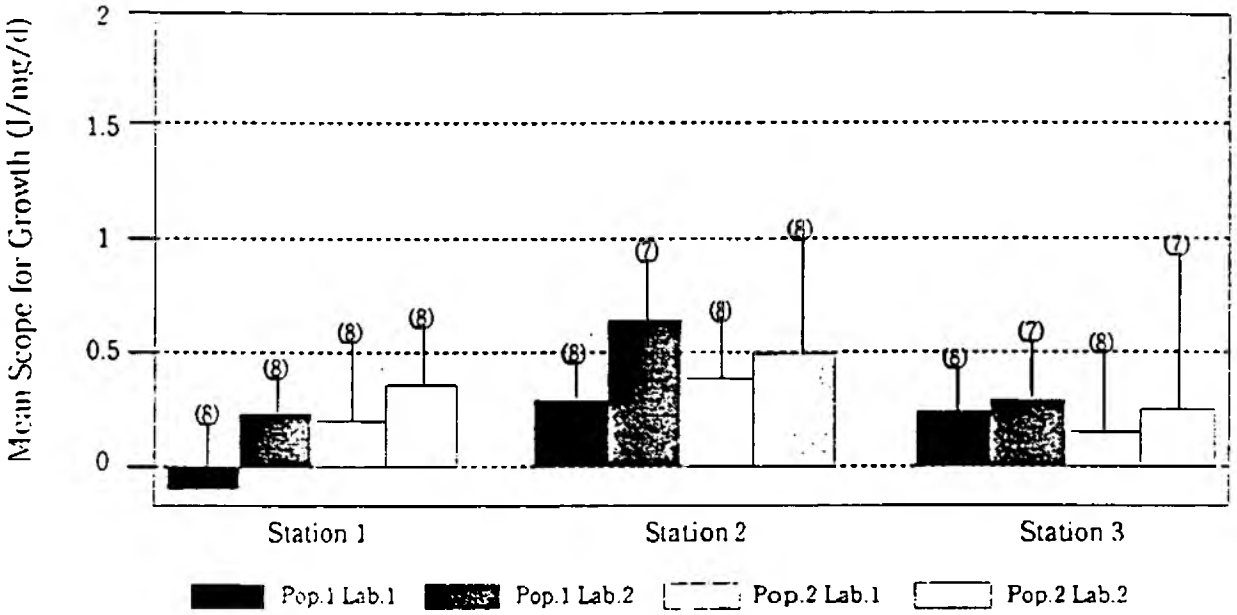
Values = Mean energy consumed, 95% confidence interval and (n)

Figure A4.2 Respiration rate of *Gammarus pulex* at three stations in the Colne catchment.



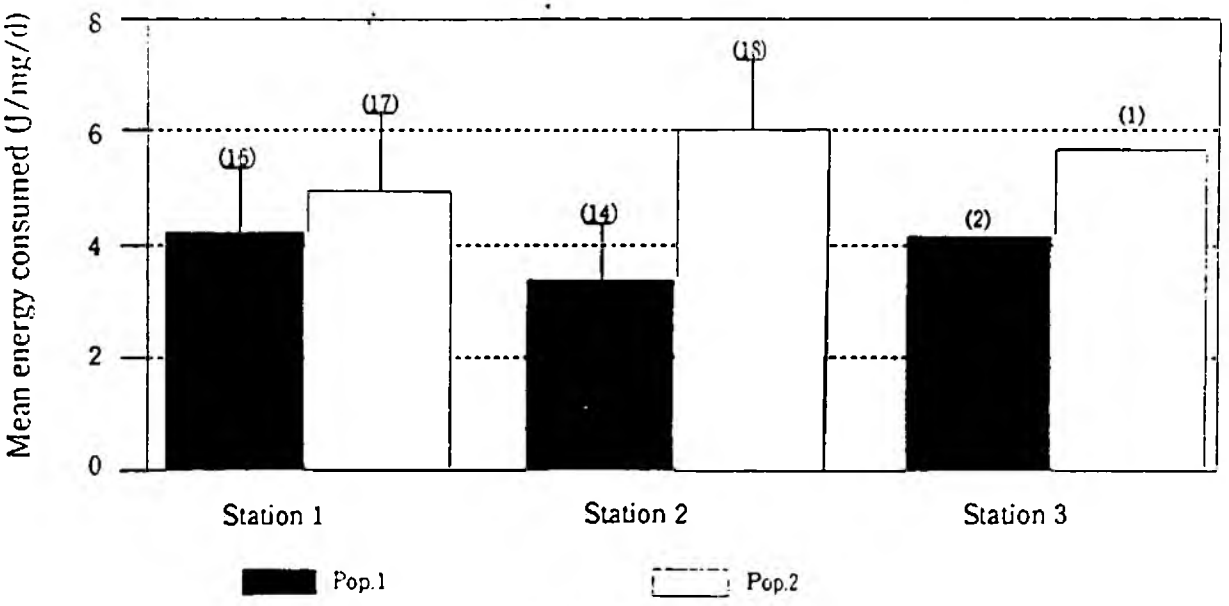
Values = Mean respiration rate, 95% confidence interval and (n)

Figure A4.3 Scope for Growth of *Gammarus pulex* from three stations in the Colne catchment.



Values - Mean Scope for Growth, 95% confidence interval and (n)

Figure A4.4 Persistence of field effects on the feeding of *Gammarus pulex* : Colne.



Values - Mean energy consumed, 95% confidence interval and (n)

A4.2 LEA CATCHMENT

A4.2.1 Chemical and biological sampling

a) Physico-chemical characteristics

The results from the three stations are presented in Table A4.12. None of the determinands measured at any of the three stations exceeded an established or proposed EQS. Ammonia concentrations at Station 2 were, however, considerably higher in the retrieval sample than in the deployment sample. Iron and manganese levels were also higher at Station 2, while zinc levels were highest at Station 3. The flow rate at Stations 2 and 3 varied between sampling dates, and the temperature range at these stations was higher than at Station 1. The conductivity of the water at Station 3 was considerably higher upon retrieval of the Gammarus, suggesting a higher concentration of dissolved ionic substances at this station, downstream from the sewage treatment works.

b) Invertebrate sampling

Results from kick samples taken at the three stations are presented in Table A4.13. Station 1, on the River Ash, possessed a considerably greater species richness than either Station 2 or 3, upstream and downstream respectively from the sewage treatment works. Gammarus were only present in the sample from Station 1.

A4.2.2 Gammarus pulex bioassays

a) Field mortality

The mortality of Gammarus deployed at the three stations is presented in Table A4.14. Both populations had a similar low or zero mortality at all three stations, suggesting that the water quality at the three field stations was not acutely lethal to Gammarus.

Table A4.12 - Physico-chemical characteristics of Lea deployment stations

Parameter	Deployment (30/5/89) Station			Retrieval (5/6/89) Station		
	1	2	3	1	2	3
Width (m)	5.4	5.0	5.0			
Depth (cm)	20.0	35.0	40.0			
Flow rate (m/s)	0.223	0.493	0.451	0.188	0.154	0.574
Temperature (min-max °C)				10-14	13-19	13.5-20
Conductivity (µmhos)	500	500	500	500	480	800
pH	8.2	8.0	7.7	8.1	8.0	7.5
Alkalinity (as $CaCO_3$)	246	171	216	260	171	232
TOC (as C)	2.36	6.04	8.78	2.4	5.3	9.1
Ammonia (as N)	<0.04	<0.04	0.12	<0.04	0.4	<0.04
Nitrate (as N)	5.03	3.45	9.10	5.62	3.6	13.8
Nitrite (as N)	0.06	0.11	0.06	0.05	0.1	0.05
SS	2.0	8.2	4.8	2.2	9.0	4.6
Chloride	30.0	46.9	74.6	34.4	48.2	94.8
Sulphate	44.5	82.7	98.3	44.2	80.5	100.0
SRP (as P)	0.120	0.004	4.130	0. 130	0.040	5.670
Calcium	120.0	103.0	122.0	124.0	103.0	127.0
Magnesium	4.35	2.93	3.88	4.38	2.98	4.26
Sodium	15.0	27.9	75.6	16.7	27.2	82.1
Potassium	3.38	3.72	12.3	3.45	3.46	14.2
Copper	<0.004	<0.004	0.008	<0.004	<0.004	0.009
Zinc	<0.003	0.009	0.041	<0.003	0.007	0.043
Chromium	<0.001	<0.001	0.002	0.008	0.01	0.006
Nickel	<0.008	<0.008	0.009	<0.008	<0.008	0.01
Cadmium	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004
Lead	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Manganese	0.008	0.042	0.012	0.009	0.041	0.009
Iron	0.041	0.079	0.049	0.042	0.094	0.05
Aluminium	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04

TOC = Total Organic Carbon
SS = Suspended Solids
SRP = Soluble Reactive Phosphorus
All results reported in mg/l unless otherwise stated.

Table A4.13 - Invertebrates found in kick samples from three stations in the Lea catchment

Taxon	Station 1	Station 2	Station 3
ANNELIDA			
Oligochaeta			
Limnodrilus hoffmeisteri	*	*	*
Potamothrix hammoniensis	*	*	*
Lumbriculus variegatus	*		
Psammoryctides barbatus	*		
Naididae	*		
Hirudinea			
Glossiphonia complanata	*	*	
Erpobdella octoculata	*	*	*
Hemiclepsis marginata			*
NEMATODA	*		
PLATYHELMINTHA			
Turbellaria			
Dugesia lugubris		*	*
Polycelis tenuis		*	
MOLLUSCA			
Gastropoda			
Potamopyrgus jenkinsi	*	*	*
Lymnaea peregra	*	*	*
Lymnaea stagnalis		*	
Planorbis planorbis	*	*	
Planorbis leucostoma		*	*
Valvata piscinalis	*		
Ancylus fluviatilis	*		
Bivalvia			
Pisidium sp.	*	*	*
Sphaerium lacustre		*	*
Sphaerium corneum	*	*	*
ARTHROPODA			
Isopoda			
Asellus aquaticus	*	*	*
Amphipoda			
Gammarus pulex	*		
Plecoptera			
Leuctra hippopus	*		
Ephemeroptera			
Ephemera danica	*		
Ephemerella ignita	*		
Habrophlebia fusca	*		
Caenis moesta	*	*	*
Baetis rhodani		*	*

Table A4.13 continued

Taxon	Station 1	Station 2	Station 3
Trichoptera			
Mystacides longicornis	*		
Athripsodes albifrons	*		
Athripsodes biliniatus	*		
Goera pilosa	*		
Limnephilus sp.	*		
Halesus sp.	*		
Hydropsyche angustipennis	*		
Tinodes weineri	*		
Ceraclea senilis		*	
Diptera			
Chironomidae	*	*	*
Simulium sp.			*
Ceratopogonidae	*		
Neuroptera			
Sialis lutaria	*		
Hemiptera			
Sigara dorsalis		*	
Corixa sp.	*	*	
Coleoptera			
Agabus didymus			*
Dytiscus sp.			*
Elmis aenea	*		
Limnius volckmari	*		
Hydroporus sp.	*		
Deronectes depressus elegans	*		
Haliplus ruficollis		*	
Hydracarina			
Hygrobates longipalpis	*		
Lebertia sp.	*		
Hydrodromida despiciens	*		
Copepoda	*	*	*
BMWP Score	145	60	50

Table A4.14 - Percentage mortality of caged Gammarus at three stations in the Lea catchment

Station	Population 1	Population 2
1	4	2
2	0	0
3	4	4

b) Feeding rate in the field

i) Three-station analysis

Population 1 ate significantly less at Station 2, upstream from the sewage treatment works, than at Station 1, the reference station, ($q=4.837$, $P<0.01$) (Figure A4.5, Table A4.15). Population 1 also ate less at Station 2 than Population 2 ($q=5.071$, $P<0.005$). There was a significant difference between laboratories in the measurement of feeding rate with Laboratory 1 consistently measuring a higher rate than Laboratory 2. No difference was found in the feeding rate of animals between baskets for either population within any of the three stations (highest F for any population at any station=2.29, $P>0.05$). These results show that the feeding rate of only one population differed significantly between stations.

Table A4.15 - Results of ANOVA performed on the feeding rate of Gammarus in the Lea trial - three station analysis

Source of variation	df	Sum of squares	Mean square	F	P	Significance level
Station	2	10.988	5.494	2.65	0.072	
Population	1	4.335	4.335	2.09	0.149	
Laboratory	1	58.605	58.605	28.28	<0.001	***
Station.Population	2	33.935	16.968	8.19	<0.001	***
Station.Laboratory	2	2.938	1.469	0.71	0.493	
Population.Laboratory	1	0.495	0.495	0.24	0.626	
Station.Pop.Lab	2	3.134	1.567	0.76	0.470	
Residual	274	567.906	2.073			
Total	285	682.316				

*** = highly significant (P <0.001)

ii) Two-station analysis

Because Station 1, the reference site, was remote from the other two stations a separate analysis was run for just the upstream and downstream stations. The results from the analysis of feeding rates at the upstream and downstream stations alone do not differ greatly from the three-station analysis (Table A4.16). A population main effect becomes apparent, but this does not affect the interpretation of the results, because a higher-order interaction between station and population also exists. When a Tukey test is performed on the components of the station by population interactions, however, an interesting difference from the three-station analysis emerges. In the latter no significant differences could be detected between the upstream and the downstream stations. In the two-station analysis there is not only an interpopulation difference at Station 2 ($q=4.978$, $P<0.005$), but also a significant difference in feeding rate between Stations 2 and 3: Population 1 fed less at Station 2 than at Station 3 ($q=5.138$, $P<0.005$).

Table A4.16 - Results of ANOVA performed on the feeding rate of Gammarus in the Lea trial - two station analysis

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	1	9.142	9.142	4.25	0.041	*
Population	1	17.653	17.653	8.21	0.005	**
Laboratory	1	65.755	65.755	30.60	<0.001	***
Station.Population	1	9.967	9.967	4.64	0.033	*
Station.Laboratory	1	0.720	0.720	0.33	0.563	
Population.Laboratory	1	0.328	0.328	0.15	0.697	
Station.Pop.Laboratory	1	3.695	3.695	1.72	0.191	
Residual	181	388.971	2.149			
Total	188	496.471				

*** = highly significant (P <0.001)
** = very significant (P <0.01)
* = significant (P <0.05)

c) Respiration rate of Gammarus returned from the field

i) Three-station analysis

There were no significant differences in the measurement of Gammarus respiration rate between any station, population or laboratory (Figure A4.6, Table A4.17). However, the power of this test to detect a difference between treatment groups was very low.

Table A4.17 - Results of ANOVA performed on the respiration rate of Gammarus from the Lea trial - three station analysis

Source of variation	df	Sum of squares	Mean square	F	P	Significance level
Station	2	0.00514	0.00257	0.06	0.939	
Population	1	0.04824	0.04824	1.18	0.280	
Laboratory	1	0.00440	0.00440	0.11	0.743	
Station.Population	2	0.04781	0.02391	0.59	0.559	
Station.Laboratory	2	0.05587	0.02793	0.68	0.507	
Population.Laboratory	1	0.01447	0.01447	0.35	0.553	
Station.Pop.Lab	2	0.11828	0.05914	1.45	0.241	
Residual	79	3.22458	0.04082			
Total	90	3.51886				

ii) Two-station analysis

The results from an ANOVA performed on just the upstream and downstream stations were similar to those obtained from the three-station analysis (Table A4.18). There were no significant differences in respiration rate between stations, populations or laboratories.

Table A4.18 - Results of ANOVA performed on the respiration rate of Gammarus from the Lea trial - two station analysis

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	1	0.00004	0.00004	0	0.971	
Population	1	0.01501	0.01501	0.47	0.494	
Laboratory	1	0.01388	0.01388	0.44	0.510	
Station.Population	1	0.03689	0.03689	1.17	0.285	
Station.Laboratory	1	0.04530	0.04530	1.43	0.237	
Population.Laboratory	1	0.00046	0.00046	0.01	0.904	
Station.Pop.Laboratory	1	0.07433	0.07433	2.35	0.131	
Residual	53	1.67533	0.03161			
Total	60	1.86123				

d) Scope for growth of Gammarus deployed in the field

i) Three-station analysis

There were no significant differences in the SFG of animals between stations or populations (Figure A4.7, Table A4.19). There were differences between laboratories, however, with Laboratory 2 consistently recording a lower value than Laboratory 1. No significant station by population interaction matching that found in the analysis of feeding rate could be detected for SFG. As mentioned earlier, however, the high variability of the data and the constraints on sample size dictated by the methodology meant that the chance of committing a Type II error was high. In this case there was a 65% chance of not being able to detect a real difference between treatments.

Table A4.19 - Results of ANOVA performed on the SFG of Gammarus from the Lea trial - three station analsyis

Source of variation	df	Sum of squares	Mean square	F	P	Significance level
Station	2	0.4094	0.2047	0.93	0.401	
Population	1	0.0214	0.0214	0.10	0.756	
Laboratory	1	0.9770	0.9770	4.42	0.039	*
Station.Population	2	0.7986	0.3993	1.80	0.171	
Station.Laboratory	2	0.0276	0.0138	0.06	0.939	
Population.Laboratory	1	0.1119	0.1119	0.51	0.479	
Station.Pop.Lab	2	0.0441	0.0221	0.10	0.905	
Residual	79	17.4777	0.2212			
Total	90	19.8677				

* = significant (P <0.05)

ii) Two-station analysis

An ANOVA performed on the SFG results from just the upstream and downstream stations differed slightly from the three-station analysis. In the latter, a significant difference was detected in the measurements made by the two laboratories. In the two-station analysis the difference between the laboratories fell just short of significance at the 5% level (Table A4.20).

Table A4.20 - Results of ANOVA performed in the SFG of Gammarus from the Lea trial - two station analysis

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	1	0.1503	0.1503	0.79	0.378	
Population	1	0.4276	0.4276	2.24	0.140	
Laboratory	1	0.7622	0.7622	4.00	0.051	
Station.Population	1	0.0018	0.0018	0.01	0.924	
Station.Laboratory	1	0.0015	0.0015	0.01	0.930	
Population.Laboratory	1	0.1340	0.1340	0.70	0.405	
Station.Pop.Laboratory	1	0.0183	0.0183	0.10	0.758	
Residual	53	10.0968	0.1905			
Total	60	11.5923				

e) Persistence of field effects on feeding rate of Gammarus

i) Three-station analysis

There were no significant differences in the feeding rate of animals between either sites or populations after removal from the field (Figure A4.8, Table A4.21). Both populations fed at approximately twice the field rate when in the laboratory.

Table A4.21 - Results of ANOVA performed on the feeding rate of Gammarus during the persistence of effect experiment following the Lea trial - three station analysis

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	2	11.932	5.966	2.54	0.085	
Population	1	0.082	0.082	0.04	0.852	
Station.Population	2	1.506	0.753	0.32	0.727	
Residual	79	185.744	2.351			
Total	84	199.264				

ii) Two-station analysis

The results from an ANOVA performed on only those animals taken from the upstream and downstream field stations were similar to those obtained from the three-station analysis (Table A4.22). There were no significant differences in feeding rate between either stations or populations.

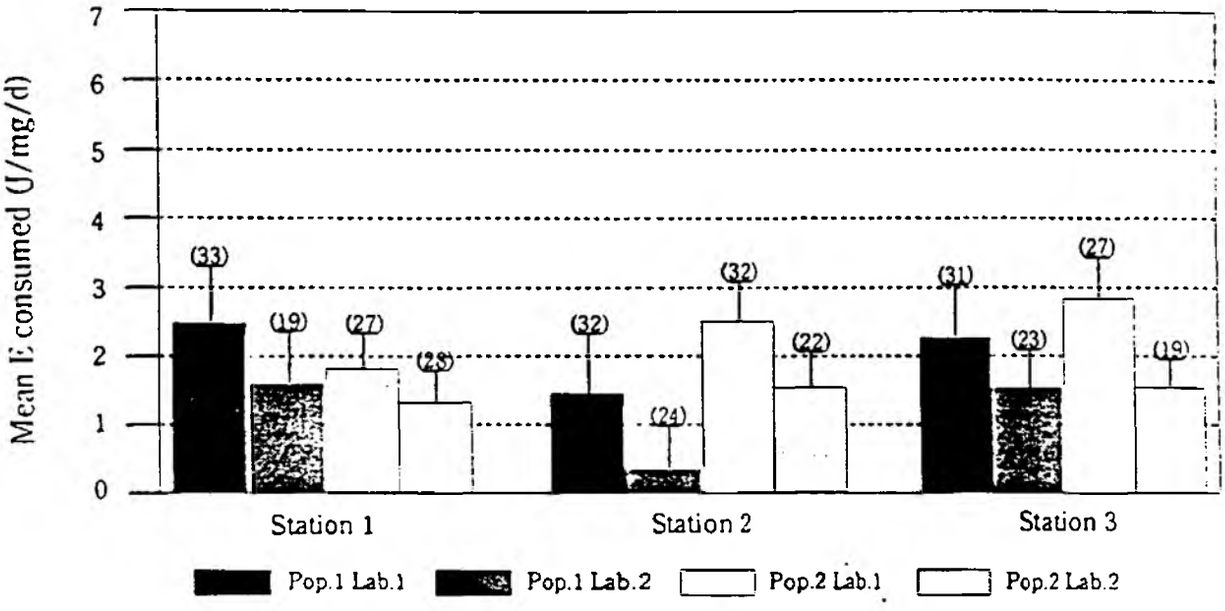
Table A4.22 - Results of ANOVA performed on the feeding rate of Gammarus during the persistence of effect experiment following the Lea trial - two station analysis

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	1	5.383	5.383	2.53	0.118	
Population	1	0.181	0.181	0.09	0.772	
Station.Population	1	0.460	0.460	0.22	0.644	
Residual	52	110.742	2.130			
Total	55	116.766				

A4.2.3 Summary of main results from the Lea catchment

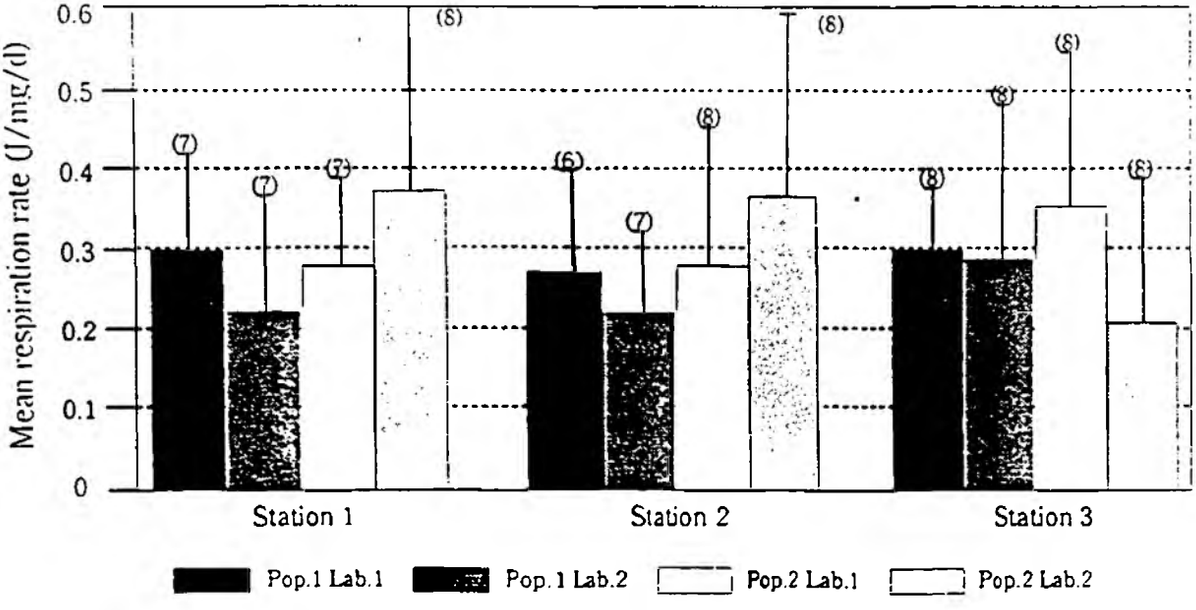
Biological sampling suggested a marked decline in water quality between Stations 1 and 2 and a further slight decline between Stations 2 and 3. No chemical determinands exceeded EQS levels at any station, although some were found at higher concentrations at Station 2. Mortality during deployment was low in both populations at all stations. Animals from Population 1 ate significantly less at Station 2 than at the other two stations. Laboratory 1 consistently measured a higher consumption rate than Laboratory 2. No significant differences could be detected between stations, populations or laboratories for respiration rate or persistence of effect, but there was a significant inter-laboratory difference in the measurement of SFG. These results suggest that the water quality upstream from the sewage treatment works was lower than that downstream during the period of deployment. The results also suggest that, in common with those from the Colne trial, the choice of a remote reference station can confuse the interpretation of results.

Figure A4.5 Energy consumed by *Gammarus pulex* at three stations in the Lea catchment.



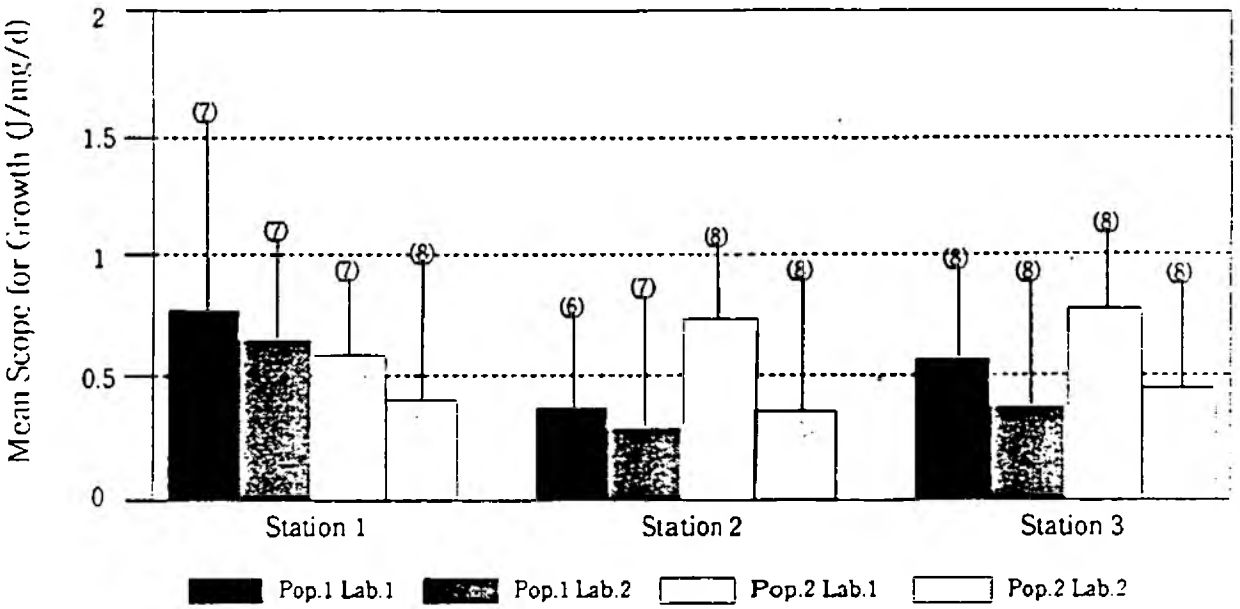
Values = Mean energy consumed, 95% confidence interval and (n)

Figure A4.6 Respiration rate of *Gammarus pulex* at three stations in the Lea catchment.



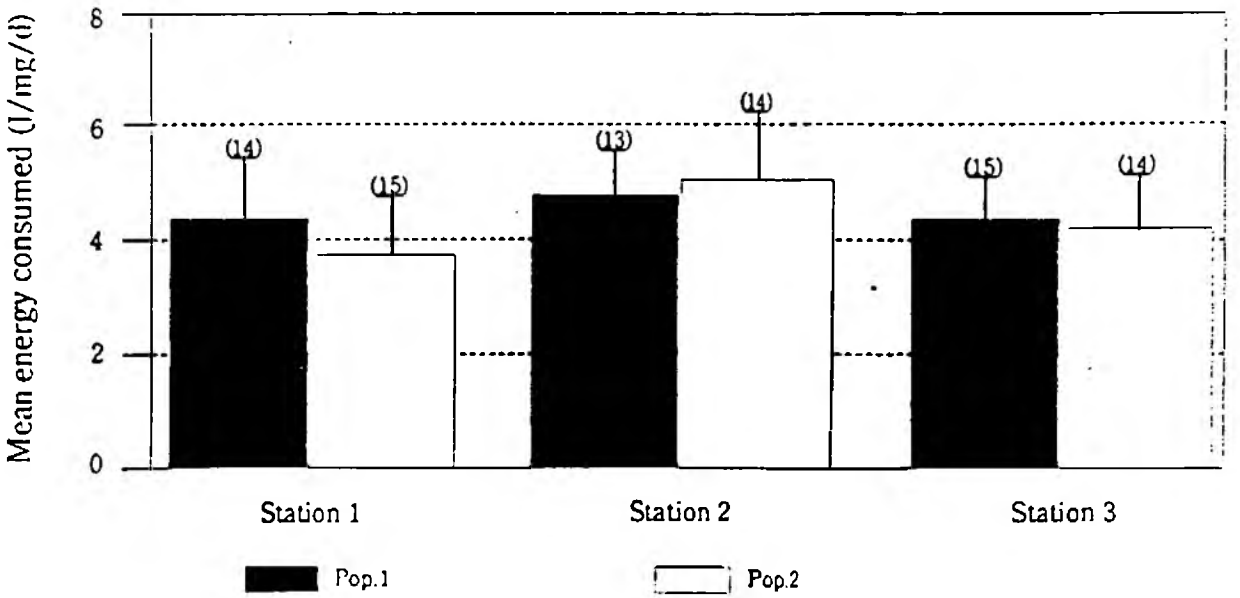
Values = Mean respiration rate, 95% confidence interval and (n)

Figure A4.7 Scope for growth of *Gammarus pulex* from three stations in the Lea catchment.



Values = Mean Scope for Growth, 95% confidence interval and (n)

Figure A4.8 Persistence of field effects on the feeding of *Gammarus pulex* : Lea.



Values = Mean energy consumed, 95% confidence interval and (n)

A4.3 WEST OKEMENT CATCHMENT

A4.3.1 Chemical and biological sampling

a) Physico-chemical characteristics

The water sample analyses from the three stations are presented in Table A4.23. Samples taken from Stations 2 and 3 contained concentrations of nickel, manganese and iron exceeding their respective EQS level. Aluminium was found at concentrations higher than the EQS at all three stations, although at Station 1 the EQS was exceeded by only a small amount. The flow rate was highest at Station 3 and lowest at Station 2, while conductivity increased from Stations 1 to 3. Temperatures were similar at all three stations.

b) Invertebrate sampling

The results from kick-samples taken at all three stations are presented in Table A4.24. Due to the large substrate particle size it proved difficult to kick-sample effectively. Hence these results are probably not a true representation of the invertebrate communities present at each station. They should, however, provide a reasonably robust basis for comparing relative changes in species richness between stations. More species of invertebrates were found at Station 1, upstream from the two discharges. Gammarus was not found at any of the stations.

Table A4.23 - Physico-chemical characteristics of West Okement deployment stations

Parameter	Deployment (26/7/89) Station			Retrieval (1/8/89) Station		
	1	2	3	1	2	3
Width (m)	3.7	6.4	3.3			
Depth (cm)	10.0	25.0	15.0			
Flow (m/s)	0.55	0.136	0.611	0.377	0.104	0.574
Temperature (min-max °C)				11.5-16	11-16	11-16
Conductivity (µmhos)	60	90	100	62	90	100
pH	7.2	6.99	6.78			
Alkalinity (as $CaCO_3$)	16.4	21.8	24.6	16.4	19.1	16.4
TOC (as C)	1.99	2.16	1.82	1.75	1.67	1.5
Ammonia (as N)	<0.012	0.019	0.022	0.033	<0.015	0.069
Nitrate (as N)	0.44	0.47	0.45	0.47	0.54	0.49
Nitrite (as N)	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
SS	0.2	2.2	2.8	5.0	0.4	2.8
Chloride	8.56	9.23	9.34	9.13	9.53	9.7
Sulphate	7.16	18.34	27.21	8.42	22.7	32.0
SRP (as P)	0.019	0.019	0.044	0.008	0.007	0.006
Calcium	5.92	8.76	10.2	7.08	10.4	12.2
Magnesium	1.36	2.05	2.58	1.56	2.35	2.96
Sodium	5.98	6.36	6.58	6.17	6.76	7.05
Potassium	0.643	0.743	0.81	0.749	0.809	0.902
Copper	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004
Zinc	0.004	0.024	0.045	0.006	0.018	0.05
Chromium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Nickel	<0.008	0.012 *	0.032 *	<0.008	0.011 *	0.036 *
Cadmium	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004
Lead	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Manganese	0.014	0.345 *	0.357 *	0.012	0.32 *	0.407 *
Iron	0.337	2.34 *	0.828	0.318	1.18	0.789
Aluminium	0.07 *	0.312 *	0.35 *	0.06 *	0.31 *	0.1 *

TOC = Total Organic Carbon
SS = Suspended Solids
SRP = Soluble Reactive Phosphorus
All results reported in mg/l unless otherwise stated. Values exceeding actual or proposed EQS levels are asterisked.

Table A4.24 - Invertebrates found in kick samples from three stations in the West Okement catchment

Taxon	Station 1	Station 2	Station 3
OLIGOCHAETA			
Stylodrilus sp.	*		*
Eiseniella teraeda	*	*	
ARTHROPODA			
Ephemeroptera			
Centroptilum luteum	*		
Plecoptera			
Leuctra geniculata	*		
Leuctra hippopus	*	*	*
Leuctra fusca	*	*	
Trichoptera			
Rhyacophila dorsalis	*	*	*
Plectropnemia conspersa	*		
Polycentropus flavomaculatus	*		
Polycentropus kingi	*	*	*
Diptera			
Protonemura meyeri	*		
Chironomidae	*	*	*
Simulium sp.	*		
Phalacrocera sp.?	*		
Thaumalea		*	
Hemiptera			
Microvelia reticulata?			*
BMWP Scores	48	32	27

A4.3.2 *Gammarus pulex* bioassays

a) Field mortality

The mortality of caged *Gammarus* at the three field stations is presented in Table A4.25. Mortality was highest in Population 1 and accounted for a quarter of animals deployed at Station 3. No animals from Population 2 died at either of Stations 1 or 3 and very few died at Station 2.

Table A4.25 - Percentage field mortality of Gammarus at three stations in the West Okement catchment

Station	Population 1	Population 2
1	10	0
2	12	2
3	25	0

b) Feeding rate in the field

There was a significant station by population interaction due to Population 1 feeding less at Stations 2 and 3 than at Station 1 (Figure A4.9, Table A4.26; $q=4.434$ and 5.926, $P<0.025$ and <0.001 respectively).

Animals from Population 2 did not show a similar trend ($q<1.852$, $p>0.05$). There was a significant population by laboratory interaction effect due to Laboratory 1 consistently measuring a higher value than Laboratory 2 for Population 1 and a lower value than Laboratory 2 for Population 2 ($q>4.005$, $P<0.025$).

A significant difference in the feeding rate of animals between baskets was found for Population 1 at Station 3 ($F=3.82$, $P=0.011$). There was no such effect for Population 2 at Station 3, and at Stations 1 and 2 neither population showed basket effects (highest $F=1.20$, $P>0.05$).

The above shows that only the feeding of Population 1 differed significantly between stations, with a reduction downstream of the two metallic discharges. There was also evidence to suggest that the positioning of baskets had an effect on the response of Population 1 at Station 3.

Table A4.26 - Results of ANOVA performed on the feeding rate of Gammarus in the West Okement trial

Source of variation	df	Sum of squares	Mean square	F	P	Significance level
Station	2	109.308	54.654	9.62	<0.001	***
Population	1	27.874	27.874	4.91	0.028	*
Laboratory	1	11.625	11.625	2.05	0.154	
Station.Population	2	69.458	34.729	6.11	0.003	**
Station.Laboratory	2	2.616	1.308	0.23	0.794	
Population.Laboratory	1	42.019	42.019	7.40	0.007	**
Station.Pop.Lab	2	0.297	0.149	0.03	0.974	
Residual	269	1527.937	5.680			
Total	280	1791.131				

*** = highly significant (P <0.001)
** = very significant (P <0.01)
* = significant (P <0.05)

c) Respiration rate of Gammarus returned from the field

Animals taken from Station 3 respired at a significantly lower rate than those taken from Station 1 ($q=3.406$, $P<0.05$; Figure A4.10, Table A4.27). There were no significant higher order interactions between station, population or laboratory.

Table A4.27 - Results of ANOVA performed on the respiration rate of Gammarus from the West Okement trial

Source of variation	df	Sum of squares	Mean square	F	P	Significance level
Station	2	0.08009	0.04005	3.15	0.048	*
Population	1	0.01778	0.01778	1.40	0.240	
Laboratory	1	0.00263	0.00263	0.21	0.650	
Station.Population	2	0.00073	0.00036	0.03	0.972	
Station.Laboratory	2	0.01032	0.00516	0.41	0.668	
Population.Laboratory	1	0.00499	0.00499	0.39	0.533	
Station.Pop.Lab	2	0.02252	0.01126	0.89	0.416	
Residual	82	1.04242	0.01271			
Total	93	1.18147				

* = significant ($P < 0.05$)

d) Scope for growth of Gammarus deployed in the field

As for respiration rate, there was a significant difference in SFG between Stations 1 and 3 (Figure A4.11, Table A4.28; $q=3.601$, $P<0.05$). Again, there were no significant interactions between the main effects of station, population or laboratory, although the chance of committing a Type II error was again high in this test (50%-60%).

Table A4.28 - Results of ANOVA performed on the SFG of Gammarus from the West Okement trial

Source of variation	df	Sum of squares	Mean square	F	P	Significance level
Station	2	2.6718	1.3359	3.33	0.041	*
Population	1	0.0558	0.0558	0.14	0.710	
Laboratory	1	0.2512	0.2512	0.63	0.431	
Station.Population	2	1.6478	0.8239	2.05	0.135	
Station.Laboratory	2	0.2983	0.1492	0.37	0.691	
Population.Laboratory	1	0.9563	0.9563	2.38	0.127	
Station.Pop.Lab	2	0.4565	0.2282	0.57	0.569	
Residual	82	32.9268	0.4015			
Total	93	39.2645				

* = significant

e) Persistence of field effects on feeding rate of Gammarus

There were no significant differences in the feeding rate of returned animals from the different stations (Figure A4.12, Table A4.29). There was a significant difference in feeding between populations for animals returned from the field, with Population 2 consuming the most. Feeding rates in the laboratory were broadly similar to those in the field for both populations.

Table A4.29 - Results of ANOVA performed on the feeding rate of Gammarus during the persistence of effect experiment following the West Okement trial

Source of variation	df	Sum of squares	Mean square	F	P	Significance level
Station	2	6.666	3.333	1.10	0.339	
Population	1	53.261	53.261	17.55	<0.001	***
Station.Population	2	11.157	5.579	1.84	0.166	
Residual	78	236.667	3.034			
Total	83	307.750				

*** = highly significant

A4.3.3 Tissue residues of heavy metals in Gammarus and leaf discs

a) Bulked samples of animals and leaf discs

The concentrations of metals found in Gammarus and leaf discs after deployment at the three field stations are presented in Table A4.30. It was not possible to analyse for aluminium due to contamination of the samples. There were higher concentrations of iron and nickel in leaf discs from Stations 2 and 3 than in those from Station 1. Station 3 discs also contained higher concentrations of manganese. Levels of manganese were appreciably higher in Gammarus from Stations 2 and 3 while those from Station 2 also accumulated a high level of iron.

Table A4.30 – Concentrations of metals in leaf and Gammarus tissue from the West Okement trial

Determinand	Station 1		Station 2		Station 3	
	Gammarus	Discs	Gammarus	Discs	Gammarus	Discs
Chromium	0.00866	0.0119	0.00097	0.00562	0.00236	0.0103
Manganese	0.04	0.71	0.12	0.62	0.13	1.4
Iron	0.2	1.8	1.2	17.4	0.8	7.1
Nickel	0.00819	0.0337	0.00153	0.139	0.00384	0.295
Copper	0.046	0.0872	0.0336	0.0722	0.0431	0.128
Zinc	0.0665	0.572	0.0584	0.405	0.0584	0.782
Cadmium	0.00272	0.00427	0.00157	0.00183	0.00243	0.00446
Lead	<0.0008	0.0259	<0.0008	0.019	<0.0008	0.0326

All results are reported in mg/g

b) Individual animal analysis

Individual animal analyses revealed significant differences in the body burdens of animals between stations for aluminium and iron (Figure A4.13, Table A4.31). Both populations from Station 3 carried a heavier aluminium load than those from Stations 1 or 2 (q=4.071 and

Table A4.31 - Results of ANOVA performed on the metal body loads of Gammarus from the West Okement trial

Metal	Source of variation	df	Sum of squares	Mean square	F	P	Sig. levels
Al	Station	2	2 770 354	1 385 177	5.168	0.0088	**
	Population	1	359 600	359 600	1.342	0.2518	
	Station.Population	2	936 271	468 135	1.747	0.1840	
	Residual	54	14 472 309	268 005			
Cr	Station	2	3.154	1.577	0.216	0.8070	
	Population	1	35.331	35.331	4.831	0.0348	*
	Station.Population	2	6.192	3.096	0.423	0.6580	
	Residual	37	270.611	7.314			
Fe	Station	2	15 032 790	7 516 395	6.155	0.0039	**
	Population	1	2 443 394	2 443 394	2.001	0.163	
	Station.Population	2	7 551 665	3 775 832	3.092	0.0535	
	Residual	54	1 221 187				
Mn	Station	2	147 379	73 689	14.410	<0.001	***
	Population	1	19 085	19 085	3.732	0.0587	
	Station.Population	2	35 730	17 865	3.494	0.0376	*
	Residual	53	271 026	5 113			
Ni	Station	2	143.74	71.87	1.914	0.0838	
	Population	1	117.64	117.64	3.133	0.1037	
	Station.Population	2	3.67	1.84	0.049	0.9523	
	Residual	43	1614.45	37.55			
Zn	Station	2	85 454	42 727	12.334	<0.001	***
	Population	1	5 568	5 568	1.607	0.2103	
	Station.Population	2	29 804	14 902	4.302	0.0185	*
	Residual	54	187 062	3 464			

* = significant (P <0.05)
** = very significant (P <0.01)
*** = highly significant (P <0.001)

3.789, P<0.025 and <0.05 respectively). Both populations from Station 3 also carried a heavier iron burden than those at Station 1 (q= 4.907, P<0.005). There was a difference between populations alone for chromium burdens, with Population 2 carrying a higher load. For manganese and zinc there was a station by population interaction, with a higher loading of both metals present in Population 2 at Station 3 (q>6.072, P<0.001). There were no significant differences between stations or populations for body burdens of nickel.

This shows that animals from Population 1 had a significantly higher aluminium body burden at Station 3, while Population 2 had significantly higher body burdens of not only aluminium, but also iron, manganese and zinc at Station 3. A relationship could be demonstrated between the tissue residues of iron and manganese and feeding rate in animals from Population 1, but not in those from Population 2 (Table A4.32). As levels of iron and manganese increased in the tissues of Population 1 their feeding rate fell.

Table A4.32 - Correlation coefficients for feeding rate against tissue residues of heavy metals in the West Okement trial

Metal	Population 1	Population 2
Aluminium	-0.31	-0.22
Chromium	0.12	0.16
Iron	-0.44*	-0.11
Manganese	-0.39*	-0.06
Nickel	-0.21	0.26
Zinc	-0.09	0.001

* = significant at P<0.05

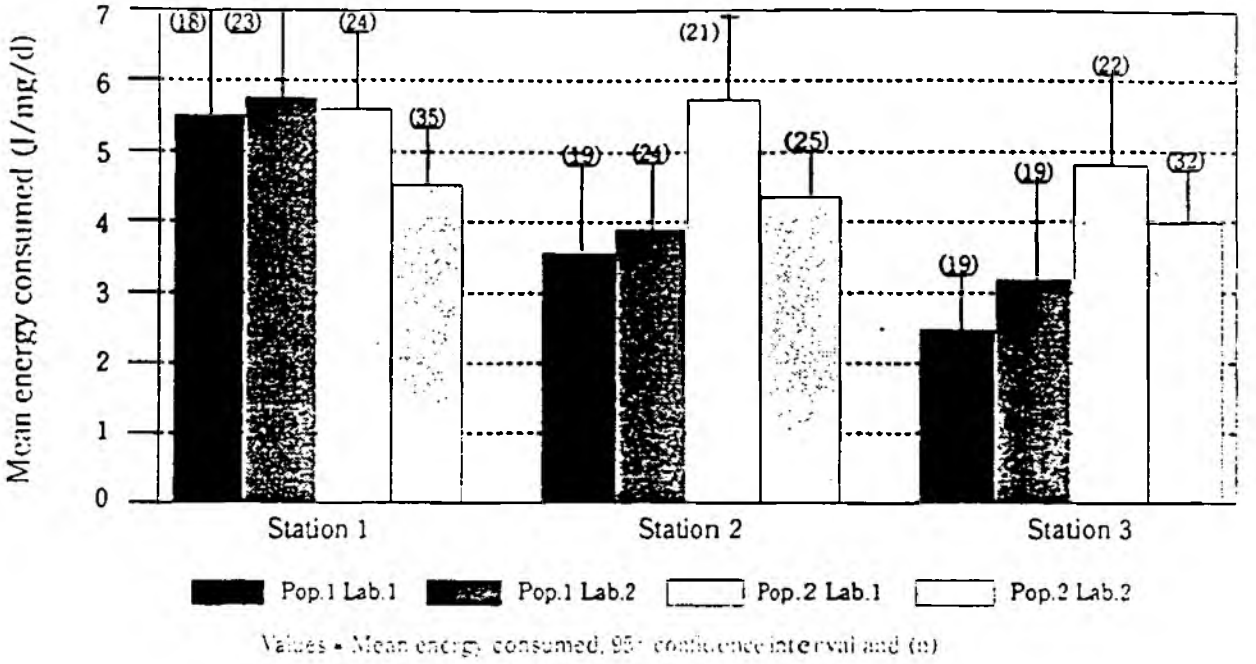
A4.3.4 Summary of main results from the West Okement catchment

Biological and chemical sampling suggested a decline in water quality between Station 1, the upstream site, and Stations 2 and 3, both downstream from metallic quarry discharges. Mortality at all three stations was low in Population 2, but accounted for 10% and 12% of Population 1 at Stations 1 and 2 respectively and 25% of Population 1 at Station 3.

Animals from Population 1 also fed and respired less at downstream stations and had a lower SFG. Animals from Population 2 did not respond in a similar fashion, despite absorbing higher concentrations of some metals.

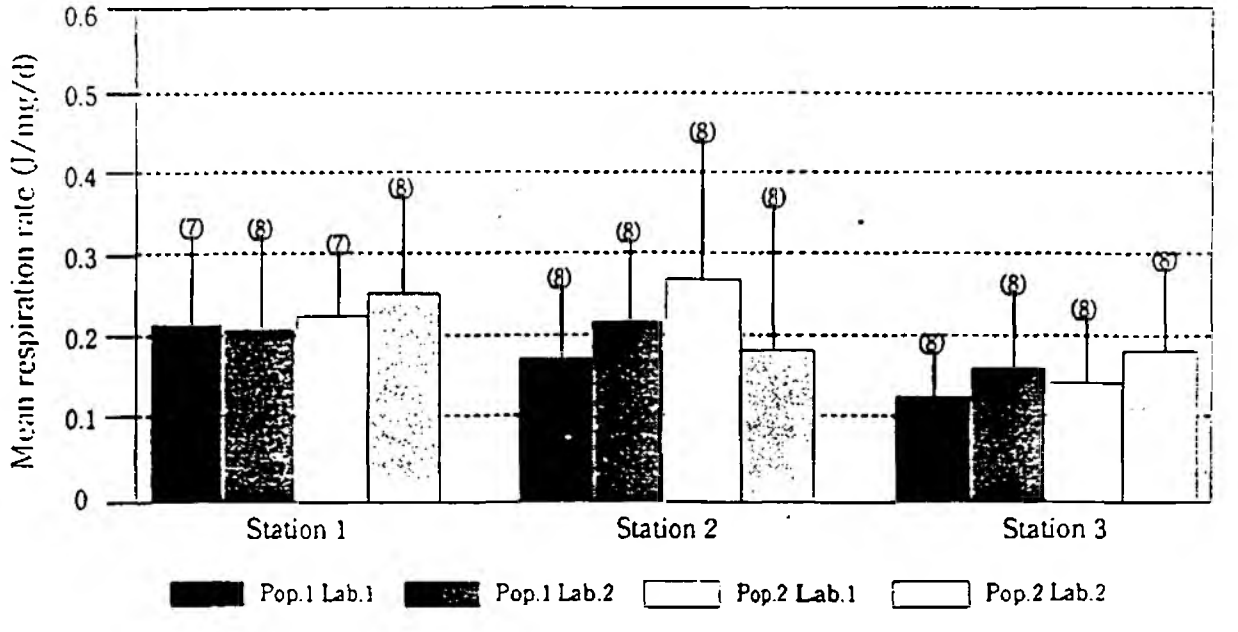
These results suggest that the quality of the water at Stations 2 and 3 was lower than at Station 1.

Figure A4.9 Energy consumed by *Gammarus pulex* at three stations in the W.Okement catchment.



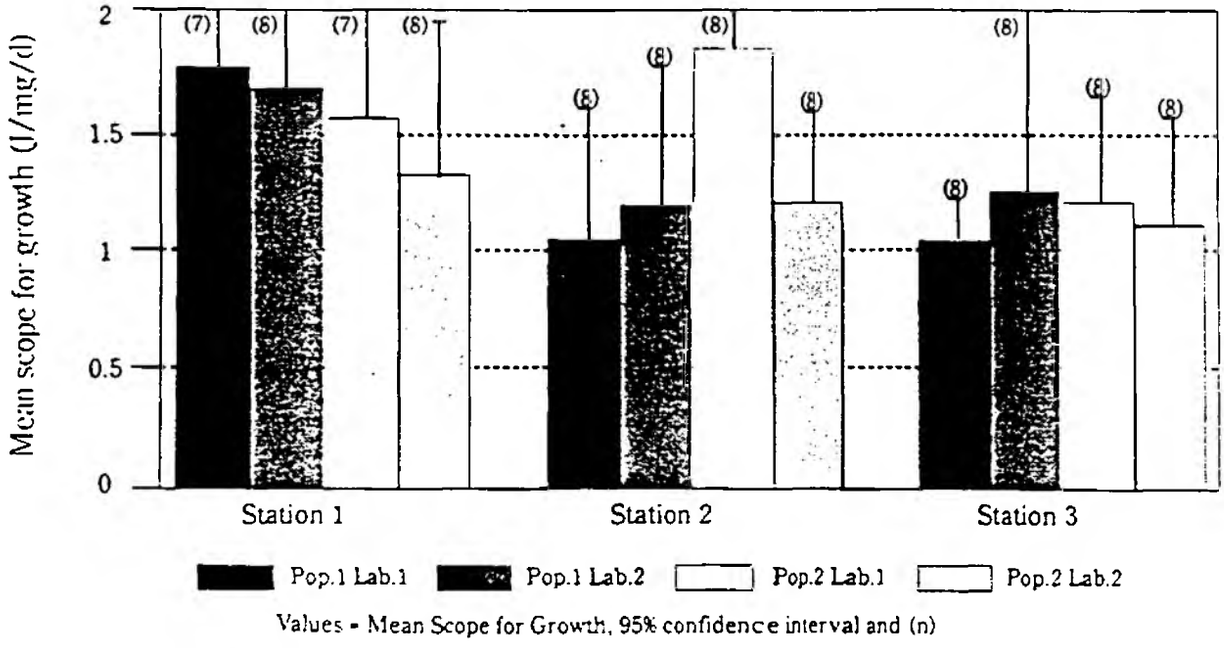
Values = Mean energy consumed, 95% confidence interval and (n)

Figure A4.10 Respiration rate of *Gammarus pulex* at three stations in the W.Okement catchment.



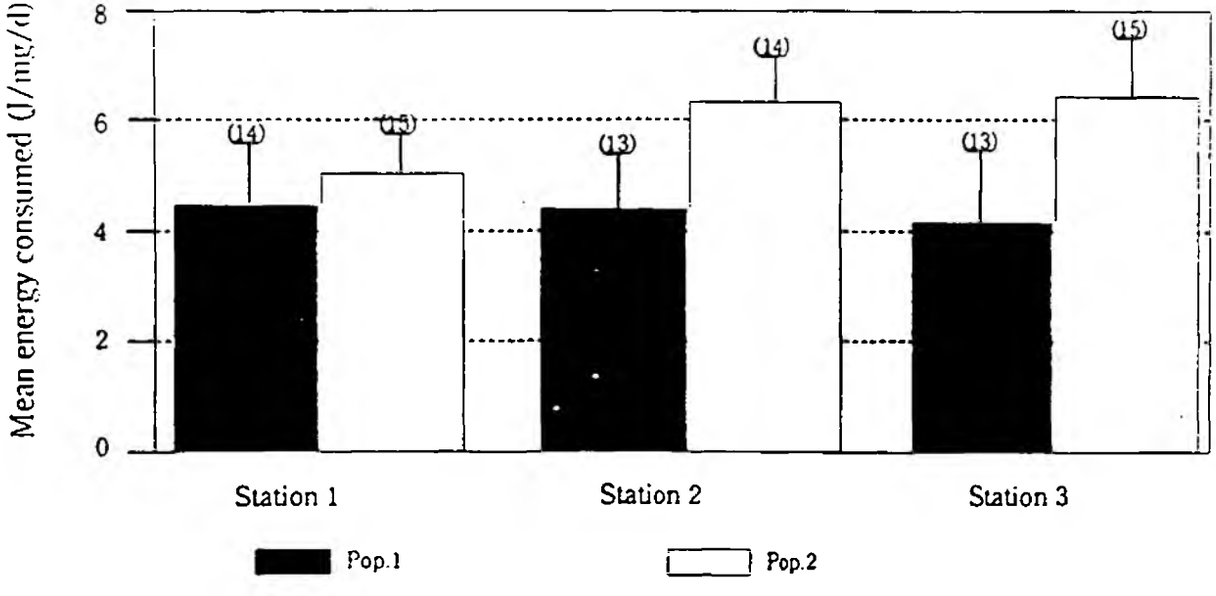
Values = Mean respiration rate, 95% confidence interval and (n)

Figure A4.11 Scope for growth of *Gammarus pulex* from three stations in the W.Okement catchment.



Values = Mean Scope for Growth, 95% confidence interval and (n)

Figure A4.12 Persistence of field effects on the feeding of *Gammarus pulex* : W. Okement.



Values = Mean energy consumed, 95% confidence interval and (n)

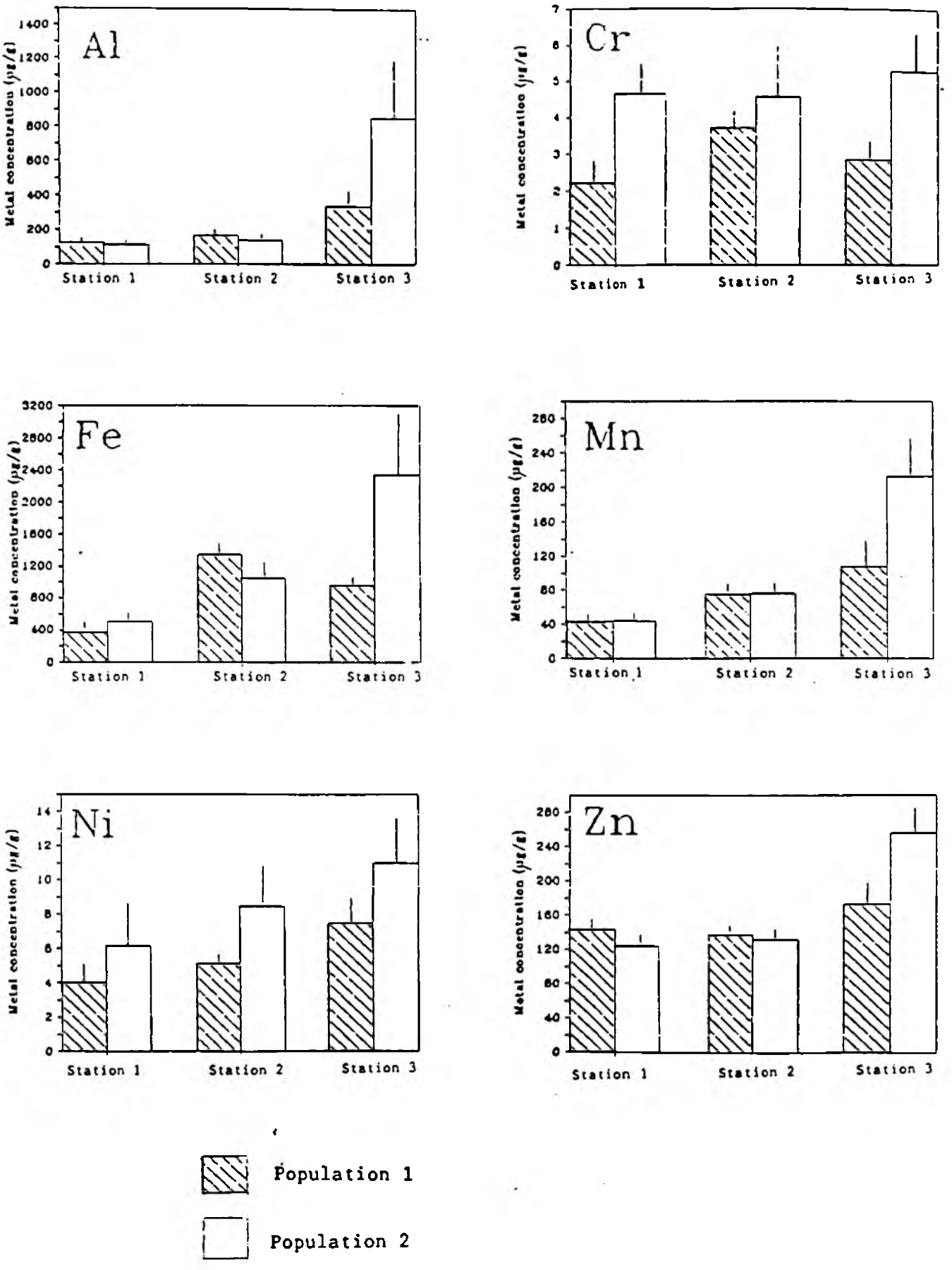


Figure A4.13 Metal body burdens of Gammarus after deployment for 6 days at three stations on the West Okement.
Values are means and one standard error.

A4.4 ERME CATCHMENT

A4.4.1 Chemical and biological sampling

a) Physico-chemical characteristics

The results of the water analyses from the three stations are presented in Table A4.33. All three stations had higher than EQS concentrations of manganese, with Stations 2 and 3 exhibiting higher concentrations than Station 1. Phenol and organochlorine levels were very low at all three stations, although phenol and dichlorophenol levels were elevated at Stations 2 and 3 at the time of deployment. The flow rate varied between stations and sampling dates, but not in any systematic way. The range of temperature recorded from Station 1 was considerably wider than that from the other two stations. This was probably due to a drop in water level over the deployment period at this station. The conductivity of the water recorded from Stations 2 and 3 was higher than that recorded from Station 1, but not by a large amount. This suggests that the difference in dissolved ionic substances between stations was low. The pH at all stations was low, but particularly so at Station 3 on deployment of the Gammarus cages, when a pH of 4.49 was measured.

During this deployment a pulse discharge was observed from the second effluent channel and samples were taken for analysis (Table A4.34). The pulse discharge contained considerably higher levels of suspended solids, copper, zinc, manganese and aluminium than that found in the normal effluent. The volume of the discharge also increased greatly during the pulse, but we were unable to quantify this, or the concentration of determinands in the receiving water.

b) Invertebrate sampling

The invertebrate species found in kick samples taken from each station are presented in Table A4.35. Stations 2 and 3 had a

Table A4.33 - Physico-chemical characteristics of Erme deployment stations

Parameter	Deployment (16/8/89) Station			Retrieval (22/8/89) Station		
	1	2	3	1	2	3
Width (m)	4.0	4.0	7.0			
Depth (cm)	20.0	48.0	29.0			
Flow rate (m/s)	0.35	0.12	-	0.10	0.052	0.122
Temperature (min-max °C)				10.5-23	14-18	15-16
Conductivity (µmhos)	60	80	80	80	100	95
pH	6.67	6.04	4.49 *	6.86	6.27	6.37
Alkalinity (as $CaCO_3$)	6	6	6	11.6	8.4	6.5
TOC (as C)	3.1	3.1	3.1	1.7	2.5	2.4
Ammonia (as N)	0.024	0.022	<0.02	<0.03	<0.03	<0.03
Nitrate (as N)	0.39	0.48	0.48	0.86	0.89	0.65
Nitrite (as N)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
SS	2.0	2.0	2.5	1.5	1.0	2.0
Chloride	11.6	11.5	11.5	12.2	11.6	11.0
Sulphate	6.6	13.4	16.6	11.4	20.3	19.6
SRP (as P)	<0.01	<0.01	<0.01	<0.03	<0.03	<0.03
Calcium	3.03	5.7	6.54	6.11	8.42	7.85
Magnesium	1.31	1.40	1.42	1.98	1.68	1.47
Sodium	7.39	7.98	8.2	9.6	9.45	9.15
Potassium	1.5	1.5	1.5	1.4	1.4	1.3
Copper	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004
Zinc	<0.003	<0.003	<0.003	0.005	<0.003	<0.003
Chromium	<0.001	<0.001	<0.001			
Nickel	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008
Cadmium	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004
Lead	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Manganese	0.009 *	0.055 *	0.072 *	0.014 *	0.092 *	0.102 *
Iron	0.153	0.156	0.141	0.057	0.06	0.069
Aluminium	0.09 *	0.1 *	0.09 *	<0.04	0.06 *	0.07 *
Organochlorine (as Cl)	0.015	0.013	0.017	0.013	0.015	0.015
Phenol	0.0006	0.00032	0.00027	0.00032	0.00033	0.00028
2-chlorophenol	0.00005	0.00006	0.00009	<0.00002	<0.00002	<0.00002
4-chlorophenol	0.00018	0.00018	0.00018	0.00012	0.00013	0.00012
Dichlorophenol	<0.00002	0.00019	0.00019	<0.00002	0.00002	<0.00002
Trichlorophenol	<0.00004	<0.00004	<0.00004	0.00032	0.0004	0.00036
Penta-chlorophenol	<0.0001	<0.0001	<0.0001	<0.00005	<0.00005	<0.00005

TOC = Total Organic Carbon
SS = Suspended Solids
SRP = Soluble Reactive Phosphorus
Results reported in mg/l unless otherwise stated. Values exceeding actual or proposed EQS levels are asterisked.

Table A4.34 - Chemical characteristics of Erme discharge effluents

Parameter	Deployment (16/8/89)			Retrieval(22/8/89)	
	Effluent 1	Effluent 2		Effluent 1	Effluent 2
	Normal	Normal	Pulse	Normal	Normal
Alkalinity (as $CaCO_3$)	5	6	5	6.7	6.0
TOC (as C)	3.3	3.3	-	2.6	2.5
Ammonia (as N)	<0.02	<0.02	0.04	<0.03	<0.03
Nitrate (as N)	0.32	0.38	0.23	0.55	0.56
Nitrite (as N)	<0.01	<0.01	0.016	<0.01	<0.01
SS	2.0	0.5	54.0	2.5	1.5
Chloride	11.4	11.6	11.8	11.0	10.8
Sulphate	20.7	20.3	19.7	24.5	20.4
SRP (as P)	<0.01	<0.01	<0.01	<0.03	<0.03
Calcium	7.81	7.46	7.33	8.97	7.63
Magnesium	1.34	1.38	1.35	1.38	1.32
Sodium	8.31	8.31	8.41	9.49	9.01
Potassium	1.5	1.5	1.5	1.3	1.3
Copper	<0.004	<0.004	0.012	<0.004	<0.004
Zinc	<0.003	<0.003	0.014	0.005	<0.003
Chromium	<0.001	<0.001	<0.001		
Nickel	<0.008	<0.008	<0.008	<0.008	<0.008
Cadmium	<0.004	<0.004	<0.004	<0.004	<0.004
Lead	<0.05	<0.05	<0.05	<0.05	<0.05
Manganese	0.091	0.086	0.319	0.115	0.102
Iron	0.131	0.128	0.892	0.059	0.06
Aluminium	0.08	0.08	1.65	0.06	0.06
Organochlorine (as Cl)	0.021	0.019	0.023	0.032	0.016
Phenol	0.00048	0.00032	0.00018	0.00044	0.00028
2-chlorophenol	0.00006	0.00006	0.00005	<0.00002	<0.00002
4-chlorophenol	0.0002	0.00018	<0.00004	0.00012	0.00019
Dichlorophenol	0.00018	<0.00002	<0.00002	<0.00002	<0.00002
Trichlorophenol	<0.00004	<0.00004	<0.00004		
Penta-chlorophenol	<0.0001	<0.0001	<0.0001	<0.00005	<0.00005

Results reported in mg/l

poorer community than Station 1 and Gammarus was not present at any station. The substrate sampled in the Erme was similar to that in the West Okement and presented the same problems. Thus the results should be regarded as a relative rather than an absolute measure of species richness.

Table A4.35 - Invertebrates found in kick samples from three stations in the Erme catchment

Taxon	Station 1	Station 2	Station 3
OLIGOCHAETA			
Tubifex tubifex		*	*
ARTHROPODA			
Ephemeroptera			
Ephemerella sp.	*		
Baetis sp.		*	
Plecoptera			
Isoperla sp.	*	*	*
Leuctra sp	*	*	*
Trichoptera			
Lepidostoma sp.	*		
Sericostoma personatum	*	*	
Philopotamus sp.	*		
Polycentropus sp.	*		
Limnephilus sp.			*
Coleoptera			
Atrichopogon sp.	*		
Hygrobia hermanni			*
Dicranota sp.			*
Diptera			
Chironomidae	*	*	*
Ceratopogonidae	*		
BMWP Scores	67	41	40

A4.4.2 *Gammarus pulex* bioassays

a) Field mortality

The mortality of caged *Gammarus* at the three field stations is presented in Table A4.36. Mortality was greater in Population 1 at all three stations, but both populations suffered their highest mortality at Station 3.

Table A4.36 - The percentage field mortality of *Gammarus* deployed at three stations in the Erme catchment

Station	Population 1	Population 2
1	8	2
2	11	2
3	23	11

b) Feeding rate in the field

There was a significant station by population interaction due to Population 1 at Station 1 feeding at a significantly higher rate than at Stations 2 or 3 ($q=4.511$ and 6.308, $P<0.05$ and <0.001 respectively; Figure A4.14, Table A4.37). The feeding of Population 2 was also depressed at Station 2 ($q=5.062$, $P<0.005$) but not at Station 3 ($q=1.811$, $P>0.05$). There were also significant differences in the response of each population at Stations 1 and 2, with Population 1 feeding more than Population 2 ($q=5.231$ and 5.246 respectively, $P<0.005$). There were significant differences in the measurement of feeding between the two laboratories, with Laboratory 2 generally measuring a lower rate than Laboratory 1.

A significant difference in the feeding rate of animals between baskets was found for Population 1 at Station 1 ($F=3.82$, $P=0.011$).

There was no such effect for Population 2 at Station 1, and at Stations 2 and 3 neither population basket effects (highest F=2.24, P>0.05).

These results show that the feeding of both populations was lower at Station 2 than at Station 1. The feeding rate of Population 2 increased at Station 3 but remained low in animals from Population 1. There was also evidence to suggest that the position of the holding baskets at Station 1 influenced the response of Population 1.

Table A4.37 - Results of ANOVA performed on the feeding rate of Gammarus in the Erme trial

Source of variation	df	Sum of squares	Mean square	F	P	Significance level
Station	2	127.059	63.530	12.74	<0.001	***
Population	1	100.680	100.680	20.20	<0.001	***
Laboratory	1	35.691	35.691	7.16	0.008	**
Station.Population	2	36.757	18.378	3.69	0.026	*
Station.Laboratory	2	5.728	2.864	0.57	0.564	
Population.Laboratory	1	7.772	7.772	1.56	0.213	
Station.Pop.Lab	2	7.120	3.560	0.71	0.491	
Residual	263	1310.984	4.985			
Total	274	1631.785				

*** = highly significant (P <0.001)
** = very significant (P <0.01)
* = significant (P <0.05)

c) Respiration rate of Gammarus returned from the field

There were no significant differences in the measurement of energy respired between any station, population or laboratory (Figure A4.15, Table A4.38), but the power of the test to detect any differences was very low.

Table A4.38 - Results of ANOVA performed on the respiration rate of Gammarus in the Erme trial

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	2	0.01944	0.00972	0.29	0.748	
Population	1	0.02275	0.02275	0.68	0.411	
Laboratory	1	0.02970	0.02970	0.89	0.348	
Station.Population	2	0.07494	0.03747	1.12	0.330	
Station.Laboratory	2	0.03151	0.01575	0.47	0.625	
Population.Laboratory	1	0.01656	0.01656	0.50	0.483	
Station.Pop.Lab	2	0.02306	0.01153	0.35	0.709	
Residual	81	2.70123	0.03335			
Total	92	2.91926				

d) Scope for growth of Gammarus deployed in the field

There were significant differences in the SFG of animals between stations and populations, and a station by population interaction fell just short of significance at the 5% level (Figure A4.16, Table A4.39). Population 1 had a lower SFG at Stations 2 and 3 than at Station 1 ($q=4.275$ and 5.144, $P<0.05$ and <0.01 respectively). There were no significant differences in the response of Population 2 at the different stations ($q<0.864$, $P>0.05$). There was also a significant difference between populations at Station 1 ($q=4.421$, $P<0.05$), with a higher SFG in Population 1 than Population 2.

Table A4.39 - Results of ANOVA performed on the SPG of Gammarus from the Erme trial

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	2	2.6105	1.3052	4.52	0.014	*
Population	1	1.1942	1.1942	4.13	0.045	*
Laboratory	1	0.6817	0.6817	2.36	0.128	
Station.Population	2	1.7450	0.8725	3.02	0.054	
Station.Laboratory	2	0.7098	0.3549	1.23	0.298	
Population.Laboratory	1	0.0046	0.0046	0.02	0.900	
Station.Pop.Lab	2	0.2617	0.1309	0.45	0.637	
Residual	81	23.4070	0.2890			
Total	92	30.6151				

* = significant (P <0.05)

e) Persistence of field effects on feeding rate of Gammarus

There were no significant differences in the energy consumed by animals between either sites or populations after removal from the field (Figure A4.17, Table A4.40). Feeding rates in the laboratory tended to be slightly higher than those found in the field.

Table A4.40 - Results of ANOVA performed on the feeding rate of Gammarus during the persistence of effect experiment following the Erme trial

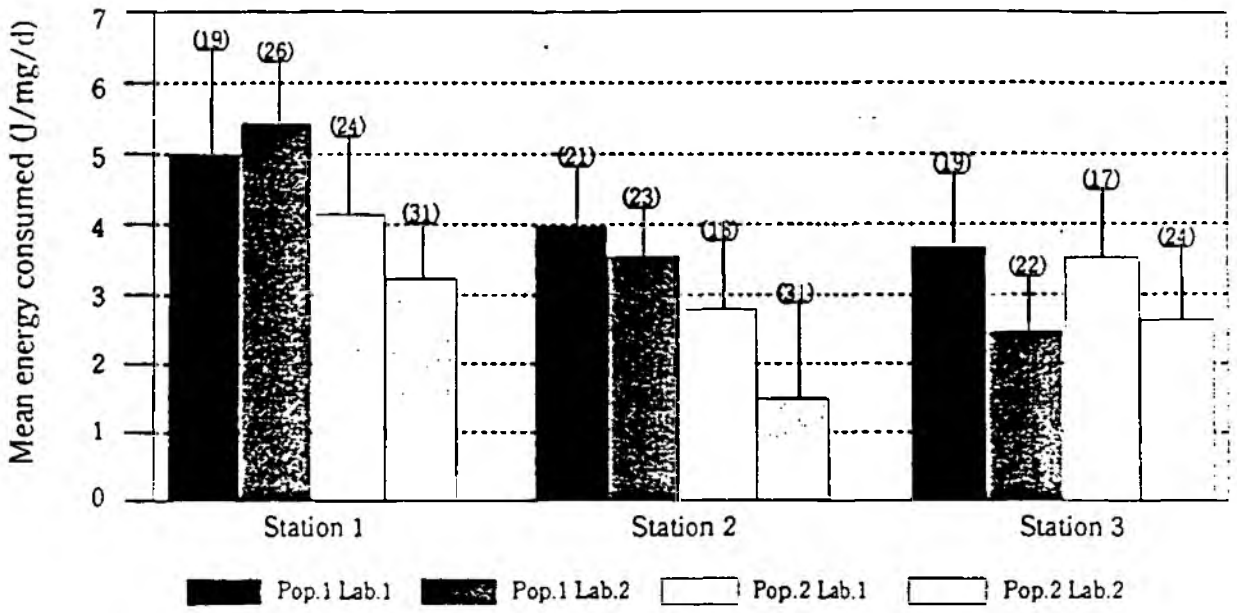
Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	2	7.062	3.531	0.92	0.401	
Population	1	5.310	5.310	1.39	0.242	
Station.Population	2	8.395	4.198	1.10	0.339	
Residual	81	309.842	3.825			
Total	86	330.610				

A4.4.3 Summary of main results from the Erme catchment

Biological sampling suggested a decline in water quality between Station 1, the upstream site, and Stations 2 and 3, both downstream from the paper mill discharges. Chemical sampling did not produce a clear indication of water quality, with all the stations exceeding EQS levels for two or more determinands. Analysis of a pulse discharge that occurred upstream from Station 3 during the deployment of Gammarus revealed high levels of some heavy metals and a low pH. The mortality in Population 2 animals was low (2%) at Stations 1 and 2, rising to 11% at Station 3. Population 1 had mortality levels of 8% and 11% respectively at Stations 1 and 2, and 23% at Station 3. The feeding rate of both populations was depressed at Station 2, downstream from the first effluent discharge. Only animals from Population 1 fed less at Station 3, downstream from the second effluent discharge. No significant differences could be detected between stations, populations or laboratories for respiration rate or persistence of field effects. The SFG of Population 1 was depressed at both downstream stations.

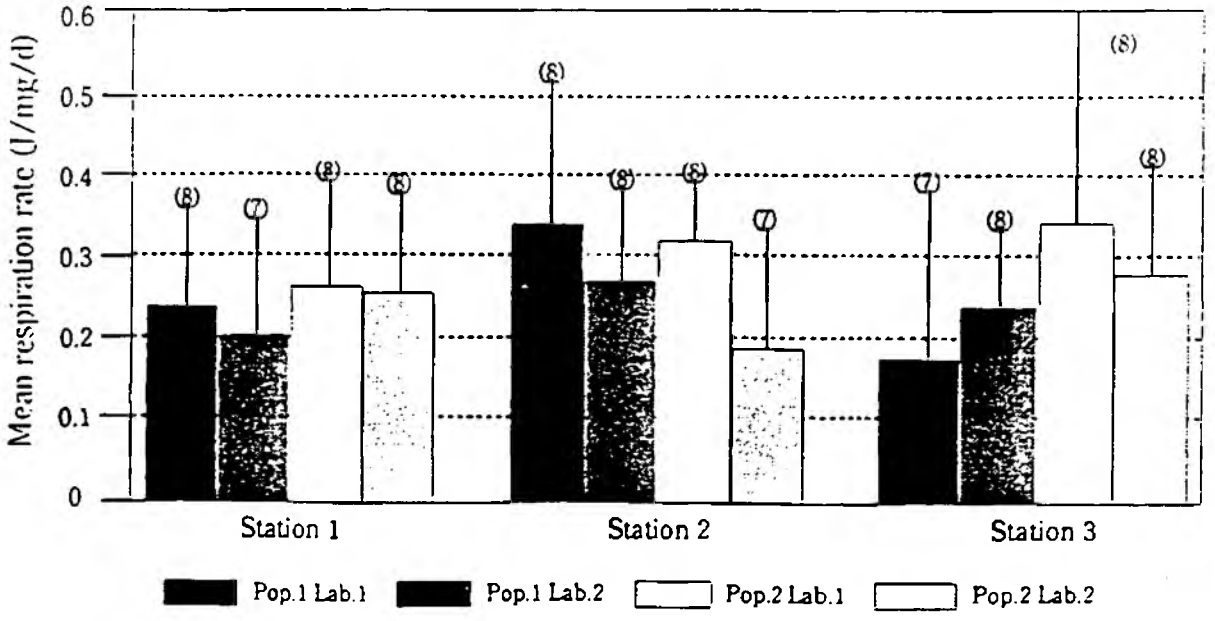
These results suggest that the water quality at both stations downstream from the paper mill discharges was lower than that found upstream.

Figure A4.14 Energy consumed by *Gammarus pulex* at three stations in the Erme catchment.



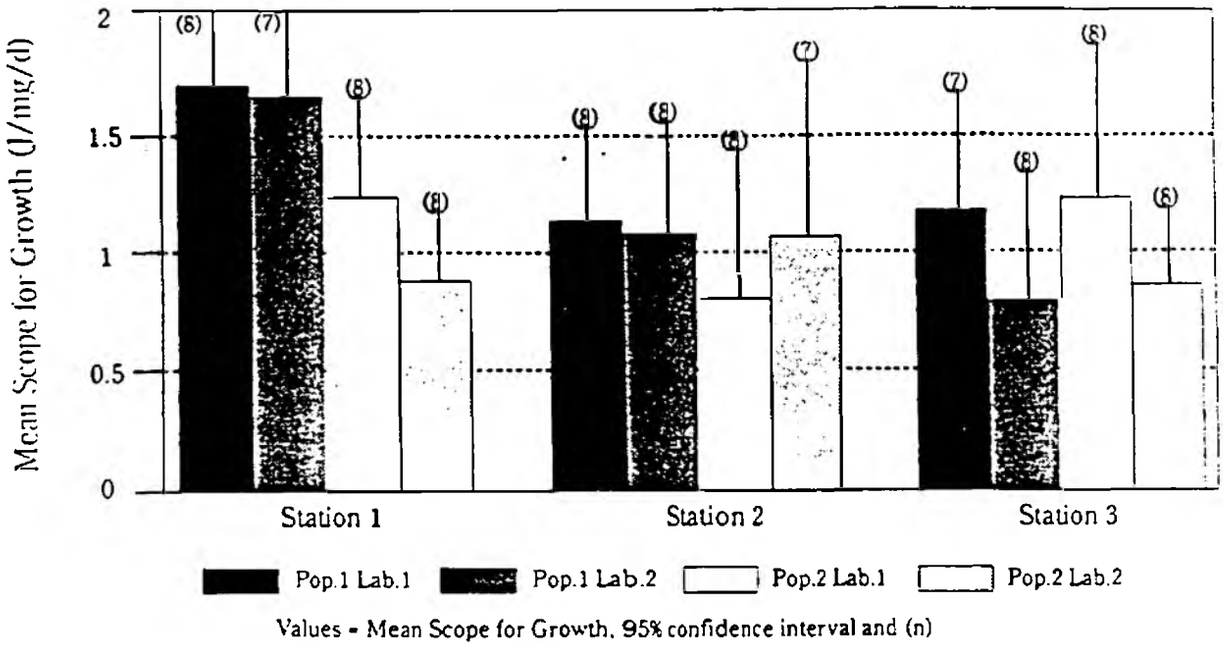
Values = Mean energy consumed, 95% confidence interval and (n)

Figure A4.15 Respiration rate of *Gammarus pulex* at three stations in the Erme catchment.



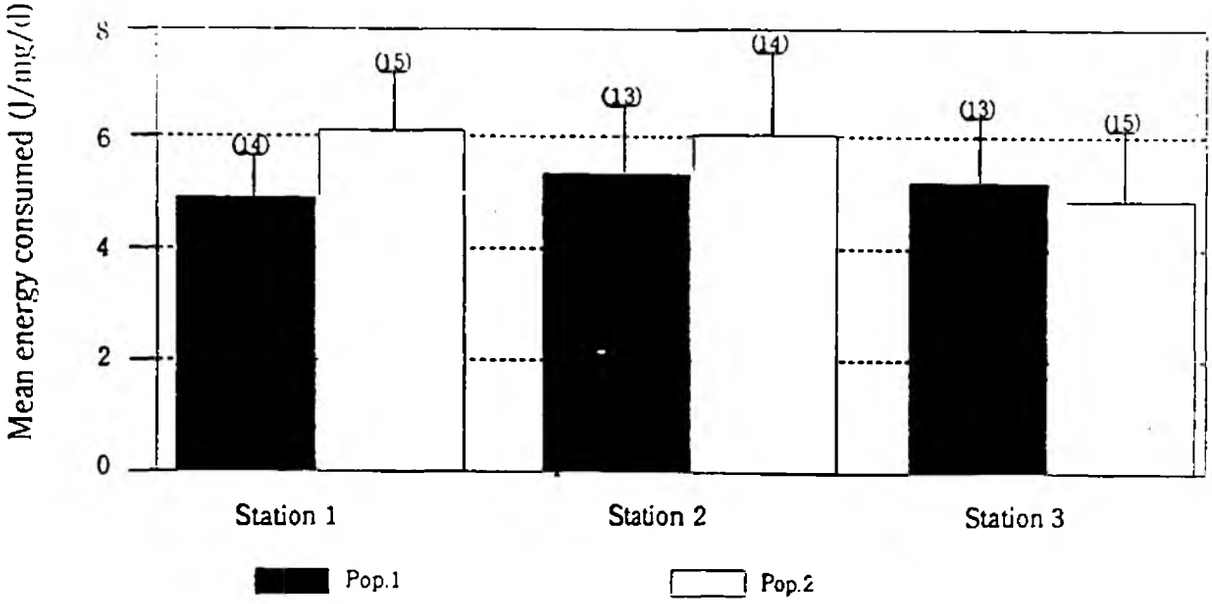
Values = Mean respiration rate, 95% confidence interval and (n)

Figure A4.16 Scope for growth of *Gammarus pulex* from three stations in the Erme catchment.



Values = Mean Scope for Growth, 95% confidence interval and (n)

Figure A4.17 Persistence of field effects on the feeding of *Gammarus pulex* : Erme.



Values = Mean energy consumed, 95% confidence interval and (n)

A4.5 HAMPSHIRE AVON CATCHMENT

A4.5.1 Chemical and biological sampling

a) Physico-chemical characteristics

The analyses of samples taken from the two stations are presented in Table A4.41. No determinands exceeded an existing or proposed EQS, but levels of ammonia were higher at Station 2 than at Station 1. All other measured parameters were very similar at the two stations.

b) Invertebrate sampling

The species of invertebrate found in kick samples taken from the two stations are presented in Table A4.42. Station 1 had a richer community than Station 2, and Gammarus were present at both stations.

A4.5.2 Gammarus pulex bioassays

a) Field mortality

The mortality of caged Gammarus at the two field stations is presented in Table A4.43. Mortality in both populations was low, but Population 1 displayed a lower mortality at Station 2 than at Station 1 while Population 3 showed a higher increase.

Table A4.41 - Physico-chemical characteristics of Hampshire Avon deployment stations

Parameter	Deployment (15/9/89) Station		Retrieval (21/9/89) Station	
	1	2	1	2
Width (m)	10	5		
Depth (cm)	112	150		
Flow rate (m/s)	0.22	0.24	0.36	0.24
Temperature (min-max °C)			14-16	14-18
Conductivity (µmhos)	410	410	429	430
pH	7.62	7.45	7.37	7.33
Alkalinity (as $CaCO_3$)	215	220	235	259
TOC (as C)	3.0	3.3	2.3	2.6
Ammonia (as N)	0.15	0.35	0.068	0.387
Nitrate (as N)	4.36	4.32	4.5	4.3
Nitrite (as N)	0.11	0.12	<0.5	<0.05
SS	0.6	2.0	2.2	8.6
Chloride	20.4	24.8	24.4	24.6
Sulphate	21.5	21.7	23.7	24.0
SRP (as P)	0.48	0.53		
Calcium	94.7	97.5	103.0	101.0
Magnesium	2.14	2.21	2.27	2.29
Sodium	13.8	14.2	14.0	13.7
Potassium	3.0	3.2	2.9	2.9
Copper	<0.004	<0.004	<0.004	<0.004
Zinc	0.008	0.01	0.005	0.007
Chromium	0.001	0.001	0.002	0.002
Nickel	<0.01	<0.01	<0.01	<0.01
Cadmium	<0.004	<0.004	<0.004	<0.004
Lead	<0.05	<0.05	<0.05	<0.05
Manganese	0.003	0.007	0.007	0.011
Iron	0.017	0.045	0.03	0.043
Aluminium	<0.04	<0.04	<0.04	<0.04

SS = Suspended Solids
TOC = Total Organic Carbon
SRP = Soluble Reactive Phosphorus
All results reported in mg/l unless otherwise stated.
No values exceeded actual or proposed EQS levels.

Table A4.42 - Invertebrates present in kick samples from two stations on the Hampshire Avon

Taxon	Station 1	Station 2
ANNELIDA		
Oligochaeta		
Lumbriculus variegatus	*	
Lumbricus terrestris	*	
Tubifex tubifex	*	*
Hirudinea		
Glossiphonia complanata	*	
Glossiphonia heteroclita	*	
MOLLUSCA		
Gastropoda		
Ancylus lacustris	*	
Viviparus viviparus		*
Potamopyrgus jenkinsi	*	
Lymnaea trunculata	*	
Planorbis leucostoma	*	
Planorbis laevis	*	
Planorbis carinatus		*
Lymnaea peregra		*
Segmentina complanata		*
Bivalvia		
Sphaerium corneum	*	
Pisidium sp.	*	
ARTHROPODA		
Isopoda		
Asellus aquaticus		*
Amphipoda		
Gammarus pulex	*	*
Trichoptera		
Brachycentrus subnubilis	*	
Philopotamidae		*
Limnephilus sp.	*	
Stenophylax sp.	*	
Polycentropodidae		*
Hemiptera		
Aphelocheirus aestivalis	*	
Corixa sp.		*
BMWP Score	59	42

Table A4.43 - Field mortality of Gammarus at two stations on the Hampshire Avon

Station	Population 1	Population 3
1	10	5
2	4	12

b) Feeding rate in the field

There was a significant station by population by laboratory interaction due to Laboratory 2 measuring a significantly lower feeding rate for Population 3 at Station 2 than at Station 1 ($q=5.534$, $P<0.005$; Figure A4.18, Table A4.44). No differences were found in the feeding rate of either population between baskets within either station (highest $F=2.22$, $P>0.05$).

Table A4.44 - Results of ANOVA performed on the feeding rate of Gammarus in the Hampshire Avon trial

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	1	59.066	59.066	13.70	<0.001	***
Population	1	4.778	4.778	1.11	0.294	
Laboratory	1	13.830	13.830	3.21	0.076	
Station.Population	1	5.741	5.741	1.33	0.251	
Station.Laboratory	1	0.167	0.167	0.04	0.844	
Population.Laboratory	1	5.708	5.708	1.32	0.252	
Station.Pop.Lab	1	24.571	24.571	5.70	0.018	*
Residual	138	595.077	4.312			
Total	145	708.902				

*** = highly significant (P <0.001)
* = significant (P <0.05)

c) Respiration rate of Gammarus returned from the field

There was a significant station by population by laboratory interaction due to Laboratory 2 measuring a low respiration rate for Population 3 at Station 2 (Figure A4.19, Table A4.45; Scheffé procedure comparing Station 2, Population 1, Laboratory 2 set with the remaining three Population 3 measurement sets: S=3.14, P<0.01).

Table A4.45 - Results of ANOVA performed on the respiration rate of Gammarus from the Hampshire Avon trial

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	1	0.00008	0.00008	0.01	0.944	
Population	1	0.01538	0.01538	0.95	0.334	
Laboratory	1	0.00748	0.00748	0.46	0.500	
Station.Population	1	0.07805	0.07805	4.80	0.031	*
Station.Laboratory	1	0.01166	0.01166	0.72	0.400	
Population.Laboratory	1	0.01864	0.01864	1.15	0.287	
Station.Pop.Lab	1	0.11246	0.11246	6.92	0.010	**
Residual	79	1.28406	0.01625			
Total	86	1.52781				

** = very significant (P <0.01)
* = significant (P <0.05)

d) Scope for growth of Gammarus deployed in the field

There was a significantly lower SFG downstream from the discharge at Station 2 (Figure A4.20, Table A4.46). No station by population by laboratory interaction was found despite a power of 70% in this test.

Table A4.46 - Results of ANOVA performed on the SFG of Gammarus from the Hampshire Avon trial

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	1	2.8432	2.8432	7.63	0.007	**
Population	1	0.3104	0.3104	0.83	0.364	
Laboratory	1	0.9305	0.9305	2.50	0.118	
Station.Population	1	0.3703	0.3703	0.99	0.322	
Station.Laboratory	1	0.5235	0.5235	1.41	0.239	
Population.Laboratory	1	0.1934	0.1934	0.52	0.473	
Station.Pop.Laboratory	1	0.9907	0.9907	2.66	0.107	
Residual	79	29.4198	0.3724			
Total	86	35.5817				

** = very significant (P <0.01)

e) Persistence of field effects on the feeding rate of Gammarus

Animals belonging to Population 3 ate significantly less than those from Population 1 after their return from the field (Figure A4.21, Table A4.47). There were no significant differences between stations. Feeding rates in the laboratory tended to be higher than that measured in the field, except for Population 3, for which the reverse was true at Station 1.

Table A4.47 - Results of ANOVA performed on the feeding rate of Gammarus during the persistence of effect experiment following the Hampshire Avon trial

Source of variation	df	Sum of square	Mean square	F	P	Significance level
Station	1	8.014	8.014	1.60	0.213	
Population	1	139.255	139.255	27.72	<0.001	***
Station.Population	1	13.442	13.442	2.68	0.109	
Residual	47	236.142	5.024			
Total	50	396.853				

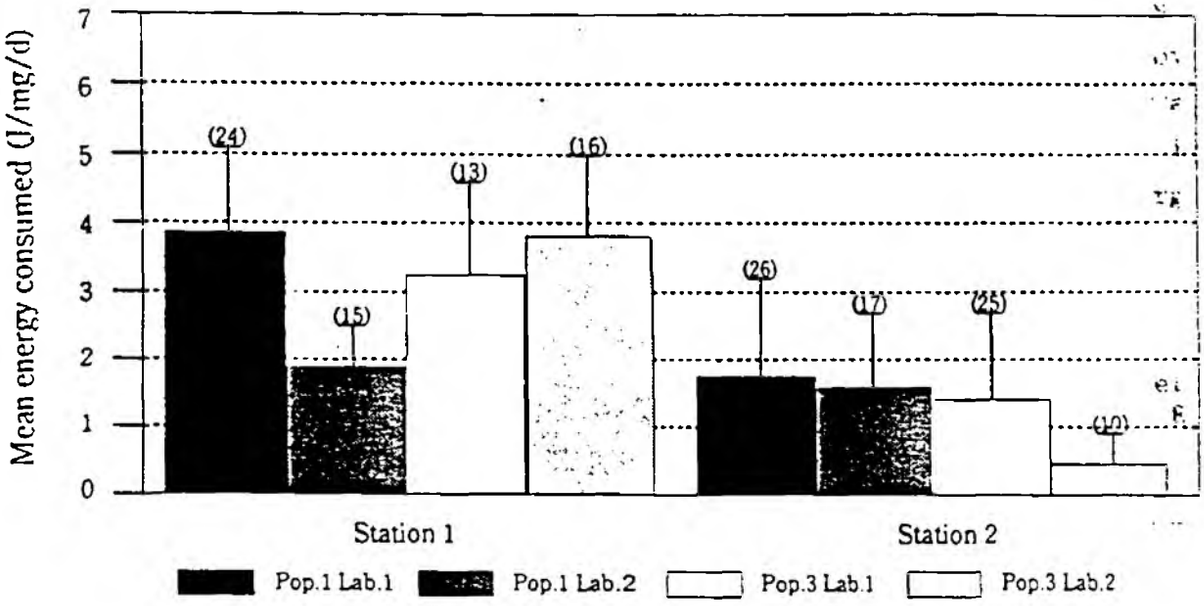
*** = highly significant (P <0.001)

A4.5.3 Summary of main results from the Hampshire Avon catchment

Biological sampling suggested a decline in water quality between Station 1, the upstream site, and Station 2, downstream from the fish farm. None of the chemical determinands that were analysed exceeded EQS levels. Patterns of Gammarus mortality were unusual. At Station 1, 10% of Population 1 and 5% of Population 3 died during the deployment period. At Station 2, a reversal occurred, with 4% of Population 1 and 12% of Population 3 dying during deployment. Although the mean feeding rate of both populations was lower at the downstream stations, the only significant difference was measured by Laboratory 2 on animals from Population 3. Laboratory 2 found a similar difference when measuring the respiration rate of Population 3. In the estimation of SFG only a difference between stations could be detected, with animals displaying a lower SFG at the downstream station.

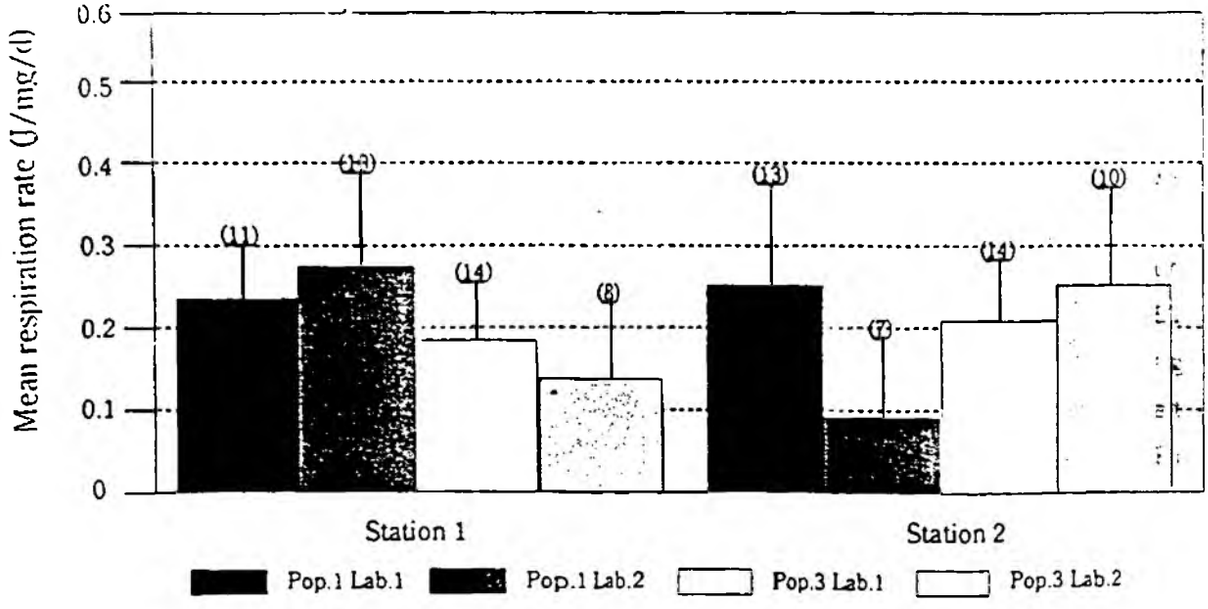
The results from this trial, although confusing, do suggest a deterioration in water quality downstream from the fish farm.

Figure A4.18 Energy consumed by *Gammarus pulex* at two stations in the Avon catchment.



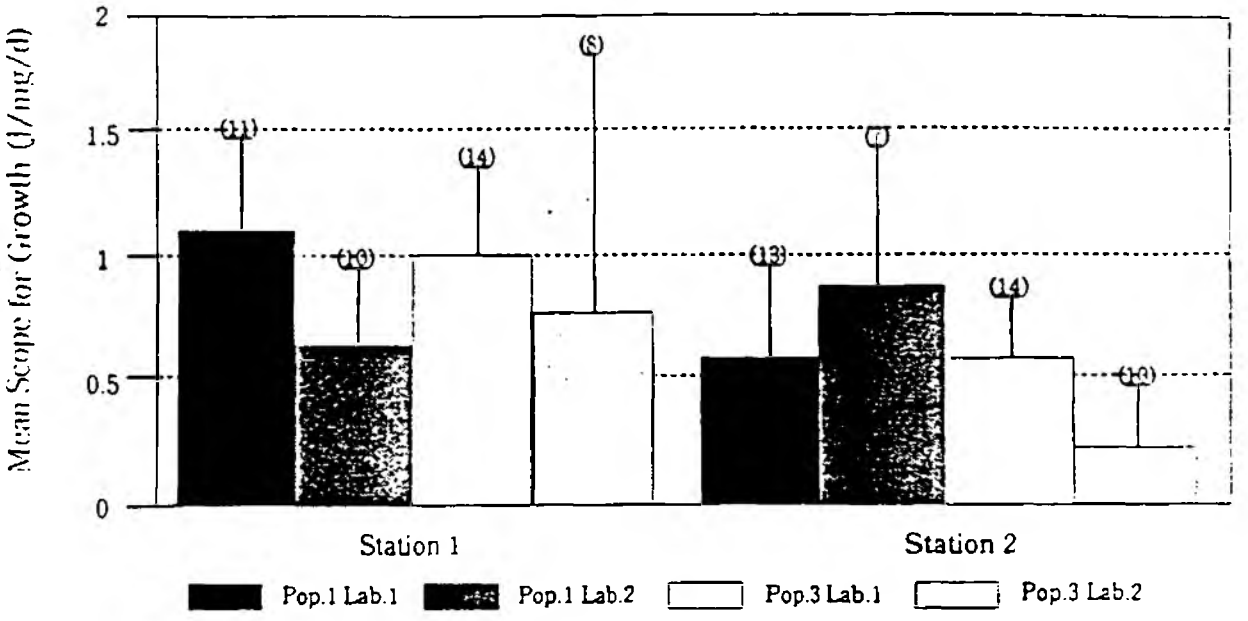
Values = Mean energy consumed, 95% confidence interval and (n)

Figure A4.19 Respiration rate of *Gammarus pulex* from two stations in the Avon catchment.



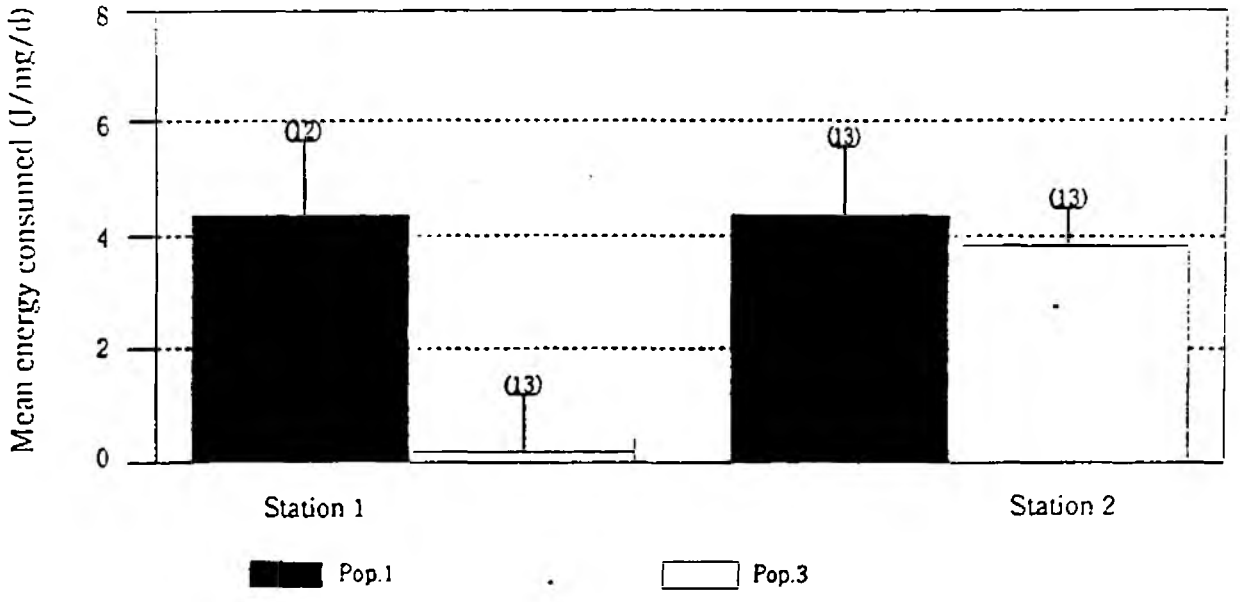
Values = Mean respiration rate, 95% confidence interval and (n)

Figure A4.20 Scope for growth of *Gammarus pulex* from two stations in the Avon catchment.



Values = Mean Scope for Growth, 95% confidence interval and (n)

Figure A4.21 Persistence of field effects on the feeding of *Gammarus pulex* : Avon.



Values = Mean energy consumed, 95% confidence interval and (n)

A4.6 SUMMARY OF GAMMARUS ENERGETICS RESULTS

Table A4.48 summarises the significant main effects and interactions detected in the analysis of feeding and respiration rate, SFG and persistence of field effects from the five field trials.

Significant differences in the feeding rate of animals in different treatment groups were found in all the trials. Both populations of Gammarus fed less at a downstream station in the Colne and Erme trials, while only Population 1 fed less at the downstream stations in the West Okement trial. In all the above, a depression in feeding rate matched the results obtained from invertebrate and chemical sampling. On the Lea trial, Population 1 fed less upstream from the discharge although the invertebrate samples suggested that water quality was slightly lower at the downstream station. Chemical samples taken from the Lea were equivocal, but suggested that water quality may have been poorer at the upstream station during the period of Gammarus deployment. The results from the Hampshire Avon were more confusing than those obtained from the previous four trials. Only Laboratory 2 measured a significant reduction in feeding rate for Population 3, the WRc-cultured Gammarus, at the downstream station.

Significant differences in the feeding rate of Gammarus between baskets at the same station occurred only twice (7% of the total). This suggests that small-scale changes of environment within a station are unlikely to affect deployed Gammarus in the majority of cases.

Significant differences in respiration rate were only found on the West Okement and Hampshire Avon trials. Estimates of SFG differed significantly between stations on all but the Lea trial, supporting most of the results from the feeding rate analyses.

The power of the tests of significance was low in the analyses of respiration rate and SFG, due to high variation and a small sample size. This may explain why fewer significant differences were detected using

Table A4.48 - Summary of Gammarus energetics analyses

Trial	Source of difference						
	Station	Pop	Lab	Station.Pop	Station.Lab	Pop.Lab	Station.Pop.Lab
A - Significant differences in feeding rate in the field							
Colne	*			(*)	(*)	[*]	
Lea	[*]	[**]	***	***			
Okement	***	*		**		**	
Erme	***	***	**	*			
Avon	***						*
B - Significant differences in respiration rate							
Colne							
Lea							
Okement	*						
Erme							
Avon				*			**
C - Significant differences in SFG							
Colne	*		(*)				
Lea			(*)				
Okement	*						
Erme	*	*					
Avon	**						
D - Significant differences in feeding rate in persistence of effects experiment							
Colne		**					
Lea							
Okement		***					
Erme							
Avon		***					

* - P <0.05 ** - P <0.01 *** - P <0.001

() - only significant in three-station analysis [] - only significant in two-station analysis

the respiration rate and SFG data than were detected using the feeding rate data.

Effects on feeding rate detected in the field did not persist over a six-day feeding test in the laboratory in any of the trials. Significant differences in the feeding rates of the two populations were apparent however, in three of the trials. Feeding rate in the laboratory was generally higher than that measured in the field, especially for the Colne and Lea trials. The level of consumption in the laboratory was also more consistent across trials, with mean feeding rate generally varying between 4-6 J/mg/day.

Table A4.49 - The biomass (µg fungus/mg leaf disc) of Cladosporium on leaf discs before deployment. Values are means and (standard errors), n=10 for each group

Trial	Laboratory 1	Laboratory 2
Colne	30.12 (14.75)	64.48 (7.12)
Lea	64.54 (24.89)	113.69 (18.06)
West Okement	111.30 (40.87)	29.02 (9.55)
Erme	61.52 (10.68)	27.69 (8.14)
Hampshire Avon	43.06 (17.59)	76.92 (9.2)

A4.7 ANALYSIS ACROSS DIFFERENT DEPLOYMENTS

A4.7.1 Variation in food quality

There was considerable variation in the biomass of Cladosporium fungus in leaf discs (Table A4.49). This variation was both within and between laboratories, but the laboratory differences were significant only in the Erme Trial (Student's t-test, $t=2.56$, $P<0.05$). The variation in leaf discs prepared by Laboratory 2 was less than Laboratory 1 in all trials.

There were no significant changes in the fungal biomass on leaves over the period of deployment at any station in any of the five trials ($F<1.2$, $P>0.05$), suggesting that any differences in water quality between stations did not have an effect upon the biomass of Cladosporium.

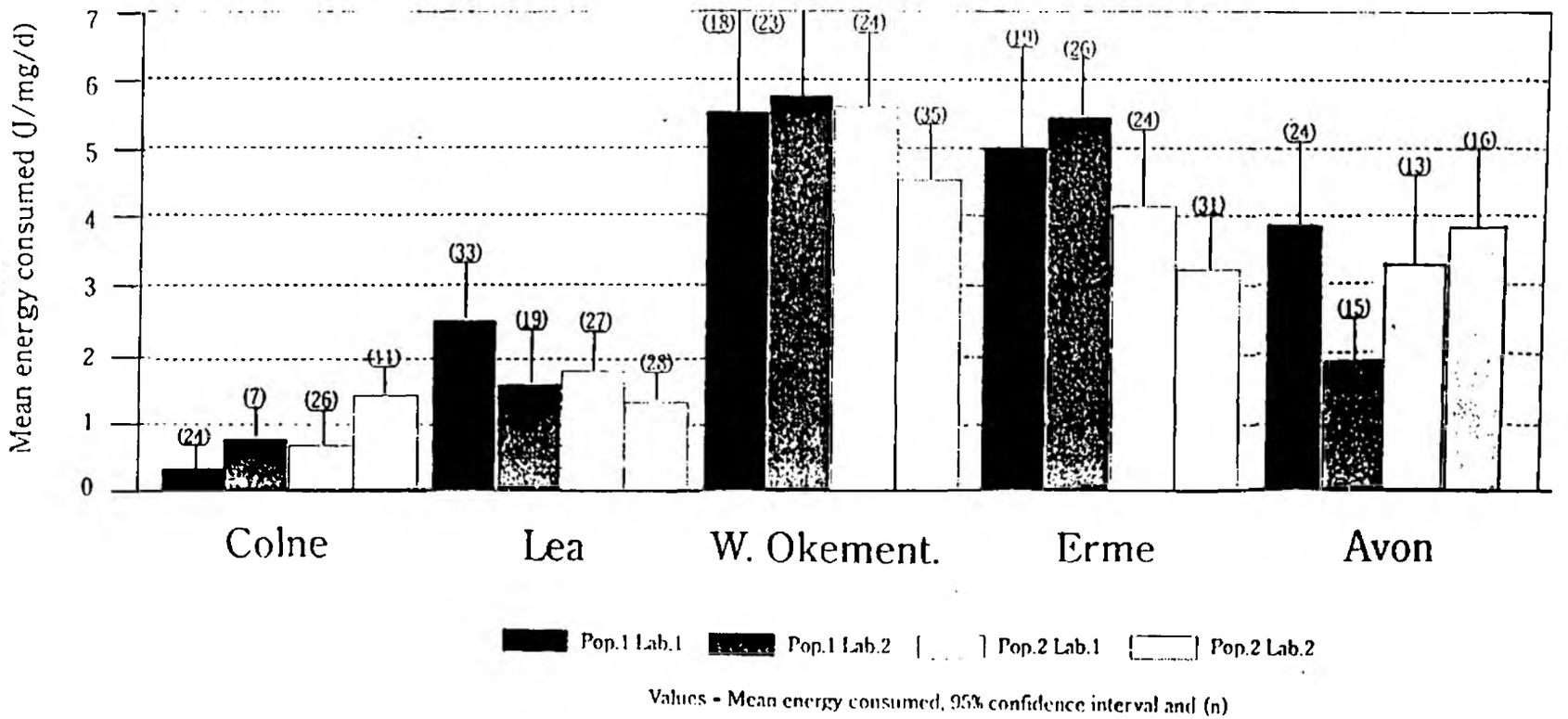
A4.7.2 Variation in feeding rate at "clean" sites

The feeding rate of animals at Station 1 in all trials is plotted in Figure A4.22. Feeding differed considerably between trials. This may have been due to either seasonal or station-specific effects.

A4.7.3 Variation in the weight change of control leaf discs

The leaf correction factors used to control for weight change in leaf discs deployed in the field are given with an estimation of their variance in Table A4.50.

Figure A4.22 **The Feeding rate of Gammarus at 'clean' stations in the five field trials**



Values = Mean energy consumed, 95% confidence interval and (n)

Table A4.50 - Leaf correction factors for the food of each population at each station in every trial. Values are means and (standard deviations)

Trial	Station	Population 1 food	Population 2 food
Colne	1	0.993 (0.033)	1.018 (0.018)
	2	0.997 (0.095)	1.022 (0.024)
	3	1.048 (0.091)	1.081 (0.085)
Lea	1	0.972 (0.104)	0.942 (0.025)
	2	0.967 (0.028)	1.003 (0.025)
	3	0.916 (0.037)	0.958 (0.036)
West Okement	1	0.917 (0.043)	0.921 (0.059)
	2	0.954 (0.031)	0.949 (0.024)
	3	0.967 (0.033)	0.949 (0.027)
Erme	1	0.931 (0.019)	0.943 (0.019)
	2	0.956 (0.035)	0.942 (0.078)
	3	0.956 (0.016)	0.964 (0.043)
Hampshire Avon	1	0.915 (0.041)	1.004 (0.097)
	2	1.059 (0.079)	1.035 (0.138)

The greatest difference in leaf correction factors between populations was at Station 1 on the Hampshire Avon. Here there was a difference of 0.089 between the means.

The effect of such a difference on the determination of feeding rate can be illustrated with reference to a hypothetical 7 mg animal that consumes 10 mg of food over a 6 day period. Using a correction factor of 0.915 the calculated feeding rate is 3.82 J/mg/d, compared with 5.193 J/mg/d for a correction factor of 1.004.

Although this is an extreme example, it does illustrate the fact that a small change in the leaf correction factor may change the overall result considerably. However, given the high variation in feeding rate observed within treatment groups, it is unlikely that even the above

difference could alter the significance of the differences observed in the field trials (see Section 4.7.5).

A4.7.4 The relationship between feeding rate and production of faeces

The regression equations describing the relationship between feeding rate and faeces produced for Populations 1 and 2 in the persistence of effect experiments are, respectively:

Population 1 $F = 0.5811C + 0.545$ (accounts for 64.4% variance) Equation 5

Population 2 $F = 0.6169C + 0.623$ (accounts for 60.3% variance) Equation 6

These linear regression equations were found to fit the data better than any polynomial. The equations for the two populations are plotted in Figure A4.23 alongside the line generated by the Maltby and Naylor equation ($F=0.74C-0.303$; $r^2=91.8\%$). All three lines are similar, although the Maltby and Naylor equation predicts a lower production of faeces at low feeding rates. An estimation of faecal production is important only in the calculation of SFG. These results suggest that at normal feeding rates the Maltby and Naylor equation provides a reasonable approximation of faecal production.

A4.7.5 Optimum sample size for the measurement of feeding rate

Positive coefficients of variation (CV) for feeding rate varied from 36.53% (Laboratory 2, Population 2, Station 2, West Okement) to 417.8% (Laboratory 2, Population 1, Station 2, Lea).

The mean CVs across treatment groups and the range of CVs in each trial are given in Table A4.51.

Figure A4.23 **The relationship between energy consumed and energy lost in faeces for three populations of Gammarus pulex**

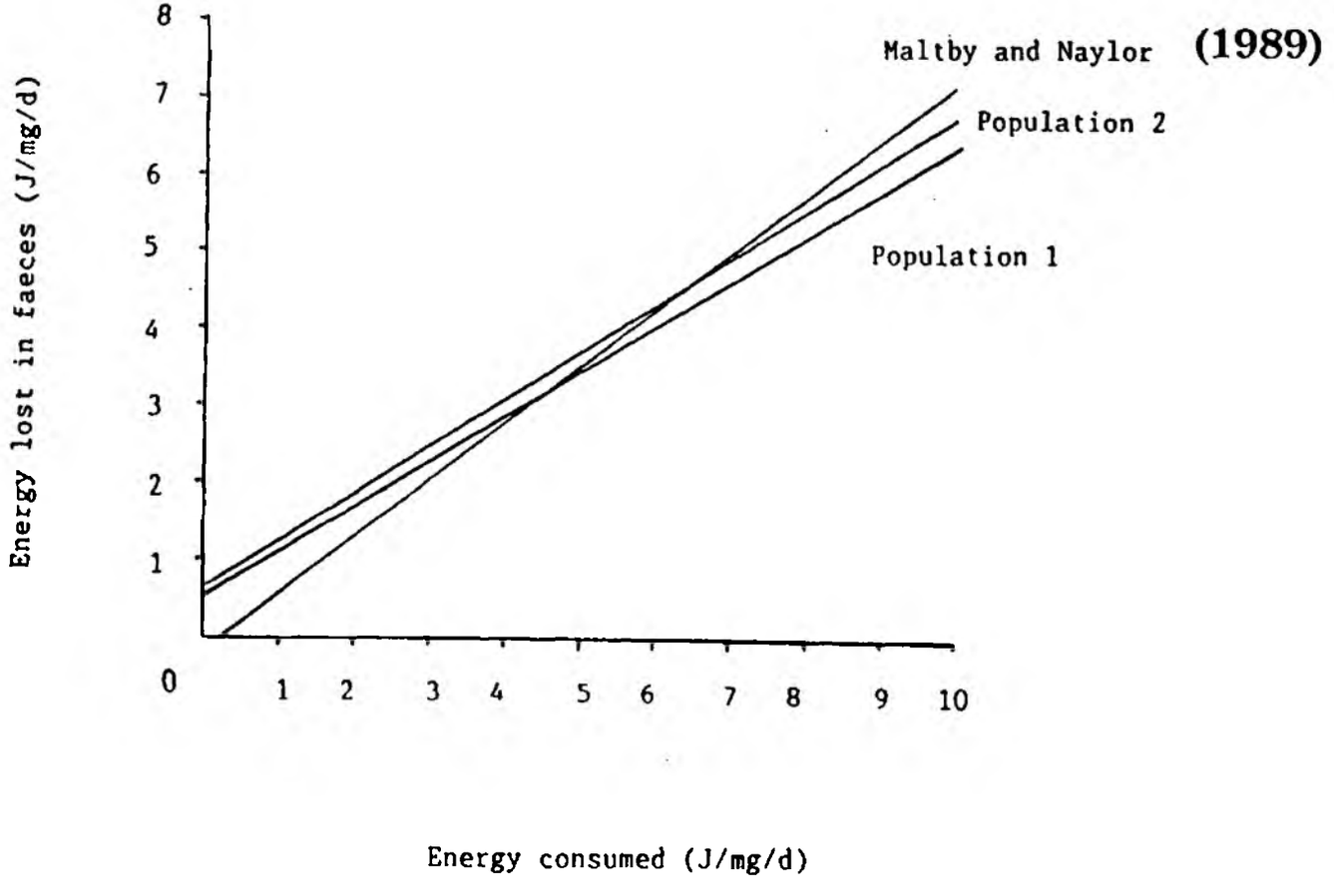


Table A4.51 - Coefficients of variation of feeding rate of Gammarus in the five field trials

Trial	Mean CV	S.D.	Range of CVs
Colne	107.8	59.5	44.6 - 215.3
Lea	107.5	96.4	41.2 - 417.8
West Okement	55.2	12.7	36.5 - 69.8
Erme	68.5	47.6	40.12- 223.7
Hampshire Avon	112.9	48.4	41.31- 214.2

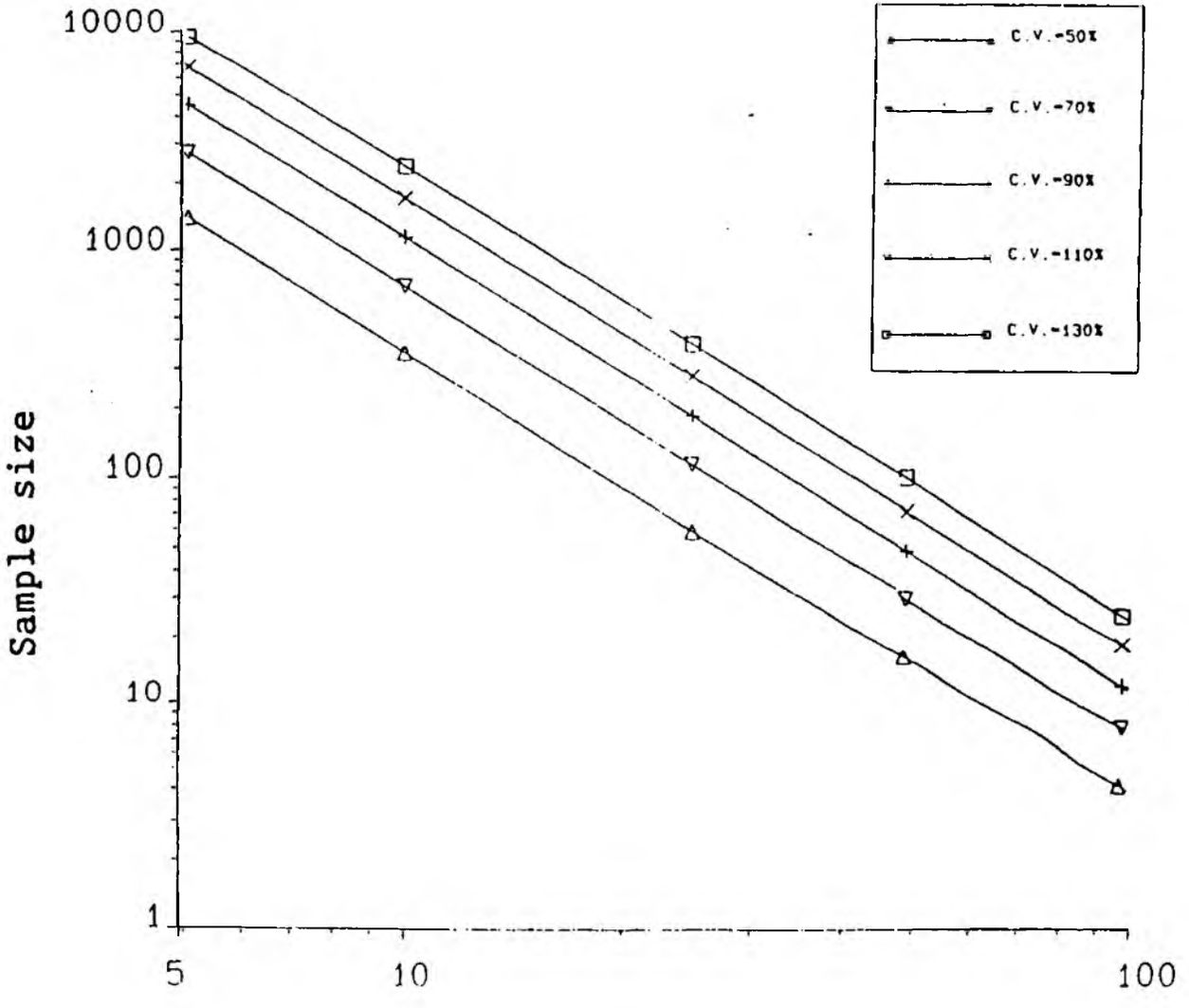
Coefficients of variation varied considerably across treatment groups within trials. The least variation (ie the smallest range and standard deviation of CVs) was on the West Okement whilst the greatest was on the Lea. All the trials achieved a CV of around 40% for at least one treatment group. Figure A4.24 shows the sample sizes necessary for detecting a given percentage difference between means, with a power of 75% over a range of CVs from 50% to 130%. From this it is clear that with the CVs in these trials only relatively large differences between treatment groups could have been detected with the sample sizes deployed. With a sample size of 56 per station the minimum detectable difference between treatments is 27% for a CV of 50%, and 70% for a CV of 130%.

SECTION A5 - DISCUSSION

A5.1 THE EFFECT OF STRESSORS ON THE FEEDING RATE OF G. PULEX

One of the primary objectives of this series of field trials with Gammarus was to investigate the response of animals to different field stressors. However the simple upstream, downstream methodology used in these trials was not sufficiently rigorous to ascribe any differences in the response of Gammarus at the different stations to the particular discharges under investigation. This is because of the problem of

Figure A4.24 Sample sizes necessary in order to detect differences between treatment group means at five levels of variation. (Significance level = 5%, power of test = 75%, number of treatment groups = 3).



pseudoreplication, first articulated by Hurlbert (1984), which is particularly relevant to upstream, downstream, or before-and-after impact analyses (Underwood 1989). Pseudoreplication occurs in these types of experiment because it is impossible to allocate treatments randomly. Samples are taken from, or bioassays are placed, either above or below the relevant discharge and so the impact of the discharge is not spatially randomised. The necessary outcome of such an experimental design is that although an impact may be registered downstream from the discharge, one has not controlled for other variables such as substrate type, flow rate or temperature. Certain experimental designs may partially overcome this problem (eg Stewart-Oates et al 1986), but it is important to realise that single upstream, downstream designs, although providing a useful indication of possible problems resulting from discharges, are not sufficiently rigorous to confirm cause and effect.

Notwithstanding the above, the responses of Gammarus from the five trials suggest that water quality may have a significant effect upon feeding rate and SFG. In four of the five trials at least one of the two populations showed a difference in feeding rate between one or more stations. In the Colne, West Okement and Erme trials feeding was lower downstream from the discharge. In the Lea feeding was lower at the upstream station than it was downstream from the sewage treatment works. In the Hampshire Avon trial both populations had reduced feeding rates at the downstream site although this was only significant for measurements made by one laboratory on one population.

It is not clear which stressors were responsible for reducing the feeding rate of Gammarus in the Lea and Hampshire Avon trials. The high ammonia levels at the Lea upstream station and the Avon downstream station makes ammonia a possible candidate although levels of unionised ammonia were lower than those causing a reduction in Gammarus feeding rate in laboratory experiments (Maltby and Naylor 1989). Heavy metals were the most likely stressors in the three remaining trials. In the Colne trials, levels of lead in water and leaf disc samples from Station 3 were higher than the level found to cause 100% mortality of Gammarus over a 28 day period under laboratory conditions (Zencirci 1980).

Several other metals were relatively high at Station 3, especially iron and aluminium, but these were unlikely to have been a major factor in the reduction of feeding. In the West Okement and Erme trials, metals were again the most likely stressors although in both trials only Population 1 was affected. Very high levels of iron, manganese and aluminium were found at Station 2 and high levels of nickel, manganese and aluminium were found at Station 3. The leaf and Gammarus tissue burdens indicate that animals were able at least partially to control their intake of metals, although whether this was by choosing not to feed upon contaminated material, or by regulating the metal contaminants by sequestration, immobilisation or excretion is unclear. It is known that aquatic fungi accumulate at least some heavy metals by surface adsorption and that this may have a profound effect upon the viability of the Gammarus that feed upon decaying leaf material (Duddridge and Wainvright 1980). Gammarus are also able to detect and avoid toxic food (Maltby, unpublished data), and invertebrates can associate the effects of toxic stress with a particular food for a number of days (Lee and Bernays 1990). It is therefore possible that if metal-induced stress was responsible for reduced feeding at the metal-rich stations it was due to either:

i) direct uptake of contaminants by Gammarus through the water column leading to toxic stress,

ii) contaminated food discs leading to food avoidance,

iii) direct, continuous toxic impact by ingestion,

iv) initial ingestion and toxic impact followed by learned avoidance.

A5.2 THE RELATIONSHIP BETWEEN MORTALITY AND FEEDING RATE

The relationship between mortality and feeding rate is not clear. In the Colne, West Okement and Erme trials, Population 1 animals had a higher mortality than Population 2 animals. In the Lea trial both populations had a low mortality at all stations. In the Hampshire Avon

trial mortality was higher in Population 1 than in Population 2 at the upstream station but lower at the downstream station.

Both mortality and feeding rate data suggest that Population 1 was the most sensitive of the two populations, but at Station 2 on the Lea, where Population 1 fed less, mortality was zero. Similarly, at Station 2 on the Erme, where the feeding rate of Population 2 was depressed, mortality was lower than at Station 3, where no depression in feeding rate could be detected. Such results suggest that feeding rate is more sensitive than mortality as an indicator of water quality.

Mortality may, however, influence the sensitivity of a sublethal measurement such as feeding rate. In the West Okement and Erme trials more than 20% of Population 1 died at Station 3. This may have influenced the distribution of results in one of two opposing ways. If the animals that died were those that were most sensitive to the effects of water quality on feeding rate, then the mean feeding rate for that treatment group would have increased. If, however, the animals that died were those that consumed the most food and thereby ingested the most toxicants, the mean feeding rate would have been reduced for that treatment group. In the former case it may not be possible to detect a depression in feeding rate and in the latter the detection of a difference may be due to an artificial distribution. If therefore seems sensible to use both lethal and sublethal measurements as a combined tool for the investigation of water quality and to use caution when interpreting the results of tests in which there is a high mortality.

A5.3 DIFFERENCES IN THE RESPONSE OF POPULATIONS

It is not obvious why a difference occurred in the response of the two populations or why this difference was not consistent between trials. A possible explanation is that Population 2 was obtained from a stream in which levels of iron and aluminium were higher than the levels found in the source stream of Population 1. Iron and aluminium were two of the main contaminants in the West Okement and Erme trials and Population 2 may have become adapted either genetically or phenotypically to

withstand higher levels of these metals than Population 1. Metal tolerance induced by pollution is a common phenomenon in aquatic systems (Luoma 1977), and has been found in populations of Gammarus (Howell 1985). Gammarus collected from wild populations at different sites also vary in individual metal body burdens (Table A5.1). The animals from Population 2 deployed at West Okement, although suffering no depression in feeding rates do not appear to have regulated metal accumulation at metal-rich stations any better than animals from Population 1. Mortality was considerably greater for Population 1 animals (25% at Station 3), although their body burdens were lower. This suggests that Population 2 animals had adapted to metal contamination and were able to accumulate a greater burden of metals before sustaining any toxic damage. Further evidence of the lower susceptibility of the Population 2 animals is supplied by the fact that although there was a significant negative relationship between body burdens of iron and manganese and feeding rate for Population 1, no such relationship existed for Population 2. The relative tolerance of Population 2 could be tested further by leaving animals from that population at metal-rich sites for a longer period. If regulation of metals does not occur then it is likely that sublethal stress followed by mortality would occur in this population also, but over a longer time scale.

Table A5.1 - Range of body burdens for several metals found in G. pulex from a number of British sites (all values $\mu g\ g^{-1}$)

Metal	Range				Author
Cu	55.3	(35.4-86.3)	- 100	(88.5-114)	* Rainbow and Moore (1986)
Pb	298	(197-449)	- 322	(260-400)	* Rainbow and Moore (1986)
Fe	469	(296-741)			* Rainbow and Moore (1986)
Zn	61.5	(57.3-65.9)	- 128	(110-150)	* Rainbow and Moore (1986)
	73.6	(5.8)	- 141.9	(18.6)	+ Howell (1985)
Cd	0.43	(0.04)	- 0.74	(0.09)	+ Howell (1985)

* - mean values and 95% confidence interval
\+ - mean values and standard errors

It is worth emphasising the problem of ascribing the response of an *in situ* bioassay to particular contaminants. The difficulties presented by pseudoreplication have already been mentioned. A further problem involves the circularity of basing the interpretation of a bioassay on chemical data. It was suggested in the introduction that one of the main reasons for using a bioassay is to overcome problems inherent in chemical monitoring. A good bioassay should provide an integrated response to water quality, and this may involve a response to unmeasured contaminants, to contaminants present at concentrations below the level of analytical detection or to the combined effect of contaminants that would not alone be regarded as stressors. It is therefore unrealistic to assume that correlations between individual determinands in water and biological response will be obvious in every situation.

A5.4 DIFFERENCES IN THE MEASUREMENT OF RESPONSE BY LABORATORIES

In all five field trials differences in the measurement of feeding rate between the two laboratories occurred either as a main effect (ie there was a consistent difference across stations and populations in measurements by the two laboratories), or as an interaction with either station or population. It is of some concern that the two laboratories involved in this exercise did not achieve a greater concordance in their measurement of response. However, responses measured by each laboratory were in the same direction in each trial. Further work is necessary to elucidate the differences in the methods used by the two laboratories.

A5.5 REPEATABILITY AND VARIATION OF FEEDING RATE

The demonstration of a high level of repeatability of response when animals are exposed to the same treatment is a necessary stage in the validation of any test. This was not attempted in this series of trials, but comparison of the feeding rate at 'clean' stations between trials shows that this technique does not provide an absolute, repeatable result between different field sites. This is to be expected and could have been due to either seasonal or inter-site differences. *G. pulex* feeds less at low temperature (Nilsson 1974), but no obvious

correlation existed between feeding rate and water temperature at the five clean stations. Similarly, there was no strong seasonal effect in animals from the clean stations used in the persistence of effect experiments. Such results suggest that the quality of the different clean stations was the most important factor influencing feeding rate, although more work is required to test this hypothesis.

An important factor in any robust bioassay is a low level of variation within treatment groups. Within-treatment group variability was high in all of the field trials, but compared reasonably well with similar, 'relevant' bioassays (eg Stay et al 1989, Schimmel et al 1989, Martin et al 1985). High variability of response in any test system is undesirable because it limits the precision with which differences can be detected. It is almost certainly most useful to have a bioassay that can detect relatively small differences in the response of different treatment groups, although it is important to establish the relevance of the differences and not become over-precise: small differences in response may be statistically significant but ecologically unimportant (Schaeffer et al 1987). In the five field trials the 'best' coefficients of variation achieved in all the trials were around the 40% level. If one assumes that a difference in feeding rate of 25% between stations is of 'biological significance' then it would require a sample size of approximately 80 animals per station to be able to detect a difference of this magnitude at the 5% significance level with a power of 80% (Sokal and Rohlf 1981). The deployment of only 20 animals per station would necessitate the reduction of the CV to approximately 19% in order to have the same probability of detecting the above difference. It is unrealistic to suggest that such a low CV could be achieved. However it should be possible to reduce all treatment group CVs to at least 40% so that a 35% difference in feeding could be detected with sample sizes of about 40 animals at each station. It is clear that the precision of this test relies critically upon the level of variation with treatment groups and so it is important to look at the sources of variation and ways in which it can be reduced.

A5.6 VARIATION IN FOOD MATERIAL

Food material may well be a source of variation. Fungal biomass varied considerably within and between treatment groups and this could have affected the palatability of the food provided. It is known that fungus is an important component in the diet of Gammarus (Bärlocher and Kendrick 1973a and 1973b, Kostalos and Seymour 1976, Willoughby and Sutcliffe 1976, Sutcliffe et al 1981). Maltby et al (1990a) have demonstrated a lower variation in feeding rate for G. pulex fed upon leaf material colonised by fungi compared with those fed upon sterile leaf material. It seems from this that fungus, or some extract from fungus, must be an important component of any Gammarus feeding test. More efficient methods for standardising inoculum size on leaf or other substrate material should be explored. Leaf thickness may also affect the feeding rate of G. pulex (Sutcliffe et al 1981). Standardising food quality might be achieved by investigating suitable artificial substrata, such as filter paper, and the inoculation of these substrata with homogenised fungal extracts.

A5.7 INDIVIDUAL VARIATION

Normal biological variation due to phenotypic or genetic differences between individual Gammarus may also be an important factor in the variable feeding response observed in the field. It may be possible to reduce such variation by culturing test Gammarus in the laboratory. There are however a number of problems associated with such an approach. It can be costly and labour-intensive and ecological relevance may be reduced with the loss of genetic diversity. Furthermore, the cultured population (Population 3) deployed on the Hampshire Avon trial displayed high variation and a peculiar response when investigated for persistence of effect. It seems sensible, therefore, to continue collecting Gammarus from wild populations, with rigorous quality control to guard against the use of parasitised or otherwise damaged individuals. Further investigations are also required into differences in response between, and variation within, different populations and the possibility

of reducing variation through short-term culturing of the F1 generation produced by wild parental stock.

A5.8 PERSISTENCE OF FIELD EFFECTS

No persistence of field effects could be detected in any of the trials, although an accident precluded a full investigation into persistence of effects from the Colne trial. There are two possible explanations for this lack of persistence. The stressors responsible for reducing feeding in the field may have induced a behavioural response in the Gammarus and not have caused a physiological change; alternatively, the depuration rate of accumulated toxicants may have been too rapid to be detectable using a six day feeding test. If the latter were true then feeding may have been depressed briefly, at the beginning of the persistence experiment. Such a reduction would not have been detected in this test. There is evidence from other work to support this idea. G. pulex exposed to a number of difference stressors for just one day showed a depression in feeding rate compared with controls not only during a contaminant pulse, but also on the day after the pulse had passed (McCahon et al 1989). The question of whether field effects persist is an important one because it determines the uses and limitations of a feeding test. If it were possible to measure a persistent effect then much of the labour incurred in a field deployment could be omitted. Measured food material would not need to be supplied to the test Gammarus in the field and a feeding test in the laboratory would be all that was required to detect differences in the feeding rates of animals from different field stations. Further investigation into contaminant accumulation and depuration rates in G. pulex and the effect that these may have upon feeding rate are thus of great importance in the development of this test. Differences were found in the response of the different populations in the persistence of effect experiments. After the Colne and the West Okement trials Population 2 ate more than Population 1; after the Hampshire Avon trial Population 3 ate considerably less than Population 1. The reasons for such differences in the response of populations are unclear. If they were due to animals from Population 2 having acclimated to the water

conditions at WRc, where the persistence of effect experiments were run, then one would have expected a more consistent response between field trials. However, the feeding behaviour of the two populations did not differ significantly upon return from either the Lea or the Erme trials. Also, one might have expected Population 3 animals, cultured at WRc for over two years, to have fed at a high rate when returned to conditions similar to their original culture medium. Again, this did not occur. Although the causes of the above differences are unclear, these results do confirm the intrinsic differences that exist between populations in their response to the environment.

A5.9 THE MEASUREMENT OF RESPIRATION AND SCOPE FOR GROWTH

One of the objectives of these trials was to assess the relative values of the different components of the scope for growth suite of tests. A reduction in SFG was apparent in only three of the trials, the Colne, West Okement and Hampshire Avon. A reduction in respiration rate could be detected only in the West Okement trial. Generally, the power of the ANOVA tests used to analyse the results of the respiration rate and SFG investigations was too low to be able either to confirm or reject the null hypothesis that there was no difference in the response of treatment groups (Hayes 1987). It is difficult to see how one could increase the power of the analysis without a very large increase in sample size. This would involve a concomitant increase in the number of animals respired, an expensive, labour-intensive and technically difficult operation. Given the small contribution that respiration rate makes to the overall calculation of a single Gammarus energy budget, and the correspondingly large contribution made by the feeding rate measurement, it seems most sensible to adopt feeding rate as the most sensitive and cost-effective measure of a gammarid's energetic response to stress.

A5.10 THE ECOLOGICAL RELEVANCE OF GAMMARUS BIOASSAYS

Probably the most important question to answer when using any bioassay is, 'What is the ecological relevance of the response?' G. pulex is a

member of many lotic communities in the UK and is one of the most important species in the functional group responsible for shredding decaying leaf material (Chergui and Pattee 1988). In many systems such material is the major source of energy and so any disruption in the activity of these detritivores may have a considerable impact upon higher trophic levels by reducing the direct or indirect availability of food. The problem of limited food availability is not only that, at its simplest, less food means fewer animals but also that a shortage of food, even if only temporary, is likely to increase the susceptibility of an ecosystem to chemical stress (Kooijman and Metz 1984). It therefore seems likely that a reduction in the viability of Gammarus populations in the field would have a profound impact upon the whole lotic community.

Further questions arise from this. Is it possible to demonstrate the likelihood of population level effects from the response of individuals? Does a reduction in feeding rate or SFG automatically lead to a lower population level, or the elimination of Gammarus entirely? Populations and communities are controlled by many homoeostatic mechanisms that may lead to resilience in the face of stress. It is possible that if female Gammarus are stressed they will alter the manner in which they partition energy, allocating a greater proportion to the production of offspring and less to their own body growth and maintenance. Preliminary evidence suggests, however, that when female Gammarus are subjected to stress in the laboratory they produce fewer, smaller offspring (Maltby and Naylor 1990). If this were to occur in the field then it could lead to a lower population level, although a reduction in density-dependent competition and emigration may have a mitigating influence. Perhaps a factor of greater practical importance than subtle population level change is the extent to which the different sexes and life history stages of Gammarus may be affected by stress (Buikema and Benfield 1979). Female reproductive condition, the stage reached in the moult cycle and the age of the animal all appear to be important, with females, juveniles and moulting animals showing far greater susceptibility than sexually-mature inter-moult males (McCahon and Pascoe 1988a, 1988b, 1988c). Thus any reduction in the feeding rate of the mature males used in the test may

well represent the lowest degree of response expected from a normal population of Gammarus comprising males, females and juveniles. A reduction in the feeding rate of males could therefore indicate the potential elimination of the species from the station at which it is deployed due to the acute mortality of toxicants to females and juveniles. Gammarus is also more likely to enter the drift when stressed, with recent observations in artificial streams suggesting that drift may occur before any effect on feeding rate is apparent (Mitchell, Shell Research Limited, pers comm).

The feeding rate of Gammarus may be used as an 'early-warning' of potential environmental impact before it occurs, or as a more specifically targeted research tool to explain particular instances of damage. Toxicity testing has not traditionally been widely used by regulatory authorities in the UK. Instead, routine chemical sampling and biological monitoring have provided the bulk of information upon which decisions are based. It is clear that with the ever increasing number of chemicals released into the aquatic environment it is neither cost-effective nor even possible to guard against ecological damage through chemistry alone. Benthic sampling is also insufficient to guard against damage since it is, by definition, a record of what species are present and can only tell the biologist what has happened in the past, not what is about to occur. Benthic monitoring, especially when the results of this technique are translated into biotic scores or indices, has been shown to be inappropriate for guarding against chemical pollution (Slooff 1983). The tolerance of different invertebrate species to chemicals differ markedly and are not closely related to taxonomic lines (Engstrom-Heg et al 1978). However, the comparison of feeding rates with BMWP scores at the upstream and downstream stations in these trials produced compatible results on all but the Lea trials. The discrepancies on the Lea trial may have been due to a short-term difference in water quality masking long-term trends.

It is essential to integrate routine chemical and biological monitoring with ecotoxicological investigations, as it is through the latter that cause-and-effect relationships may be established. The River

Invertebrate Prediction and Classification System (RIVPACS) developed by the Institute of Freshwater Ecology may provide the opportunity for just such an integration (Wright et al 1989). If the predictions of species that should be present at particular stations are demonstrated to be accurate for clean sites, then it should be possible to use ecotoxicological techniques to demonstrate cause and effect at sites where RIVPACS has suggested that species may be absent due to poor water quality. The Gammarus feeding test is the type of bioassay that could be particularly useful in such circumstances.

SECTION A6 - CONCLUSIONS

1. The feeding rate of G. pulex can indicate differences in quality between stations upstream and downstream from different types of effluent discharge.

2. The feeding rate response depends upon the population of Gammarus used in the bioassay. Some populations may be more tolerant than others to certain contaminants. This could be due to adaptation caused by prior exposure to toxicants.

3. Different laboratories may measure a different level of response for reasons that are, at present, unclear. This is a matter of some concern, but in the series of field trials reported would not have led to the two laboratories drawing different conclusions when comparing feeding rates upstream and downstream from a discharge.

4. Within group variation is high, but not significantly higher than in many other 'relevant' bioassays. There are some indications that variation could be reduced by fairly minor changes to the methodology.

5. The feeding rate of G. pulex is a relative and not an absolute measure of water quality. Environmental factors other than

contamination are likely to vary between river catchments and have some influence on the response of test animals.

6. Effects on feeding rate observed in the field did not persist in animals returned to the laboratory. This suggests either that the field response was behavioural, or that the depuration rates are too rapid to detect using a six-day feeding test.

7. The feeding rate of G. pulex is of ecological relevance in many lotic systems and may be useful either as an 'early-warning' system of potential environmental impact, or as a tool for explaining causes after ecological damage has occurred.

8. The measurement of respiration rate is costly, labour-intensive, technically difficult and subject to high variation. Respiration rate values contribute only a small amount to the overall energy budget equation in comparison to feeding rate.

SECTION A7 - RECOMMENDATIONS

1. The measurement of respiration rate and the subsequent calculation of scope for growth should be abandoned. The feeding rate of G. pulex is sufficiently sensitive to differences in water quality to stand alone as a bioassay.

2. A deployment methodology should be developed that overcomes some of the major problems presented by pseudoreplication. This methodology should also seek to reduce within-treatment group variation. Experimental work is needed to test a number of different systems.

3. A range of artificial food materials should be tested for use in lieu of fungal-inoculated alder leaf discs. The objectives of such tests should be to reduce both labour costs in the production, and variability in the quality, of food material.

4. Differences between populations of G. pulex in the response of their feeding rate to different contaminants should be investigated further, initially in the laboratory. Experiments should include an investigation into whether variation between populations is due to genetic or phenotypic adaptation.

5. The uptake and depuration rates of various contaminants in G. pulex should be investigated with a view to establishing a link between contaminant body burden and feeding rate.

6. The effect of a reduction in feeding rate upon populations of wild or semi-wild G. pulex should be investigated further. Any population-level effects should be related to potential community-level effects due to energy or nutrient limitation.

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APPENDIX 1

DERIVATION OF THE EQUATION USED TO CALCULATE THE RESPIRATION RATE OF *GAMMARUS PULEX* (EQUATION 4)

From Wrona and Davis (1984),

$$MO_2 = \frac{(O_1 - O_2) * A * F}{W}$$

where MO_2 = Weight-specific oxygen uptake (µmol/mg/h)
A = O_2 solubility at 15 °C (2.01 µmol/l/torr)
F = Flow rate (l/hr)
O_1 = Dissolved oxygen entering respirometry chamber (torr)
O_2 = Dissolved oxygen leaving respirometry chamber (torr)
W = Dry weight of animal (mg)

and,

$$R = MO_2 * 24 * (22.41 \times 10^{-6}) * (21 \times 10^3)$$

where R = Respiration rate (J/mg/d)
24 = Conversion from hours to days
22.41×10^{-6} = Conversion from µmol to litres
21×10^3 = Oxyjoule equivalent (Elliott and Davidson 1975)

Therefore,

$$R = \frac{(O_1 - O_2) * 2.01 * 24 * (22.41 \times 10^{-6}) * (21 \times 10^3) * F}{W} \qquad \text{Equation 7}$$

A 250 µl sampling syringe fills in T sec

$$= \frac{250}{1000 * 1000} \text{ litres in } \frac{T}{60 * 60} \text{ hours}$$

$$= \frac{250 * 60 * 60}{1000 * 1000 * T} \text{ l/h}$$

$$\therefore F = \frac{0.9}{T}$$

Substituting into Equation 7.

$$R = \frac{(O_1 - O_2) * 2.01 * 24 * (22.41 \times 10^{-6}) * (21 \times 10^3) * 0.9}{W * T}$$

$$= \frac{20.432\ (O_1 - O_2)}{W * T} \qquad \text{Equation 4}$$

SECTION B - THE MEASUREMENT OF GLUTATHIONE-S-TRANSFERASE ACTIVITY IN SPHAERIUM CORNEUM

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SUMMARY

Bioassays can provide a sensitive and cost-effective means of monitoring the effects of aquatic pollution, particularly, in situations where traditional chemical-based assessment would not be adequate. The multi isozymic glutathione-s-transferase (GST) enzyme system is widely distributed among invertebrate and vertebrate species and has a key role in the detoxification of accumulated toxicants. This report describes a study into an assay for the measurement of GST activity in Sphaerium corneum. The aim is to establish the technique as a biochemical index of sub-lethal toxicant stress for routine use.

Initially tissue samples from groups of Sphaerium were used and the effects of different tissue and enzyme extract storage regimes on the assay procedure were investigated. An accurate and reproducible biochemical test capable of measuring GST activity in small tissue samples has now been developed. Group tissue samples were also used to study the effects of reproductive state, season and toxicant exposure on GST induction. The reproductive status of animals apparently had no effect on baseline GST activity, which was also measurable in young Sphaerium removed from ovigerous females. A fluctuating pattern of GST activity was measured in animals from the Salterhebble population throughout the year, though the seasonal variation only appeared to be correlated with water temperature. During toxicant exposure, induction of GST has been measured in response to both organochlorine compounds (the pesticides lindane, permethrin and tecnazene) and the heavy metal zinc.

However in the study it was apparent that animals of similar shell size had varying tissue weights. The effect of body size (as tissue wet weight) on GST activity was therefore investigated in individuals from four sampling sites. An allometric size-effect was found in all the Sphaerium populations and a normalisation procedure has been derived, whereby total GST activities in individuals are standardised to those for a 40 mg tissue wet weight animal.

On the basis of the size-effect study, in-depth toxicant dosing experiments and assessments of the effects of collection and transport to and within the laboratory on GST induction have been made on individual tissue samples, the results of which were normalised. An induction of GST activity following field collection and transportation to the laboratory was found, which was significantly elevated above initial levels after a 48 h lag phase. The elevated tissue GST activities declined to basal levels after 96-192 hours, indicating that animals collected from the field should be acclimated for at least 120 hours before conducting dosing experiments. In contrast the movement of animals between tanks in the laboratory had no significant effect on GST induction. The nature of GST induction in individual Sphaerium during exposure to a range of lindane concentrations (100-10 000 ng/l) is currently under investigation. The studies are being conducted at 4, 8, 12 and 16 °C to assess the extent to which GST induction is temperature dependent.

On the basis of this study the enzyme glutathione-s-transferase has been identified as a potentially useful biochemical index of physical or pollution stress. In addition, due to the nature of GST induction this technique apparently has the potential to indicate prior exposure to episodic events. A number of populations now need to be investigated in order to characterise the range of response patterns which might be encountered during routine application.

SECTION B1 - INTRODUCTION

In recent years the use of bioassays for monitoring and controlling effluent discharges has been increasingly advocated (Mackay et al 1989). A range of physiological and biochemical techniques (eg scope for growth, lysosomal enzyme activity) have been investigated with the long term aim of deploying certain of these methods for assessing continuous and episodic pollution events.

The enzyme system glutathione-s-transferase has attracted interest as a method for the detection of biological events since chemical stress has been shown to result in GST induction in aquatic (Boryslawskyj et al 1988; Lee and Keeran 1988) and mammalian (Schram and Robertson 1985) species.

The enzyme system has a central role in the biotransformation of xenobiotics, with GSTs catalysing any biochemical reaction in which the tripeptide, glutathione (GSH) acts as a nucleophilic agent and conjugates with electrophilic metabolites and metabolically produced oxidising agents. Glutathione or, more strictly, the thiolate anion (GSO) provides electrons for nucleophilic attack on reactive compounds containing an atom with positive, or partial positive charge. The process, (shown below) serves to protect the numerous nucleophilic centres of vital endogenous macromolecules, such as nucleic acids and proteins, from the toxic effects of highly reactive electrophiles. As a result of the molecular weight of glutathione (MW=307) the GSH-toxicant conjugate can be preferentially secreted by the biliary system, which processes molecules of molecular weight 300-500 according to the species.

Electrophile Glutathione Glutathione-conjugate

The ubiquity of the GST enzyme system in plant and animal species was first identified by Booth et al (1961). In the past 28 years these multi-isozyme systems have received considerable attention from pharmacologists, geneticists, biochemists, clinicians and, not least, toxicologists. A considerable proportion of the initial studies were conducted on GST systems in mammalian groups such as sheep (Reddy et al 1983), pigs (Williamson et al 1986) and man (Kamisaka and Habig 1975). It rapidly became apparent from these isolation and characterisation studies that similarities in structure and function existed between species.

Initial work on GST in aquatic organisms was carried out on the blue crab, Callinectes sapidus (Tate and Herf 1977). However, a considerable proportion of studies conducted have centred on fish species such as the rainbow trout (Nimmo 1985), plaice (George and Young 1988), atlantic salmon (Ramage and Nimmo 1984) and the little skate (Foureman and Bend 1982).

In invertebrate species, GST has recently been purified and characterised in the snails Helix adspersa, Indoplanorbis exustus, Cerithiden obtusa and Elizia orbicularis (Balabaskaran et al 1986). Lee and Keeran (1987) have measured GST activity in the hepatopancreas of the blue crab, Callinectes sapidus and digestive gland cytosol of the marine gastropods, Nassarius obsoletus and Cerethium floridanium (Lee and Keeran 1988).

Studies by Boryslawskyj (1990) at the Huddersfield laboratory have shown GST induction in the freshwater bivalve mollusc, Sphaerium corneum following laboratory and field exposure to the organochlorine pesticides dieldrin and lindane. On this basis, the use of a GST assay as a routine in situ bioassay for the assessment of both heavy metal and organic pollution has been investigated. The main objectives of the research programme were to:

1) establish a dose-response relationship between the level of GST induction and concentration for a range of toxic substances;

2) investigate the detailed dynamics of GST response to one compound, in terms of the time lag for response induction and the time to attain tissue and response equilibrium;

3) investigate seasonal variation in the levels of GST activity.

The study has also investigated the effects of extrinsic physico-chemical parameters such as water temperature, pH and ionic composition, and intrinsic factors such as body size and reproductive status, on GST levels. Furthermore, in addition to studies on Sphaerium, preliminary investigations have been carried out on Gammarus pulex to assess the wider applicability of the assay procedure. The research programme was conducted with the overall objective of establishing a reproducible, robust and accurate assay procedure which could be applied to a range of aquatic species commonly used for in situ assessments of pollution events.

SECTION B2 - MATERIALS AND METHODS

B2.1 SAMPLING PROCEDURES

B2.1.1 Sampling sites

In the study, populations of Sphaerium corneum from Malham Tarn, Worsborough Reservoir, Salterhebble, Elland and the Goit have been used. The sampling site locations are shown in Figure B1 and below is a brief description of the character of each location.

a) Worsborough Reservoir

The reservoir is situated approximately 60 m above sea level, 1 mile from Worsborough town (5 miles south of Barnsley, South Yorks). Animals were dredged from the Rockley Dike which runs alongside and eventually meets the outflow of the reservoir. The river is 12-15 cm deep with a moderate flow and a coarse-medium grained bed.

b) Malham Tarn

The tarn is located 400 m above sea level, approximately 2 miles from Malham village (10 miles north east of Settle, North Yorks). Sphaerium were taken from a stream flowing south from the tarn. This rapidly flowing stream is 3-4 cm in depth with a coarse-medium grained bed.

c) Salterhebble

The Salterhebble site is 3/4 mile upstream from the Elland site on the Calder & Hebble navigation canal. There is no flow in the canal and the river bed is of coarse-grained material.

d) Elland

The Elland site is situated approximately 30 m above sea level, 4 miles north of Huddersfield. Animals were taken from an over-flow basin (stone flagged base) in the Elland section of the Calder & Hebble navigation canal. The moderately flowing river is 12-15 cm deep and the sediment bed is medium-fine grained.

e) Goit

The Goit site is located on the Polytechnic site where the River Colne meets the Huddersfield narrow canal. The river bed is 1-1.2 m in depth with a moderate flow over a medium-fine grained bed.

The Gammarus pulex used in this study were supplied by WRc and were obtained either from a laboratory culture maintained at Medmenham or from Haseley Brook, Oxfordshire.

B2.1.2 Collection of animals from sampling sites

Animals were dredged from the bottom of each site using a 25 cm x 30 cm nylon net with a stainless steel surround, and placed in a clean

insulated flask. During transport back to the laboratory this flask was baffled to prevent spillage and undue mechanical stress to the animals.

B2.1.3 Collection of water samples at the sampling sites

On each occasion animals were collected, a water sample was taken and the water temperature recorded. The water was stored in a thoroughly cleaned 5 litre brown glass Winchester bottle, which was rinsed 3 times with site water before taking the sample.

On return to the laboratory, the pH of the water sample was taken and a hexane extraction carried out (Section B2.1.2). The remaining water sample was stored at 4 °C until anion concentrations were measured (Section B2.2.6).

B2.2 ASSAY PROCEDURE

B2.2.1 Extraction of GST from tissue samples

In the extraction of GST from groups (5-10 mussels) or individual Sphaerium corneum the tissue mass was treated in the same manner. After careful removal, the tissue mass was placed in a pre-weighed vial maintained on ice. The tissue was weighed and sonicated (35 watts power) in 4.0 ml of 0.1M potassium phosphate (kpi) pH 6.5 buffer for 15 seconds. In Gammarus pulex samples, ultrasonic tissue disruption was replaced by mechanical homogenisation, using a micro-homogeniser attachment (capacity 5 ml) to the MSE overhead drive homogeniser. The group (10 animals) or individual tissue samples were homogenised for 30 seconds at maximum speed in 3.5 ml of 0.1M kpi buffer (pH 6.5).

In all cases, the suspension was centrifuged at 100 000 g for 30 minutes and the resulting supernatant decanted and retained. The pellet was also retained for possible tissue burden analysis.

B2.2.2 Storage conditions

In the course of laboratory or field experiments it may not always be possible to immediately analyse animals sampled at a given time for inherent GST activity. Therefore the effects of storage of tissue samples or enzyme extracts either on ice (0 °C), frozen (-20 °C) or in liquid nitrogen (-96 °C) was investigated in order to ascertain the extent to which these conditions affect GST levels compared with initially measured values.

a) Effect of storage of tissue in liquid nitrogen (-96 °C).

The experimental procedures used were as follows:

1) Immediate extraction and assay,

2) Extraction and assay after the tissue had been stored in ice cold buffer (0 °C) for a fixed time period,

3) Extraction and assay after tissue has been stored in liquid nitrogen (-96 °C) for a fixed time period.

Following removal from the liquid nitrogen, the tissue samples were thawed, blotted dry and then weighed. The GST extraction procedure was carried out and measured enzyme levels were compared with initial values.

b) Effect of storage of enzyme extract on ice (0 °C) or frozen (-20 °C).

GST was extracted as previously described (Section B2.2.1) and stored in a plastic vial. An initial assay was carried out and the remaining enzyme extracts were stored overnight either unfrozen at 0 °C or frozen at -20 °C. After 15 hours the extracts held on ice or frozen were reassayed and the measured GST activity was compared with the initial rate.

B2.2.3 Samples for GLC analysis of organochlorine compounds

In all analyses, thoroughly cleaned glassware was used. Extraction vessels were initially rinsed with 2.0 ml of n-Hexane (pesticide grade) before being oven dried at 55 °C.

a) Water samples

A 200 ml aliquot of water sample was shaken with 5.0 ml of n-Hexane (pesticide grade) for 30 seconds and alloved to separate for 60 minutes. The hexane layer was removed using a glass Pasteur pipette and stored in a stoppered glass tube. A small amount of $MgSO_4$ was added where necessary in order to 'crack' any emulsion that may have formed during the procedure. All extractions were carried out on duplicate samples.

b) Tissue samples (proposed)

In the analysis of tissue burdens, the 8-9 individual pellets resulting from the 100 000 g centrifugation in the GST extraction process (Section B2.1.1) will be used. The individual pellets will be carefully combined and sonicated in 5.0 ml of n-Hexane (pesticide grade) for 15 seconds and then centrifuged at 7000 g for 10 minutes. The resulting supernatant will then be analysed as for water samples.

B2.3 ANALYTICAL METHODS

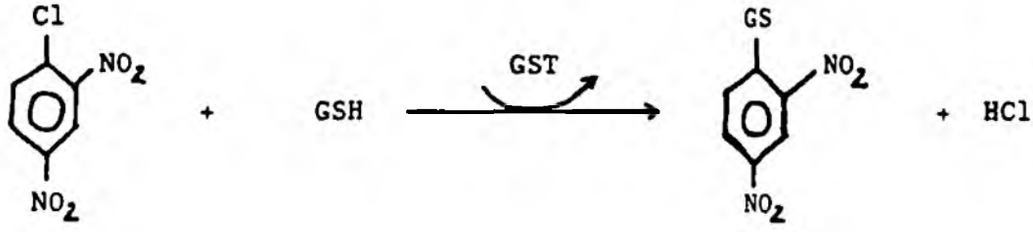
B2.3.1 Assay of GST activity

The assay method used for GST activity was based on that described by Habig and Jakoby (1981). The assay mixture consisted of the following:

0.10 ml aliquot of the supernatant extract
0.75 ml of 0.1M kpi buffer (pH 6.5)
0.10 ml of 20 mM reduced glutathione solution (Sigma Chemical Co)

0.05 ml aliquot of 50.0 mM 1-chloro-2,4-dinitrobenzene substrate (CDNB, Aldrich) in 95.0% ethanol

The rate of GSH - CDNB conjugate formation, as shown below, was followed for 3 minutes at 340 nm with a path length of 1 cm. A Beckmann DU-65 spectrophotometer (at a constant 25.0 °C) connected to a chart recorder was used.



CDNB Glutathione Glutathione-conjugate

B2.3.2 Assay of total protein content

The assay procedure used was a modification of the Biuret method (Gornall et al 1949).

a) Preparation of Biuret reagent

The reagent was prepared by adding 150 ml of 40% NaOH solution dropwise to 40.0 ml of 1% $CuSO_4.7H_2O$. The resulting solution was filtered through a No 3 sinter under vacuum, to remove any precipitate, and stored in a plastic bottle until required.

b) Assay procedure

A mixture of enzyme extract and water was added and mixed with 0.40 ml of Biuret reagent to maintain a total reaction volume of 1.20 ml. The absorbance of this solution was then recorded at 310

and 390 nm using a Beckmann DU-65 spectrophotometer. The influence of nucleic acid interference on the procedure was accounted for by repeating the assay and replacing the biuret reagent with an equivalent volume of glass-distilled water (GDW). Protein concentrations were obtained from a calibration curve constructed using bovine serum albumin BSA, Sigma Chemical Co, as the protein standard.

Previous studies have described GST specific activity in terms of μkatals per mg of protein, based on protein determinations carried out using the Biuret and the Pierce development of the Bradford dye binding technique. However work in this laboratory has shown that these methods have proved less reliable when compared to the Lowry method (Lowry et al 1951) and the ratio of absorbancies at 280/260 technique (Warburg and Christian 1941).

The experimental error associated with both the Lowry and 280/260 methods has been found to be considerably greater than that inherent in the GST assay. A study was therefore undertaken in which the correlation between the protein content (as determined by the Biuret method) and wet weight of extracted tissue was examined.

B2.3.3 Calculation of GST specific activity

In this example the calculation of the GST level in a 38.0 mg wet tissue weight animal will be considered:

For an assay yielding absorbance changes per minute at 340 nm of 0.0959 and 0.0941 units for the replicates

Mean absorbance change (Abs.340/min) = 0.0950

$$\text{Enzyme activity} = \frac{\text{Abs 340/min}}{\text{mM absorbance coefficient for CDNB}} = \frac{0.0950}{9.6}$$

$= 9.896 \times 10^{-3}$ mMoles/min/l

$= \frac{9.896 \times 10^{-3}}{60}$

$= 1.649 \times 10^{-4}$ mMoles/sec/l

Correcting for a total assay volume of 1.0 ml

$\frac{1.649 \times 10^{-4} \times 1.0}{1000} = 1.649 \times 10^{-7}$ mMoles/sec/per ml of assay volume

Measured activity $= 1.649 \times 10^{-7} \times 1000$ µMoles/sec/ml of assay volume

$= 1.649 \times 10^{-4}$ µMoles/sec/ml of assay volume

Specific activity is expressed as µkatals/mg (tissue or protein), by definition

1 katal = 1 mole substrate used/sec

1 µkatal = 1 µmole substrate used/sec

Measured activity $= 1.649 \times 10^{-4}$ µkatals/sec

This enzyme activity represents that from the volume of extract used, 0.10 cm.

Activity/ml $= 1.649 \times 10^{-4} \times 10$

Specific activity $= 1.649 \times 10^{-3}$ µkatals/ml

For an individual of 38.0 mg wet tissue weight extracted in 4.0 ml of extraction buffer

$$\text{Tissue weight/ml} = \frac{38.0}{4.0} = 9.50 \text{ mg/ml}$$

$$\text{Specific activity} = \frac{1.649 \times 10^{-3}}{9.50} \text{ µkatals/mg}$$

$$= 1.736 \times 10^{-4} \text{ µkatals/mg}$$

GST levels are frequently quoted within this report as total GST levels (µKatals) calculated as:

Total GST activity = Specific activity x Tissue wet weight

$$= 1.736 \times 38.0$$

$$= 66.12 \times 10^{-4} \text{ µkatals}$$

B2.3.4 GLC analysis

Extracted tissue or water samples were analysed using a J & W Fused Silica Megabore capillary column on a Pye Unicam gas chromatograph with Electron Capture Detector (column temperature = 180.0 °C, carrier gas He, flow rate = 5 ml/min). Integration analysis of the triplicate analyses of each sample was carried out using a Spectra Physics SP4270 integrator.

Pesticide calibration standards were prepared fresh daily from a concentrated stock standard mix of 0.1 mg/ml in n-Hexane.

B2.3.5 Ion-chromatography analysis

The water samples were initially filtered under vacuum through a 0.45 µm cellulose nitrate membrane filter to remove any solid debris. Triplicate analysis of each sample was then carried out using a Dionex Ion Pac AS-400 anion exchange column with Dionex Advanced Chromatography

module (eluant = 0.75 mM $NaHCO_3$/2.0 mM Na_2CO_3, regenerant = 25 mM H_2SO_4, column flow rate = 2.0 ml/min). The integration analysis of the replicate samples was carried out using a Spectra Physics SP4270 integrator.

Calibration standards were freshly prepared each week from a concentrated stock standard solution of 1000 ppm, which was replaced on a monthly basis.

B2.4 EXPERIMENTAL PROTOCOLS FOR STUDIES USING TISSUE SAMPLES FROM GROUPS OF SPHAERIUM

B2.4.1 Effect of seasonal variation on GST activity

The extent of seasonal variation in baseline GST activity in Sphaerium corneum was assessed in a 13 month study on animals from the Salterhebble population. At approximately 30 day intervals, animals were collected from the sampling site (Section B2.1.1) and GST from the combined tissue of 5 animals/sample was analysed immediately on return to the laboratory. A water sample was also taken and pH, temperature, sulphate and nitrate concentrations recorded.

B2.4.2 Effect of reproductive state on GST activity

The effect of an animal's reproductive state on baseline GST activity was investigated in animals from the Salterhebble site. The combined tissue of groups of ovigerous females and non-ovigerous animals, along with that from adults after removal of the young and recently released juvenile animals was analysed for GST activity.

B2.4.3 Effect of toxicant exposure on GST activity in grouped tissue samples

Sphaerium from the Goit population were collected and acclimated for 96 hours in 1.5 litres of canal water (from a nearby site considered to have no pesticide loading) at 9-10 °C. After this recovery period, the GST activity in the combined tissue of 5 animals was measured as the

time 0 h value. Groups of animals were then placed in separate tanks containing 1000 ng/l of the organo-chlorine compounds lindane, permethrin and tecnazene (using an acetone carrier solution) and 1000 µg/l of the heavy metals lead and zinc. The nominal concentrations in the tanks were achieved by the addition of a given volume of the appropriate stock solution which was distributed throughout the water by an initial gentle stirring with a glass rod. A static dosing regime was used and GST activity was analysed in the combined tissue of 5 animals at intervals of 24, 48 and 72 h for organochlorine compounds and 24 and 90 h for heavy metals. In the control group for organochlorine compound dosing, animals were exposed to an equal volume of the acetone carrier solution and GST activity was monitored as for experimental animals.

B2.4.4 Measurement of GST activity in grouped tissue from Gammarus pulex

Groups of Gammarus from the WRc culture population and a site at Haseley Brook, Oxfordshire were used in this study. The animals were frozen in liquid nitrogen and transported to Huddersfield where they were stored at -20 °C for 4 days prior to extraction. The organisms were thawed and weighed in groups of ten, before the GST enzyme extract was obtained using a modification of the standard procedure (Section B2.2.1). The resulting supernatant was decanted, retained and assayed for GST, following the standard method (Section B2.3.1).

B2.5 EXPERIMENTAL PROTOCOLS FOR STUDIES USING TISSUE SAMPLES FROM INDIVIDUAL SPHAERIUM

In the experiments described previously, GST activity was measured in the combined tissue mass of groups of Sphaerium of similar shell size, and expressed as µkatals/mg wet tissue weight. However it was apparent that organisms of similar shell size had varying tissue weights and there were marked differences in the combined tissue weight between groups. In view of the effect of tissue mass on weight specific GST activity an investigation of the effects of size on individual GST activity was conducted.

B2.5.1 Effect of body size on GST activity

Animals were collected and GST activity in individuals animals of varying size (15-120 mg tissue wet weight) from the Worsborough Reservoir, Malham Tarn, Elland and Goit populations was measured immediately on return to the laboratory (Section B2.2.1.). A water sample was also taken and pH, temperature and anion (SO_4^{2-}, NO_3^{2-}) concentrations were measured and recorded before storage at 4.0 °C (Section B2.1.3)

In these studies of individuals, non-ovigerous and ovigerous animals were noted in order that the effect of reproductive status on baseline GST activity in individuals could be assessed.

B2.5.2 Effect of handling on individual GST activity

The effects of handling, in terms of collection and transport from sampling sites and any subsequent transfer between tanks in the laboratory, on baseline GST levels were investigated using individuals from the Elland population.

a) Effect of collection from the field and transport to the laboratory

After collection and transportation to the laboratory, 50 animals were immediately placed in replicate 1.5 l tanks of Standard Soft Water, (SSW, Appendix B1) previously equilibrated to the site water temperature (8 °C). The experimental holding tanks were constantly aerated to maintain saturated oxygen levels.

The GST activity in 9 individual Sphaerium was analysed immediately on return to the laboratory (time 0 h). Groups of animals (n=9) were subsequently sampled from each tank after 24, 48, 72, 96 and 196 hours and the GST activity in each individual was determined. The pH of the holding water was measured every 24 hours.

b) Effect of transfer to separate holding tank

Animals were collected and transported to the laboratory as described above and acclimated for 96 hours in replicate 1.5 l tanks previously equilibrated to the site water temperature (8 °C). After this period 9 animals were initially removed and assayed for GST activity (time 0 h), following which groups of animals (n=30) were transferred to replicate tanks containing 1.5 litres of fresh previously equilibrated SSW. At intervals of 24, 48 and 72 hours animals were removed from each replicate tank and analysed for GST activity.

B2.5.3 Effect of lindane exposure on GST activity in individual tissue samples

Animals from the Elland and Goit populations were used in this study and were acclimated to the experimental conditions for 96 hours following collection and transport to the laboratory. The animals were then divided into control and treatment groups and placed in 1.5 litres of fresh temperature-equilibrated SSW.

Nine animals were removed after 96 hours and the GST activity in each was measured. Initially a static regime was used and the animals in the replicate treatment tanks were dosed with 1000 ng/l lindane, as described for the group exposure experiment. At intervals of 24 hours 8-10 animals were removed from the treatment and control tanks for GST analysis.

A range of lindane concentrations from 100 to 10 000 ng/l are being investigated to ascertain the nature of the dose-response relationship and to identify the threshold lindane concentration causing induction of GST activity.

In order to ascertain the extent to which GST induction following pollutant exposure is temperature dependent, lindane dosing experiments are now being conducted at 4, 8, 12 and 16 °C. The maintenance of a

stable lindane exposure concentration is being achieved by using a semi-static dosing regime with the replacement of media every 24 hours.

B2.6 MAINTENANCE OF SPHAERIUM IN THE LABORATORY

A recirculating aquarium system was established in order to assess the length of time that animals can be maintained in the laboratory before high mortality levels were evident. The animals were held in glass aquaria of SSW and were fed 5 day old Chlorella sp, from established cultures, twice weekly.

SECTION B3 - RESULTS AND DISCUSSION

In view of the widely varying nature of the experimental protocols investigated in this study the results obtained for discrete sections have been discussed in that section. The experimental findings have been considered in terms of application of the GST assay as a biochemical index of stress in a concluding section.

B3.1 ASSAY PROCEDURE CONDITIONS

B3.1.1 Storage conditions

a) Storage of extracted tissue in liquid nitrogen (-96.0 °C)

The effects on GST activity of storing extracted Sphaerium tissue in liquid nitrogen (-96 °C) or in ice cold buffer (0 °C) are shown in Figures B2a and b, in comparison to immediate extraction and analysis. The storage of tissue in liquid nitrogen for either 0.5 h (Figure B2a) or 4.0 h (Figure B2b) resulted in marked depressions of GST activity relative to the tissue levels in assays conducted immediately following extraction. As the process of freezing tissue in liquid nitrogen is extremely rapid it is unlikely that this denaturation is a consequence of proteolytic activity. A more plausible explanation may be the the rapid formation of ice crystals

encouraging interface denaturation. The often made assumption that freezing of tissues on site using liquid nitrogen results in unaltered enzyme levels is not apparently the case in this study.

b) Storage of enzyme extracts on ice (0 °C) and frozen (-20 °C) overnight

In Table B1 the effects on GST activity of overnight storage of enzyme extracts on ice (0 °C) and frozen (-20 °C) storage of an enzyme extract are shown. On the basis of the results obtained storage results in a reduction of GST compared to levels in enzyme extracts measured immediately following extraction. However GST extracts apparently retain a greater proportion of initial activity, in terms of activity/mg of tissue (wet mass) or protein, when stored at -20 °C (frozen) rather than at 0 °C (on ice).

Denaturation, or loss of enzymic activity can occur through the activity of proteolytic enzymes released from the cell upon extraction (through the activity of lysozymes etc). This activity may degrade the enzyme and thereby result in a loss in total activity. However, when the extract is frozen this activity will be halted. The 5.43% loss of activity on thawing of the extract may be due to surface denaturation where the protein is disrupted at the ice/water interface.

B3.1.2 Correlation between protein content and wet weight of extracted tissue

The results of the studies on the correlation between wet tissue weight and protein content conducted using animals from the Salterhebble, Elland, Goit and Malham Tarn populations are shown in Figure B3a-d. Strong correlations between protein content and tissue wet weight were apparent for each Sphaerium population. On this basis, tissue wet weight can be used in the calculation of GST specific activity (Section B2.2.2), thereby avoiding the use of the time consuming and inherently variable Biuret protein assay. This should allow for more rapid screening of individuals for GST content.

B3.2 EXPERIMENTAL STUDIES USING GROUPED TISSUE SAMPLES

B3.2.1 Seasonal variation in GST activity

The results of a 13 month seasonal variation study of baseline GST activity in groups of animals from the Salterhebble population are shown in Figure B4. The GST levels recorded on a monthly basis are represented with the physicochemical parameters of pH, temperature, and sulphate and nitrate concentrations (Appendix B2).

During the 13 month study no electron capture detector active compounds in the 5-100 pg range were measured in any water sample. The elevated GST activity measured in October 1988 was therefore not due to the presence of detectable organic pollutants at that sampling time, though it may reflect a pulse of pollution at the site before sampling, which triggered GST induction. The ability of the GST bioassay to detect episodic events not evident in water sampling is an important requirement for the routine deployment of the technique in the field.

On the basis of the results obtained it would appear that:

1) The most pronounced environmental effect on inherent GST activity is that of seasonal water temperature. GST activity, with the exception of the value for October 1988 generally followed the recorded temperature profile;

2) Within the narrow range recorded throughout this study, pH has little or no effect on the activity of GST within Sphaerium corneum. As no extreme levels were recorded it is unknown whether acid or alkaline waters would effect GST activity through an effect on cellular pH balance;

3) The levels of sulphate (SO_4^{2-}) and nitrate (NO_3^{2-}) ions apparently have no effect upon GST activity. However analysis of other anions which may exert effects has to be carried out;

Suteau et al (1985) demonstrated seasonal variations in the benzo(a)pyrene monoxygenase activity of Mytilus galloprovincialis. Seasonal differences in enzyme activity were correlated with both seasonal variations in water temperature and total body lipid, due to reproductive status. The study of the effects of seasonal variation on GST activity is being continued and tissue samples from individual Salterhebble animals have been used since October 1989.

B3.2.2 Effect of reproductive status

In Table B2 the effects of reproductive state on baseline GST activity in groups of animals from the Salterhebble population are shown. There was apparently no effect of reproductive status on grouped tissue GST activity. However, it was interesting to note that tissue from young Sphaerium contained a comparable level of GST per mg of wet tissue to that found in adults.

Table B2 - GST activity in grouped tissue samples from Sphaerium corneum of different reproductive state

Reproductive status	Grouped tissue wet weight (mg)	Mean GST activity (μkatals/mg tissue x 10^{-4})
No young present	143.90	1.23
	96.90	1.20
Young present	142.80	1.15
	52.50	1.35
Many young present	174.90	0.73
	211.50	1.14
Young removed	267.30	0.92
	168.50	1.34
Young animals	35.80	1.03
	39.50	0.89

Sphaerium corneum usually releases 6-7 well-developed live young (Boycott 1936). In the final developmental stages in the brood pouch the young feed on material passing through the aduct gill arrangement. In this study the measured GST levels in young animals may be normal inherent levels for juveniles or may be a direct consequence of material filtered by the parent.

B3.2.3 Effect of toxicant exposure on GST activity in grouped tissue samples

The effects of dosing animals with the organochlorine compounds lindane, permethrin and tecnazene (1000 ng/l) and heavy metals lead and zinc (1000 µg/l) are shown in Table B3. In all groups exposed to organochlorine compounds increases in GST activity relative to the controls were evident after 24 h, following which the extent of induction generally decreased. The considerable increase in GST activity in animals exposed to tecnazene for 72 h conflicts with the observations for lindane and permethrin and may be an erroneous result. However the patterns of GST induction recorded are strongly influenced by the large increase in GST activity in the control group at 48 hr.

A large increase in GST activity was evident following zinc dosing (1000 µg/l), which was maintained over a 90 h static exposure regime. There was no marked induction of GST following exposure to 1000 µg/l lead under the same experimental conditions.

Table B3 - The effects of exposure to organochlorine compounds (lindane, permethrin and tecnazene) and heavy metals (lead and zinc) on GST activity in grouped tissue samples

Toxicant	Time (hours)	Tissue wet weight (mg)	Mean GST activity (μkatals/mg x 10^{-5})	% change from control
ORGANOCHLORINE COMPOUNDS				
Control	0	259.10	1.23	-
	24	258.40	1.32	-
	48	186.95	1.72	-
	72	182.20	1.36	-
Lindane	24	211.95	1.61	+ 21.97
(1000 ng/l)	48	184.05	1.81	+ 5.23
	72	205.20	1.37	+ 0.74
Permethrin	24	209.45	1.52	+ 15.15
(1000 ng/l)	48	203.85	1.40	- 18.60
	72	193.30	1.49	+ 9.56
Tecnazene	24	205.20	1.54	+ 16.67
(1000 ng/l)	48	198.20	1.89	+ 9.88
	72	156.25	2.02	+ 45.53
HEAVY METALS				
Control	24	202.6	1.22	-
	90	186.4	1.26	-
Lead	24	170.0	1.27	+ 4.1
(1000 μg/l)	90	153.8	1.37	+ 8.7
Control	24	206.3	0.94	-
	90	274.8	0.67	-
Zinc	24	194.7	1.18	+ 25.5
(1000 μg/l)	90	205.0	0.78	+ 16.4

The findings are in accordance with earlier work by Boryslawskyj et al (1988), which showed significant increases in GST activity in Sphaerium corneum exposed to the organochlorine pesticides lindane and dieldrin, under laboratory and field conditions. Lee and Keeran (1988) measured a significant increase in GST activity in Callinectes sapidus following

exposure to the phenolic anti-oxidant butylated hydroxytoluene. This compound has also been shown to strongly induce hepatic GST activity in mammals (Schramm and Robertson 1985).

The general reduction in the extent of GST induction with time may reflect the reduction in exposure concentration experienced by the animals in this static regime. In the tanks, medium toxicant concentration would probably have declined due to volatilisation and uptake by the organisms. The effects of differences in combined tissue wet weight on the average GST activity of a sample are also apparent.

B3.2.4 GST activity in grouped tissue from Gammarus pulex

The GST activity measured in the combined tissue of 10 animals are shown below with the tissue mass for each group:

Population	Tissue wet weight (mg)	Mean GST activity (µkatals/mg x 10^{-5})
WRc culture	404.00	1.56
Haseley Brook	584.00	1.61

These initial analyses show that Gammarus has an appreciable inherent GST activity, with the extracts of 10 animals from each population showing an absorbance change of approximately 0.3 absorbance units/minute. On this basis an extract from a single individual should yield sufficient activity (0.03 absorbance units/minute) to be measured, providing the individual is of reasonable size (~40 mg wet weight).

The GST activities measured in Gammarus (1.5 x 10^{-5} µkatals/mg tissue wet weight) are lower than those measured in Sphaerium corneum (1.4 x 10^{-4} mg tissue wet weight). However in Sphaerium all the tissue used is metabolically active whereas in Gammarus the tissue wet weight includes a considerable exoskeleton component. On the basis that the metabolically active tissue in Gammarus is only 10% of total body weight

then GST activity of 1.5×10^{-5} can be considered comparable to levels from other aquatic organisms, for example Lymnea stagnalis, Mytilus edulis and Raja radiata (Stenersen et al 1987).

The apparent difference in GST activity between populations was minimal and was probably due to inherent variability rather than any population effect. In the development of the GST assay as a biochemical index of stress in Gammarus the validity of using tissue wet weight in the calculation of specific activity and the effect of individual size on GST levels have to be addressed.

B3.3 EXPERIMENTAL STUDIES USING INDIVIDUAL TISSUE SAMPLES

B3.3.1 Body size and GST activity

The GST levels found in individual Sphaerium from the Worsborough Reservoir, Malham Tarn, Elland and Goit populations in relation to body size (tissue wet weight) are shown in Figures B5-8 respectively. In the Worsborough (Figure B5a), Malham (Figure B6a) and Goit (Figure B8a) populations, inverse relationships between weight-specific GST activity and individual size were evident, though no clear correlation was apparent for individuals from the Elland population (Figure B7a). On representation of the total GST activity against individual tissue weight allometric relationships were evident in all populations (Figures B5b-8b), which indicates a marked size effect on GST activity.

In theory this effect could be avoided by using animals within a narrow size range. However, practically, this is an unsatisfactory solution as shell size is not a good predictor of tissue mass, and the size-effect has to be accounted for in all studies. This is possible if the GST activity of the animals can be converted to a standard body size.

In this respect a 'standard' wet tissue weight of 40.0 mg has been chosen and all individual GST activities have been normalised to this weight by means of the allometric equation

$$Y = aW^b$$

where: Y is the GST activity of the individual (μKatals),
X is the tissue wet weight (mg) and a and b are constants.

The constants are obtained from the regression equation of the logarithmically transformed GST and tissue wet weight data, where a and b represent the intercept and slope respectively:

$$\log Y = \log a + b \log X$$

The regression equations obtained for each population are shown in Figures B4c to B7c. The derived constant b can then be used to convert all GST activities to the theoretical GST activity for a standard 40 mg animal by substitution in the equation:

$$\log Yc = \log Yo - (b \log Xo - b \log Xc)$$ where

Yc = corrected GST activity
Xc = standard wet tissue weight (40.0 mg)
Yo = individual GST activity
Xo = individual wet tissue weight.

In Figures B9a to d the normalised mean total GST activity from each population are given with relevant water pH, temperature, sulphate and nitrate concentrations respectively. The mean GST activity in the Malham Tarn population was significantly higher than the levels in the other Sphaerium populations (T-test $P<0.05$). At this site only water temperature (Appendix B3) was markedly different from that at the other locations. However the low temperature recorded may be expected to result in a lower, rather than elevated GST activity than at other sites and this parameter is apparently not responsible for the GST difference. In addition no electron capture detector (ECD) active compound (organic pollutant) was identified in the water samples taken from this site, though compounds not detected by this procedure or those present before the sampling time could have caused GST induction.

B3.3.2 Effect of reproductive status

The inherent GST activity in ovigerous and non-ovigerous individuals from the Elland, Goit and Worsborough Reservoir populations are shown in Table B3. There were no apparent differences between non-ovigerous and ovigerous animals from any population. Although the observations were limited in number the group and individual data apparently indicated that reproductive state does not significantly affect an organisms baseline GST activity.

Table B4 - GST activity measured in non-ovigerous and ovigerous Sphaerium from the Elland, Goit and Worsborough Reservoir sites

Population	Reproductive state	Mean corrected GST activity (μkatals x 10^{-5})	Std dev	n	T-test
Worsborough Reservoir	Non-ovigerous	62.06	3.43	2	NS
	Ovigerous	59.92	4.09	9	
Elland	Non-ovigerous	69.76	13.88	9	NS
	Ovigerous	69.99	10.52	10	
Goit	Non-ovigerous	66.28	14.22	3	NS
	Ovigerous	63.89	9.15	7	

NS - Not significant

B3.3.3 Collection and transport of animals from the field to laboratory

The effects of collection and transport of Sphaerium corneum from the field to the laboratory on individual GST activity in the Elland population are shown in Table B5. The pattern of increasing GST activity from the initial value (time 0 h) to a maximum plateau level before declining was apparent in both experimental tanks (Figure B10).

Table B5 - GST activity in Elland individuals monitored in replicate tanks after collection and transport from the field to the laboratory

Time (hrs)	Experiment No	Mean corrected GST activity (µkatals x 10^{-5})	Std dev	n	b	Experimental mean GST activity (µkatals x 10^{-5})
0	-	54.93	5.24	7	1.27	54.93
24	1	63.01	5.10	8	1.39	63.61
	2	64.21	6.12	8	1.22	
48	1	72.19	10.05	9	0.68	76.67
	2	81.14	12.26	9	0.75	
72	1	79.19	7.00	9	0.82	81.09
	2	82.99	9.31	9	0.56	
96	1	73.98	7.77	9	0.65	72.53
	2	71.07	5.95	8	0.78	
192	1	60.22	2.44	8	0.93	62.84
	2	65.46	6.50	7	0.90	

b = gradient of linear relationship between log-log transformed GST-tissue wet weight data

Analysis of variance (ANOVA) revealed no significant differences between the replicate experiments ($F=1.74$, $P=0.182$), however a highly significant time effect was evident ($F=20.51$, $P<0.001$). A lag phase for induction was found and only after 48 hours was mean GST activity significantly higher than the basal GST activity at the initial sampling time (Dunnett's test, $P<0.001$). The 39.58% increase in GST after 48 hours reached a maximum of 47.62% at 72 hours after which GST activity declined towards the basals levels. The levels recorded at 192 hours were not significantly different from initial values (Dunnett's test, $P>0.05$). On this basis, collection and transport of the animals from their natural site to the laboratory system markedly stresses the animal, although the effect on the GST activity is only apparent after a 48 hour lag phase and persists for a further 48 h. The

implication of these findings is a requirement to allow greater than 96 h for acclimation following transportation of animals from the field to the laboratory.

The effect of mechanical action on mussels is well documented and studies by Coe (1947) in the pismo clam Tivela stultorum have shown that physical disturbances no greater than the handling involved in a shell measurement may be sufficient to induce disturbance ring formation. In Mytilus edulis changes in the water holding temperature have been shown to induce a biochemical response to this physical effect. The induction of heat shock proteins (stress proteins) have also been shown as a response to such an effect in this mussel by Steinert and Pickwell (1988). These are thought to impart stress resistance or tolerance to the cell. Although the observed increase in GST may not be directly analagous to that for heat shock proteins, it is possible that GST may be produced as a direct response to physical action upon the animal.

B3.3.4 Transfer of animals between tanks

The results of the replicate experiments for transfer of individuals between tanks are shown in Table B6. Statistical analysis showed that there was no significant difference in response between the replicate experiments ($F=0.02$, $P=0.985$), though GST activity varied significantly with time ($F=9.09$, $P<0.001$).

Table B6 - GST activity in Elland individuals monitored in replicate tanks after transfer between tanks

Time (hrs)	Experiment No	Mean corrected GST activity (µkatals x 10^{-5})	Std dev	n	b	Experimental mean GST activity (µkatals x 10^{-5})
0	-	64.91	5.06	10	1.00	64.91
24	1	53.80	3.76	10	0.89	53.90
	2	53.99	9.10	9	0.54	
48	1	52.27	4.60	9	0.88	54.32
	2	56.37	5.67	8	0.84	
72	1	59.48	7.27	9	0.84	56.89
	2	55.27	3.64	9	0.87	

The significant decreases in GST activity at all sampling times (Dunnett's test, $P<0.05$ in all cases) relative to the control value may reflect a continuing fall to basal levels following the 96 h recovery period following collection and transport. However the short-term handling stress involved in transferring animals between tanks apparently does not result in GST induction.

B3.3.5 Dosing studies on individual tissue samples

In this research study the effects of lindane exposure (1000 ng/l) on GST activity from the 'clean' Elland animals (no pre-history of organochlorine pesticide pollution) and the polluted Goit individuals (a past history of pollution stress) were compared.

The GST activity in individuals from the Goit population exposed to 1000 ng/l at 9-10 °C is shown in Table B7. Although a clear allometric relationship was evident in the control groups after 24 and 48 hours and data normalisation was possible, the lindane-exposed animals shoved no clear relationship between total GST activity and tissue weight after 24 hours and the correlation coefficient of the log-log transformed

equation was not significant (P>0.05). At 48 hours no differences were apparent between controls and lindane-dosed animals.

Table B7 - The effects of lindane exposure (1000 ng/l) on GST activity in individuals from the Goit population

Toxicant	Time (h)	Mean corrected GST activity (µkatals x 10^{-5})	Std dev	n	b	r	T-test
Control	24	65.32	10.40	13	0.78	0.71	-
	48	69.56	10.17	17	0.84	0.93	-
Lindane	24		Not standardised			0.38	NS
	48	62.56	17.49	18	0.93	0.57	NS

r = correlation coefficient of the log-log linear regression between GST and tissue wet weight.

The higher than normal variation in the mean GST activities of lindane exposed Goit individuals (as shown by the correlation coefficient r) could indicate that the extent of variation observed is stress related and the inability to standardise data reflects a divergence of response among a group.

Problems with the Goit site caused later dosing experiments to be confined to individuals from the Elland site.

The apparent correlation of GST activity with water temperature found in the seasonal variation study has resulted in the semi-static lindane exposure studies being conducted at a range of temperatures from 4-16 °C. In this way the extent to which GST induction is temperature dependent can be assessed.

In Table B8 the effects of semi-static lindane exposure (1000 ng/l) on GST activity in individual Sphaerium at 4 °C are shown. Statistical comparison of means (T-test) showed there was no significant difference ($P>0.05$) in GST activity between control and treatment animals at each sampling time throughout the 72 hours of study. On this basis there is apparently no induction of GST in lindane exposed animals at 4 °C. The observed decreases in GST activity in both groups at 72 h could be due to the effects of starvation.

Table B8 - The effects of lindane exposure (1000 ng/l) on GST activity in individuals from the Elland population at 4 °C

Toxicant	Time (h)	Mean corrected GST activity (µkatals x 10^{-5})	Std dev	n	b	T-test
Control	24	66.69	15.32	9	0.59	-
	48	65.60	6.73	9	0.84	-
	72	49.27	3.58	7	1.05	-
Lindane	24	69.72	15.17	8	0.66	NS
	48	69.77	6.88	9	0.82	NS
	72	45.34	9.57	7	0.82	NS

Temperature is probably exerting an effect on the rate of protein synthesis, with low rates of synthesis at low temperatures. Boryslawskyj (1990), in this laboratory, demonstrated a clear correlation between temperature and the accumulation of dieldrin in Sphaerium corneum in the range 5-20 °C. It was suggested that the changes in the rate of dieldrin uptake could be attributable to either the effect of temperature on dieldrin's physico-chemical equilibrium in the animal's lipoidal tissue, or on gill cilia beat frequency, which showed a positive relationship with temperature.

The investigations of the relationships between lindane concentration and the extent of GST activity are continuing at each temperature to establish threshold levels for GST induction.

SECTION B4 - CONCLUSIONS

Studies within this contract have been concerned with the development of the enzyme GST as a biochemical index of sublethal stress in Sphaerium corneum. On the basis of the results obtained the following conclusions have been drawn:

1) An accurate and reproducible biochemical test capable of measuring GST activity in tissue samples (>10 mg wet weight) from individual animals has been developed. Marked reductions in GST activity were found in tissue sample analysed after storage on ice (-28.8%) or in liquid nitrogen (-40.3%). In contrast, freezing the extracted enzyme was found to have only a minor effect on the inherent GST activity (-5.35%). A strong correlation between total protein content and wet tissue weight of individuals has been shown, obviating the requirement for the determination of tissue protein levels and allowing rapid screening of animals.

2) In all Sphaerium populations increasing total GST activity (μkatals) was found with increasing body size, as tissue wet weight. Allometric relationships of the form $Y = aX^b$ were established between total GST activity and tissue weight. This enables values from individuals of all populations to be corrected to a standardised 40 mg tissue weight animal.

3) A 13 month study on seasonal variation of GST in the Salterhebble population has indicated that water temperature may have a major role in determining baseline GST activity. No clear correlation between inherent GST activity and site water chemistry has been found in 4 populations.

4) No significant differences in basal GST activity have been found between ovigerous females and non-ovigerous animals at a number of sampling sites. The effect of reproductive state on GST induction during lindane exposure is currently being assessed.

5) The effects of collection and subsequent transport to and within the laboratory have been studied and showed:

   a) An induction of GST activity following field collection and transportation to the laboratory which was significantly elevated above initial levels after a 48 hour lag phase. The elevated tissue GST activities declined to basal levels after between 96 and 192 hours, indicating that animals collected from the field should be acclimated for at least 120 hours before conducting dosing experiments.

   b) The movement of animals between tanks in the laboratory had no significant effect on GST induction.

6) Induction of GST activity has been shown in response to exposure to the pesticides lindane, permethrin and tecnazene, and the heavy metal zinc, (though not lead) in a population of known pollution history. In addition the nature of GST induction is such that this technique appears to have the potential to indicate prior exposure to episodic events.

7) The induction of GST activity by lindane in individuals from a population with no pre-history of pollution has been shown. The effects of temperature on GST induction in individuals during lindane exposure are currently under investigation.

8) The presence of GST in Gammarus pulex at measurable levels has been demonstrated.

SECTION B5 - FUTURE WORK

1) Characterise the isozymic profile of GST in Sphaerium during non-polluted, stress and toxicant exposure conditions. During stress or toxicant exposure changes in certain isozymes may principally be responsible for the changes in total GST activity. The elucidation of these isozymes would increase the sensitivity of the assay. Furthermore, should the induction of certain isozymes be caused by given pollutants, inferences could be drawn about the history of pre-exposure of the organisms.

2) Monitor GST in a larger number of native populations, to ascertain the extent to which exposure to inducers effects inherent enzyme levels in animals from different sites. In view of the differences in the derived value of b measured in different experiments for a given population the nature of the relationship between total GST activity and tissue wet weight should be studied in large samples containing individuals of a wide size range.

3) Characterise new populations for laboratory and field studies in terms of identifying the lag phase for GST induction following collection and transport of animals from the field. Also characterise the lag phase for GST induction and the persistence of response to 'standard' pollutant exposure (given concentrations of a heavy metal and organic pollutant known to induce GST) to allow inter-population assessments. GST changes during exposure and depuration will be correlated with measured pollutant body burdens.

4) Carry out laboratory studies to ascertain the sensitivity of GST induction to various heavy metal and organic pollutants, with both acute and chronic toxicant exposure. These dose-response relationships will identify threshold concentrations of pollutants necessary for GST induction. In chronic exposure experiments, the effects of toxicant partitioning between aqueous and particulate phases will have to be examined in order to assess the relative importance of water and dietary derived toxicant sources in GST

induction. The present study has established that Sphaerium can be maintained in the laboratory on a diet of Chlorella sp. for at least a 2 month period.

5) Investigate in the laboratory the GST activity responses to effects of episodic discharges, concentrating on the relative importance of pollutant pulse magnitude, duration and frequency. On the basis of this study future deployment of individuals in field trials can be assessed. The field programme would probably be a WRc undertaking, following a successful characterisation of responses in the Huddersfield studies.

6) Establish an assay procedure at WRc and test its performance as a routine pollution monitoring tool.

7) Investigation the possibility of establishing laboratory stock cultures of Sphaerium, which could reduce the variability of response of test organisms to toxicants.

8) On determining the method has utility integrate the GST assay with other associated techniques currently deployed, such as feeding rate and biomonitoring studies in which tissue residues are measured.

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Table B1 - Effects of overnight storage on ice (0 °C) and frozen (-20 °C) on the enzyme extract GST levels based on tissue wet weight and tissue protein levels

Treatment	Initial GST activity		Final GST activity		% change
	µkatals/mg tissue wet weight x 10^{-5}	µkatals/mg tissue protein x 10^{-5}	µkatals/mg tissue wet weight x 10^{-5}	µkatals/mg tissue protein x 10^{-5}	
Overnight storage on ice (0 °C)	1.20	--	1.00	--	-16.7
	1.12	--	0.99	--	-11.6
	2.33	--	2.01	--	-13.7
	2.34	--	2.15	--	- 8.1
	--	3.65	--	3.18	-12.9
	--	4.13	--	3.23	-21.8
	--	4.29	--	3.60	-16.1
	--	4.03	--	3.27	-18.9
Overnight storage frozen (-20 °C)	1.78	--	1.65	--	- 7.3
	0.69	--	0.64	--	- 7.3
	1.51	--	1.45	--	- 4.0
	1.27	--	1.23	--	- 3.2
	--	3.65	--	3.48	- 4.7
	--	4.13	--	4.04	- 2.2
	--	4.29	--	3.64	-15.2
	--	4.03	--	5.37	+33.3

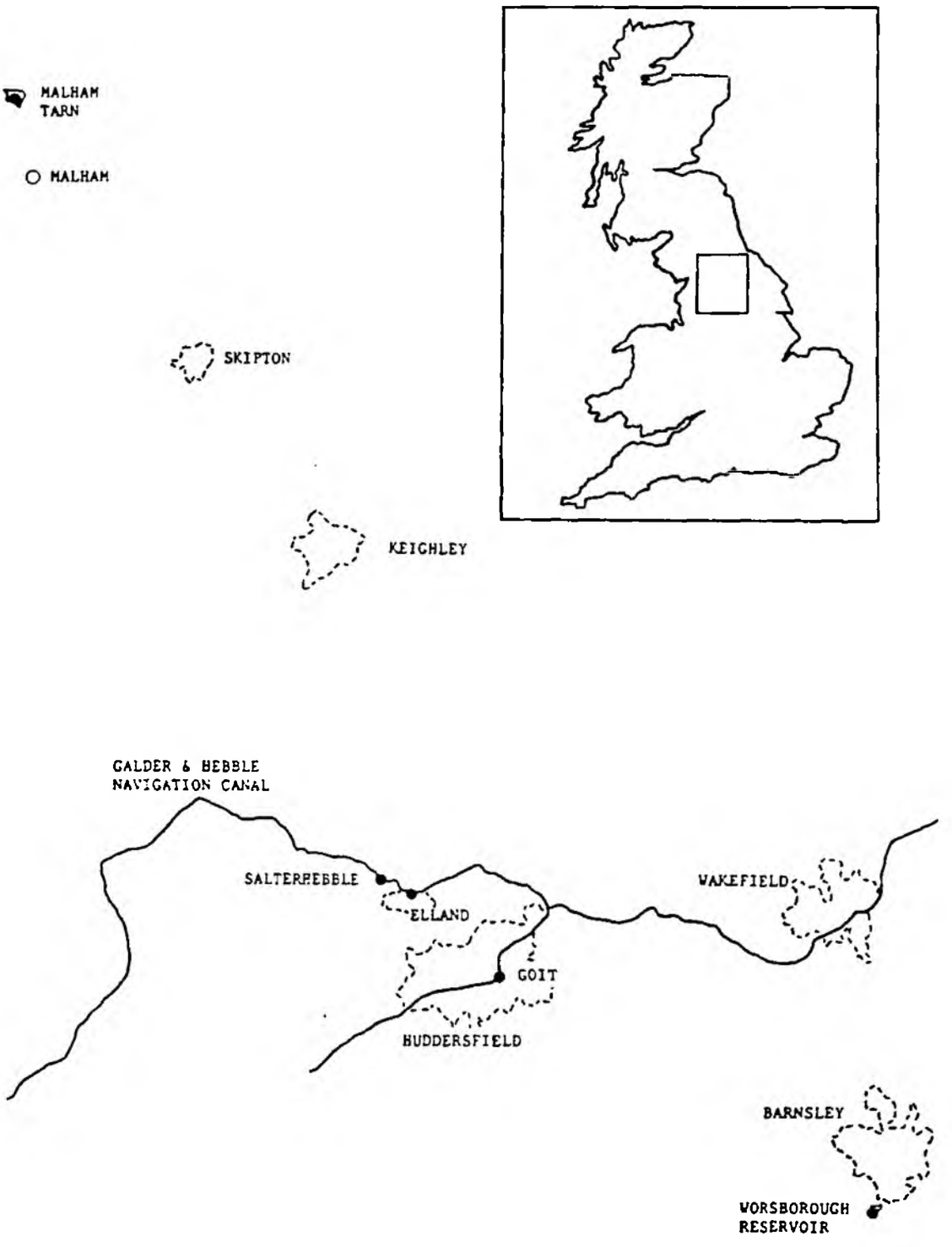


Figure B1- Locations from which Sphaerium corneum were obtained in this study.

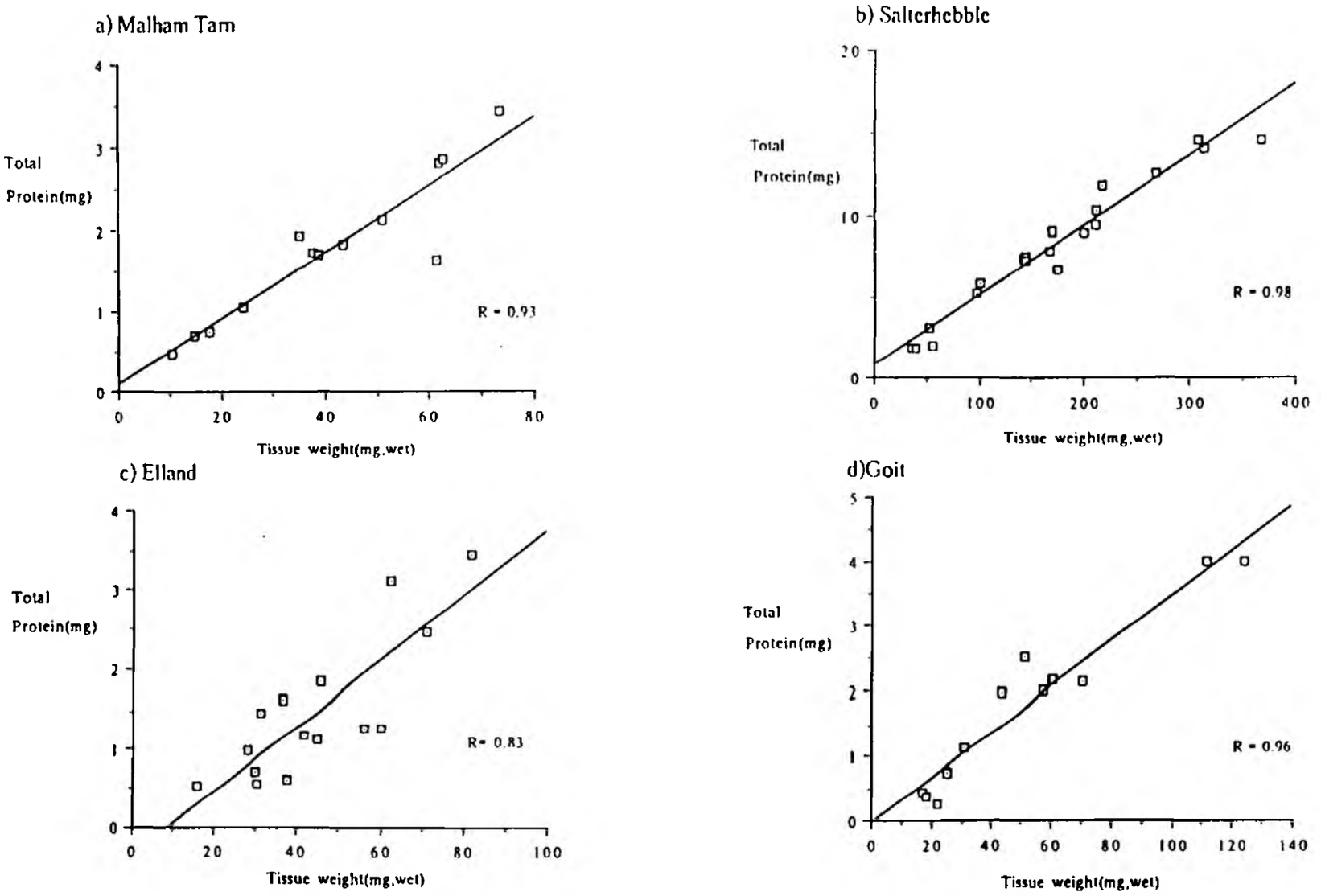
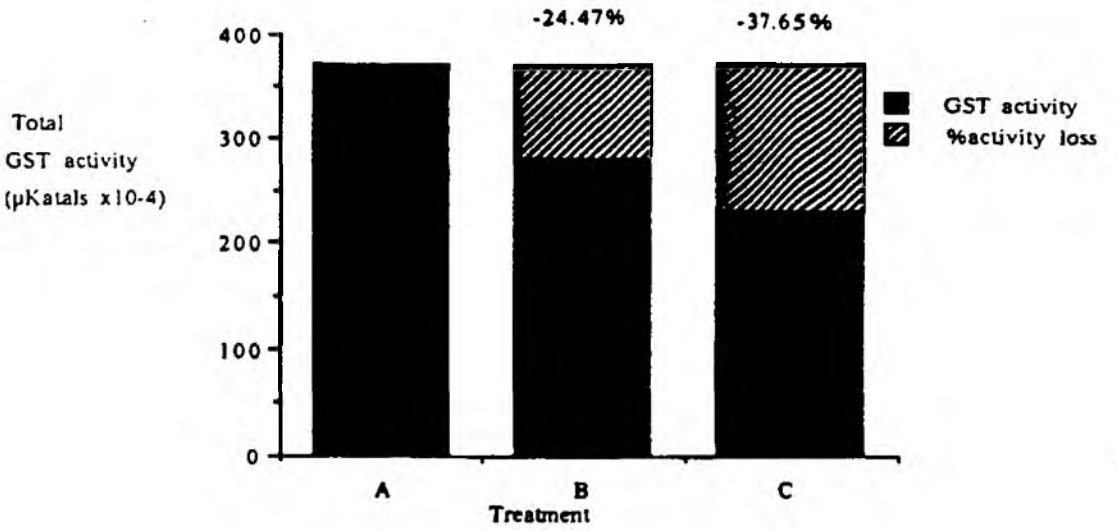
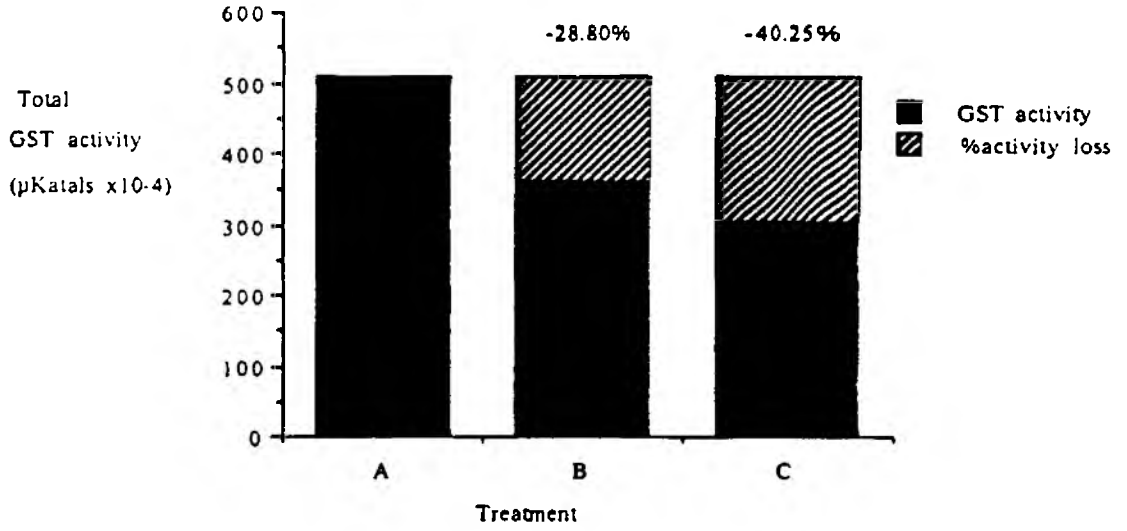


Figure B2- Correlations between tissue protein content and wet weight for the a) Malham Tarn, b) Salterhebble, c) Elland and d) Goit populations.

a)0.5h



b)4.0h



A= Immediate tissue extraction

B= Tissue in ice cold buffer(0°C)

C= Tissue in liquid nitrogen(-96°C)

Figure B3- Effects of a)0.5h and b)4.0h storage of tissue in ice cold(0°C) buffer and liquid nitrogen (-96°C) on GST levels compared to tissue analysed immediately.

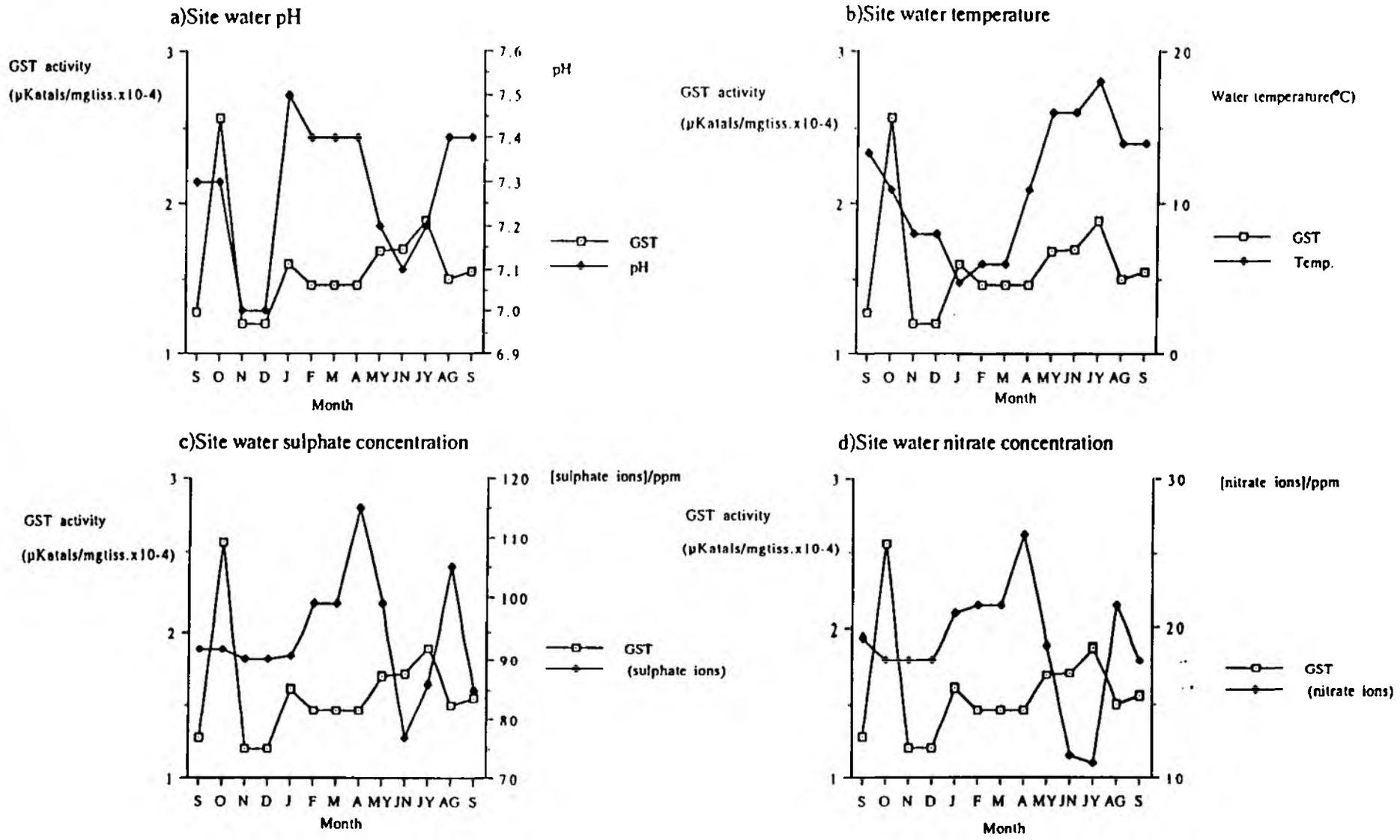


Figure B4- Seasonal variation in GST levels from Salterhebble animals in relation to a) site water pH, b) site water temperature, c) site water sulphate concentration (ppm) and d) site water nitrate concentration (ppm).

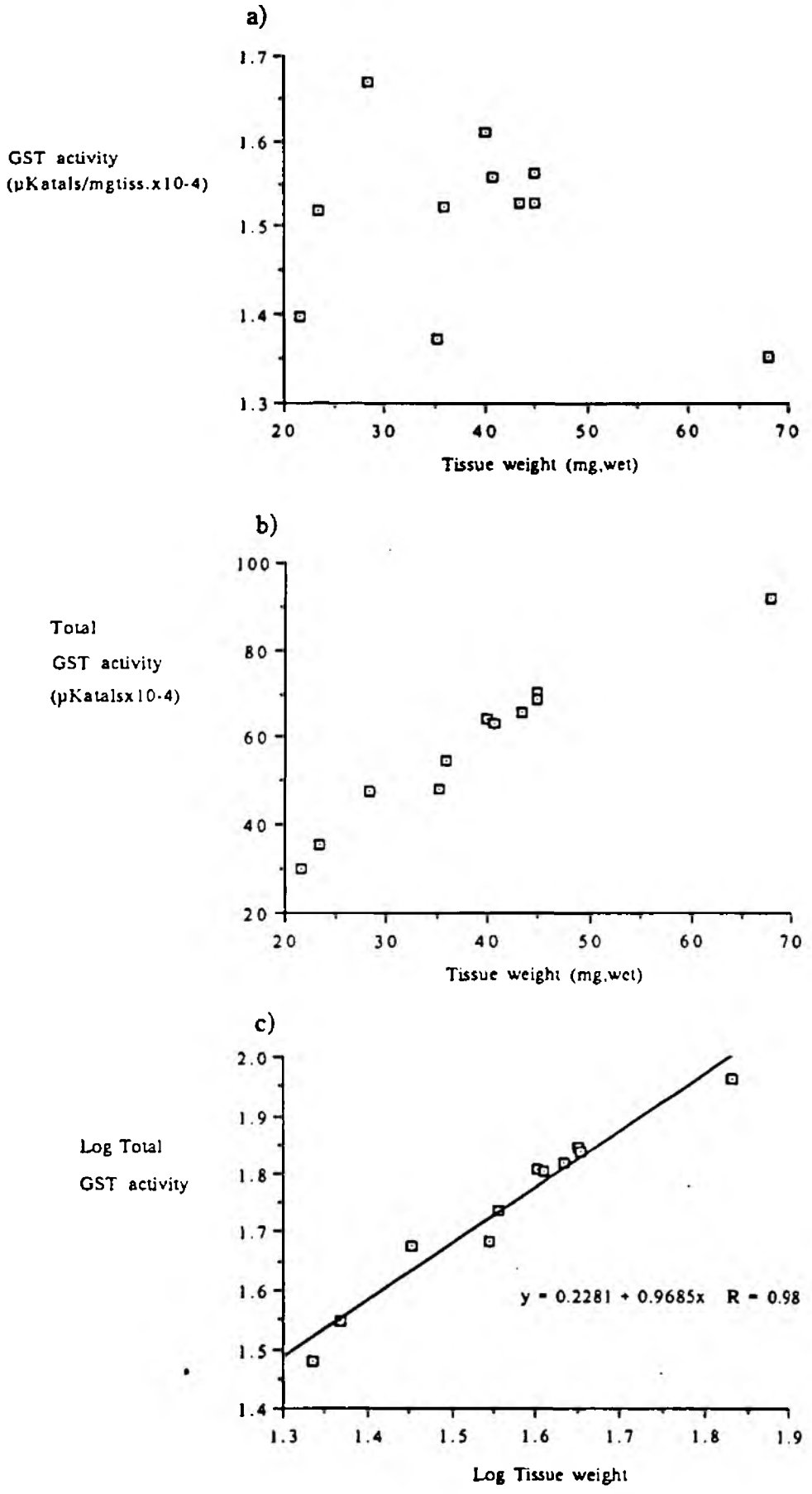


Figure B5- The a) weight specific and b) total GST levels in individual Sphaerium corneum of varying tissue weight from the Worsborough Reservoir population .In c) the logarithmically transformed total GST- tissue weight data is given.

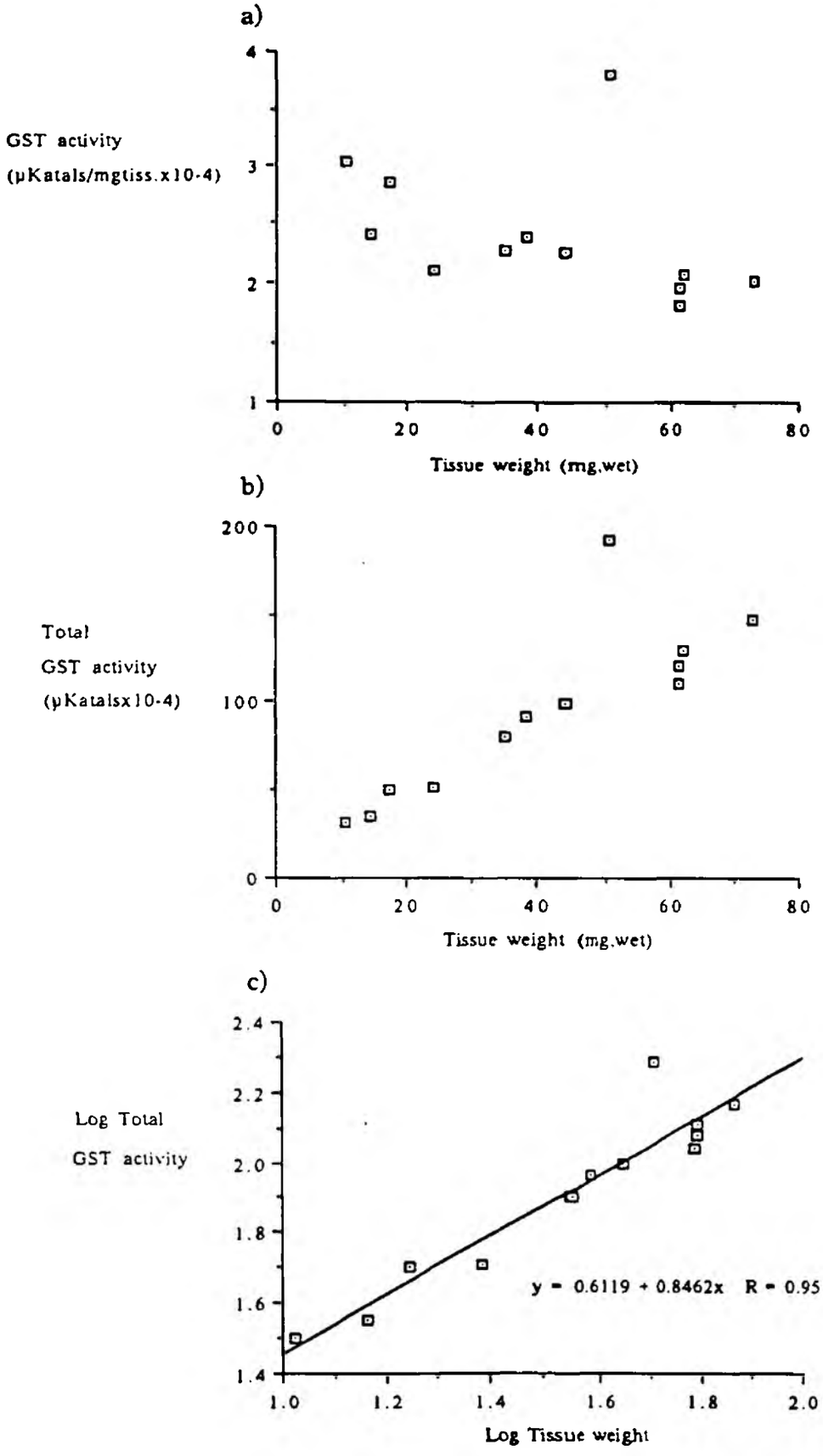


Figure B6- The a) weight specific and b) total GST levels in individual Sphaerium corneum of varying tissue weight from the Malham Tarn population .In c) the logarithmically transformed total GST- tissue weight data is given.

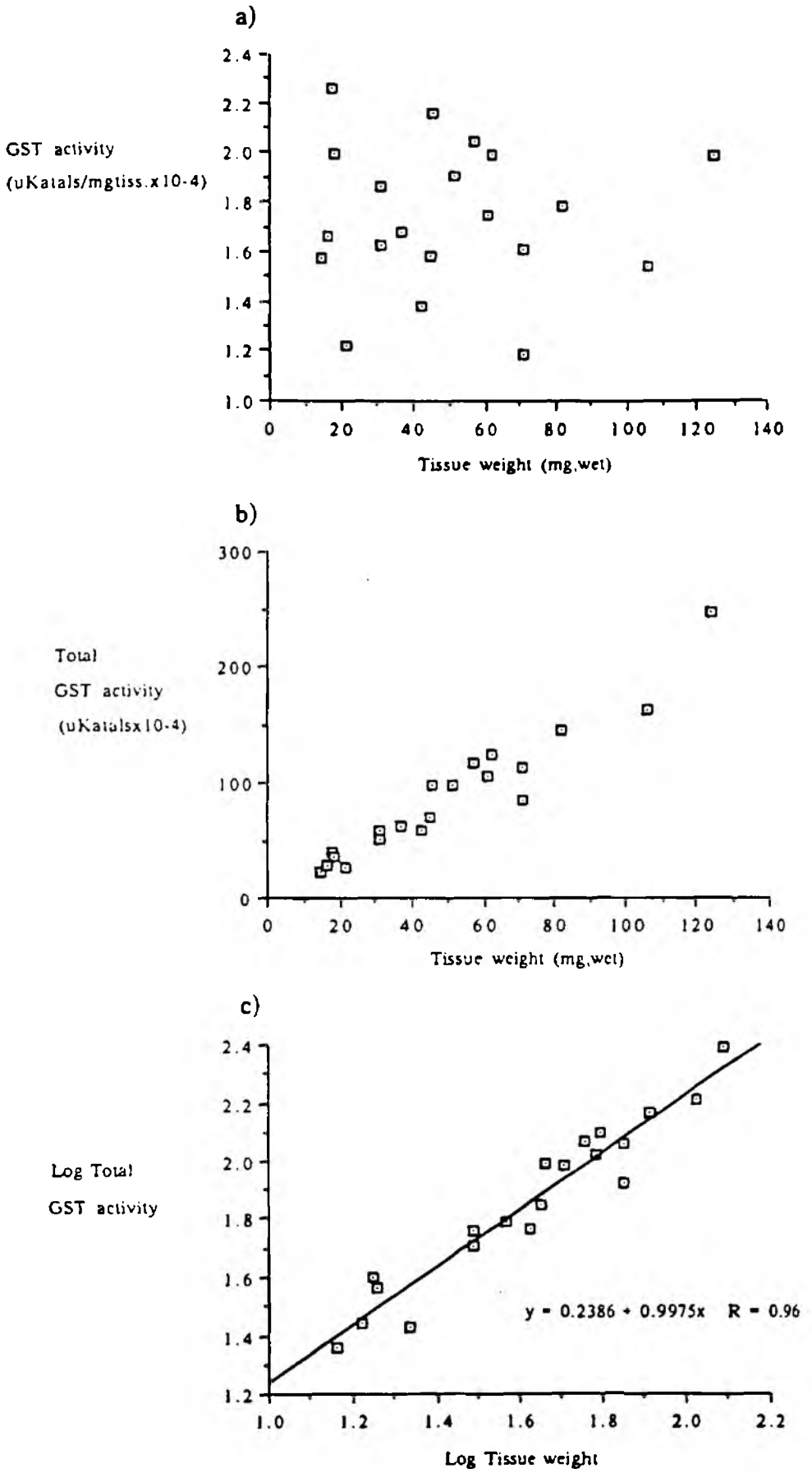


Figure B7- The a) weight specific and b) total GST levels in individual Sphaerium corneum of varying tissue weight from the Elland population .In c) the logarithmically transformed total GST- tissue weight data is given.

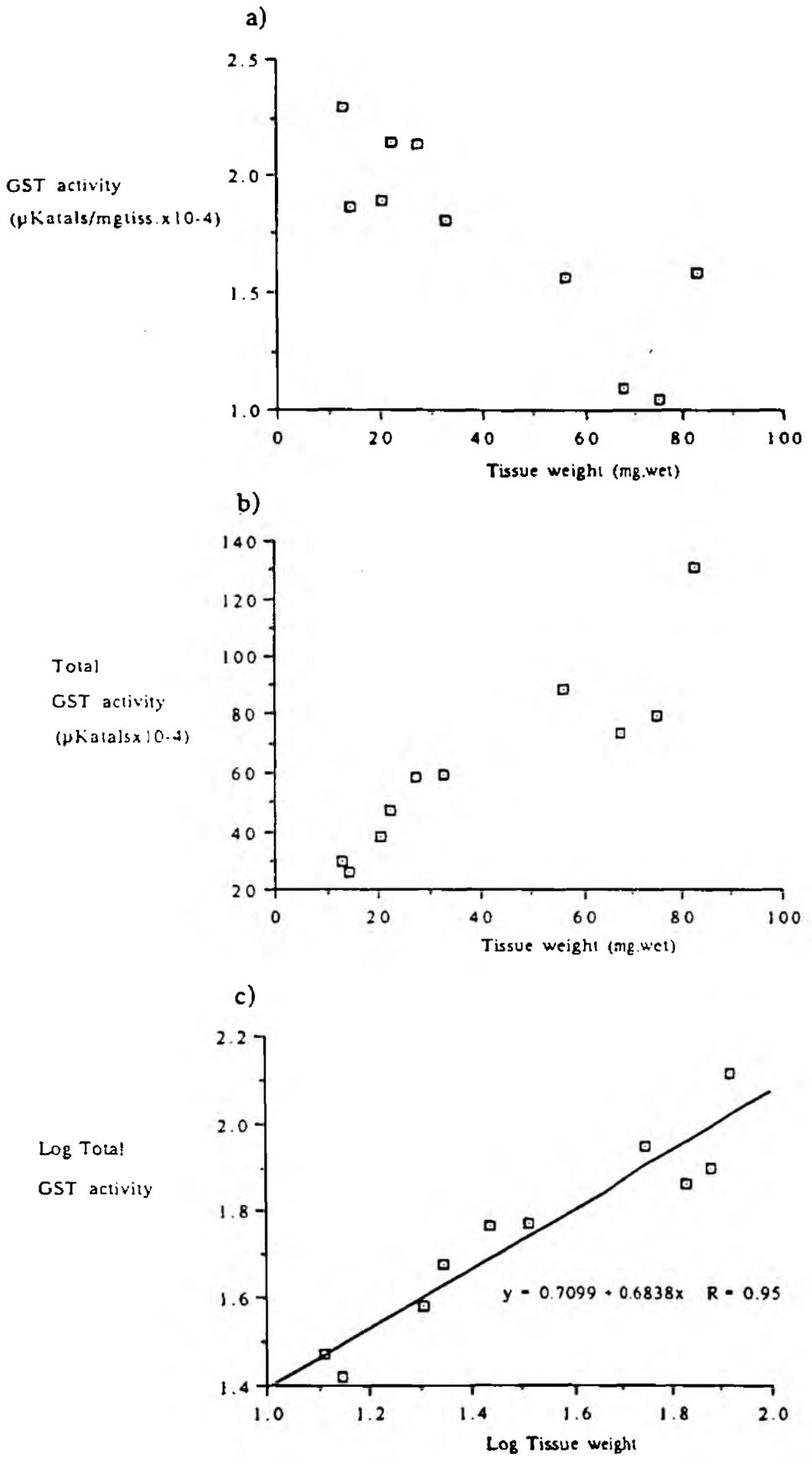


Figure B8- The a) weight specific and b) total GST levels in individual Sphaerium corneum of varying tissue weight from the Goit population .In c) the logarithmically transformed total GST- tissue weight data is given.

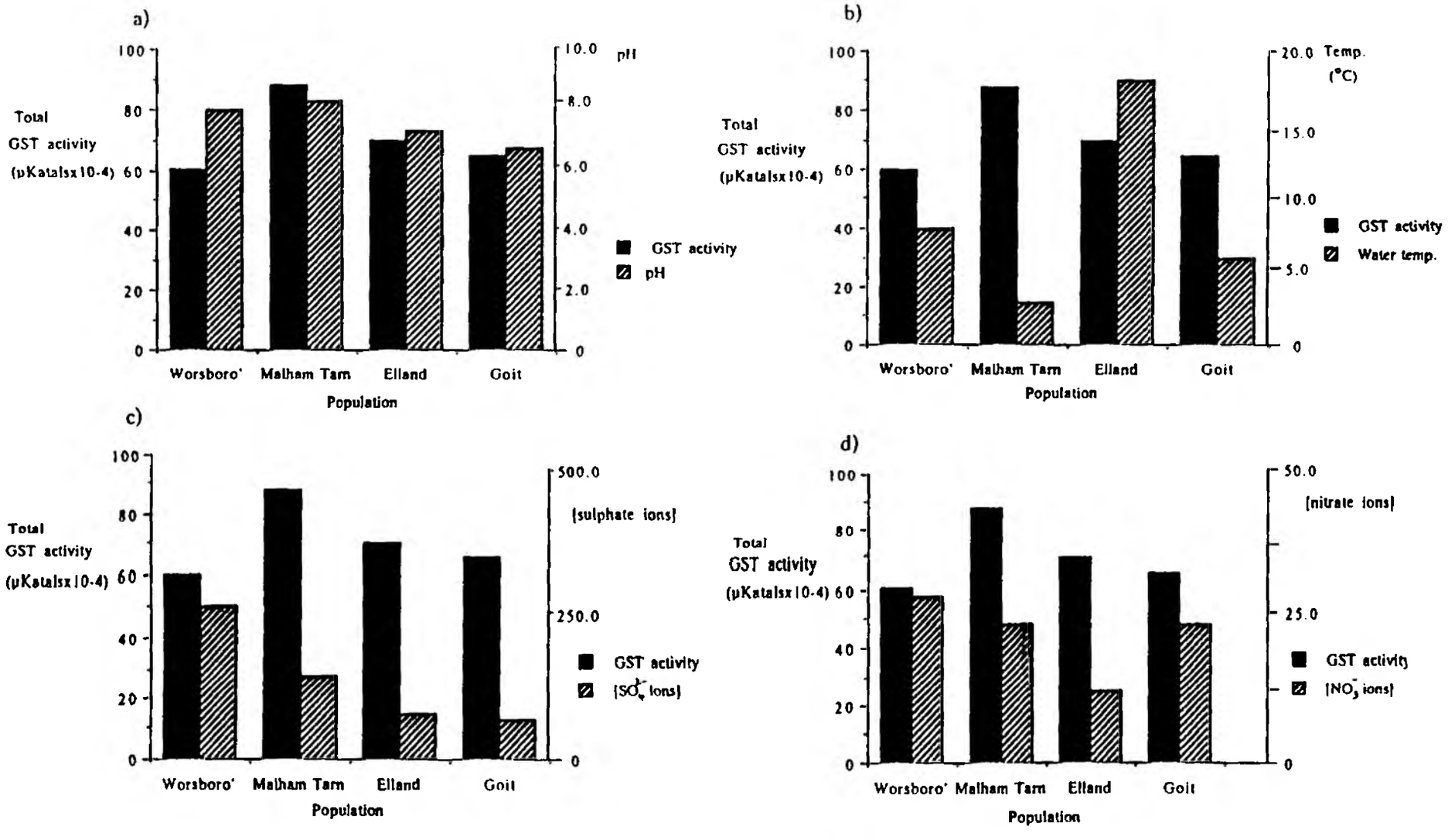


Figure B9- The mean total GST levels in Sphaerium corneum from the Worsborough Reservoir, Malham Tarn, Elland and Goit populations in relation to a) site water pH, b) site water temperature, c) site water sulphate concentration (ppm) and d) site water nitrate concentration (ppm).

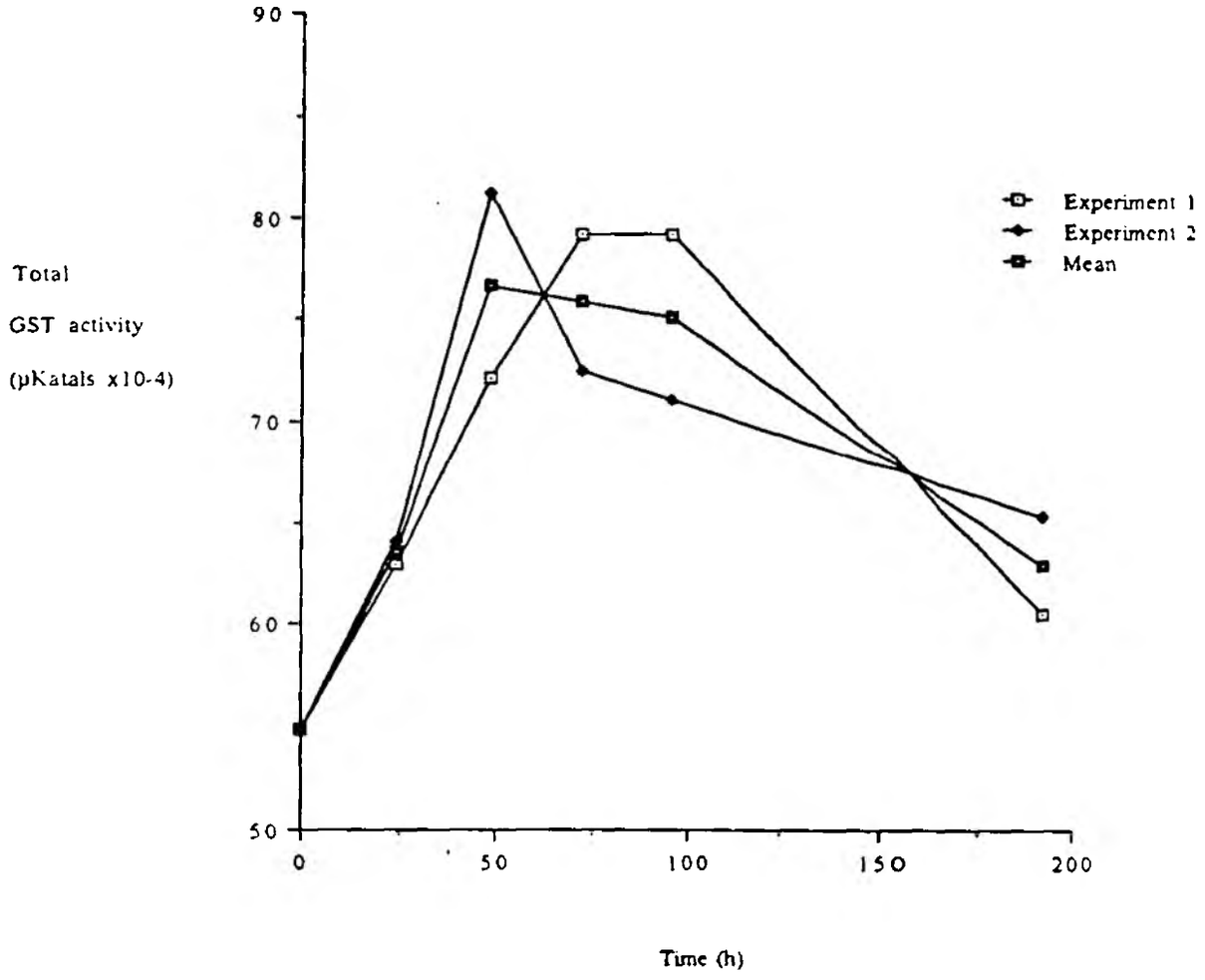


Figure B10- The effect on GST levels in Sphaerium corneum from the Elland population of collection and transfer from the field to the laboratory. The results of replicate experiments for the 192h monitoring period are given.

APPENDIX 1

PREPARATION OF STANDARD SOFT WATER (SSW)

Standard soft water was prepared by dissolving the following analar grade salts in 5 litres of Glass Distilled Water at a pH of 7.2 - 7.4.

Salt	Mass (g)
$NaHCO_3$	240.0
$CaSO_4.2H_2O$	150.0
$MgSO_4.7H_2O$	150.0
KCl	10.0

APPENDIX 2

GST ACTIVITY AND WATER CHEMISTRY DATA FOR SAMPLES TAKEN ON A MONTHLY BASIS FROM SEPT 88 TO SEPT 89 AT THE SALTERHEBBLE SITE

Month	Tissue wet weight (mg)	Mean GST activity (μkatals/mg x 10^{-4})	Water chemistry				
			pH	Temp (°C)	SO_4^{2-} (ppm)	NO_3^{2-} (ppm)	PO_4^{3-} (ppm)
Sept	33.70	1.28	7.3	13.3	92.0	19.5	ND
Oct	25.75	2.56	7.3	11.0	92.0	18.0	ND
Nov	34.55	1.20	7.0	8.0	90.0	18.0	ND
Dec	-	-	-	-	-	-	-
Jan	34.35	1.61	7.5	4.8	90.5	21.0	ND
Feb	40.66	1.46	7.4	6.0	99.0	21.5	ND
Mar	-	-	-	-	-	-	-
Apr	-	-	7.4	11.0	115.0	26.2	ND
May	48.58	1.69	7.2	16.0	99.0	19.0	ND
June	31.36	1.70	7.1	16.0	77.0	11.5	1.0
July	46.93	1.89	7.2	18.0	86.0	11.0	ND
Aug	48.60	1.50	7.4	14.0	105.0	21.5	ND
Sept	43.85	1.55	7.4	14.0	85.0	18.0	ND

APPENDIX 3

MEAN *SPHAERIUM* GST LEVELS AND WATER CHEMISTRY DATA FROM SAMPLES TAKEN AT THE WORSBOROUGH RESERVOIR, MALHAM TARN, ELLAND AND GOIT SAMPLING SITES

Population	Mean corrected GST activity (μkatals x 10^{-5})	n	Std dev	b	Water chemistry				
					pH	Temp (°C)	SO_4^{2-} (ppm)	NO_3^{2-} (ppm)	PO_4^{3-} (ppm)
Worsborough Reservoir	60.31	11	3.91	0.97	8.0	8.0	250	29.0	2.0
Malham Tarn	87.95	11	7.09	0.84	8.3	3.0	140	24.0	ND
Elland	69.55	20	11.65	1.00	7.3	18.0	75	12.5	ND
Goit	64.61	11	10.10	0.68	6.7	6.0	65	24.0	ND

SECTION C - STATUS OF OTHER BIOASSAYS UNDER DEVELOPMENT

Brian Roddie

SECTION C1 - OYSTER EMBRYO CRYOPRESERVATION

Toxicity tests based on the development of early oyster (Crassostrea gigas) embryos are becoming increasingly widely-used in assessing the environmental effects of chemicals and effluents and the quality of receiving waters. The protocol used by WRc stipulates a test duration of 24 h, and the endpoint is defined as the proportion of embryos (relative to control) successfully developing to the point where formation of the larval straight-hinge shell is complete (the D-stage). Although this test is robust and sensitive, it may occasionally be impaired by poor or variable embryo quality and viability. The selection of suitable embryo batches demands a degree of skill and experience, while the readiness with which the test can be carried out depends on access to ripe oysters or the facilities with which to condition oysters to ripeness. These constraints do not diminish the value of the test, but do limit the number and range of laboratories, locations and (sometimes) seasons in which it can be carried out.

The ability to store gametes or embryos of known quality would largely obviate these problems, and would additionally permit a very high standard of quality control and intercomparison between tests conducted at different times and in different laboratories. Oysters produce a very large number of gametes ($>10^7$), and a single pairing can provide sufficient embryos for several hundred tests.

Cryopreservation, the storage of biological material in recoverable condition at liquid nitrogen temperatures, has been recognised as a valuable tool in human and other mammalian reproductive studies and in animal husbandry. The procedure is routinely used in the storage of oyster gametes for commercial culture operations. Commercially, a high percentage recovery of viable embryos is not essential (as very large

numbers of gametes are produced), but for the purpose of toxicity testing fertilisation and developmental success must be consistently excellent.

WRc has, with the aim of establishing a readily-available ('off the shelf') and standardised test, been collaborating with a commercial biotechnology company (Cell Systems, Cambridge) in evaluating methods developed by that company for oyster embryo cryopreservation. This collaboration has largely taken the form of assessment by WRc staff of the quality of gametes from cultured stock preserved at different times and by methods which are undergoing progressive development.

Initial samples proved disappointing, and led to a period of intensive technical development by Cell Systems. This led, in 1989, to the point where 90% successful fertilisation of frozen eggs by frozen sperm was reported. Following this, arrangements were made to deliver preserved samples to WRc, so that quality (proportion fertilised and developing normally) of embryos could be formally assessed in comparison with those produced from fresh gametes. The results to date of our appraisal of preserved gametes suggest that considerable work remains to be done in respect both of ensuring that the original material is of good quality and of exercising adequate control over the freezing process. The fertilisation success and development of the most recent material examined at WRc was very poor, although this was attributed to stress experienced by the parent animals. A control test, carried out immediately afterwards on conditioned oysters maintained at the Medmenham laboratory, suggested that the freezing method was probably not yet consistent in operation. In this test, the fertilisation success and development of fresh eggs and sperm were compared with those of frozen subsamples from the same pairing. Unfrozen eggs were equally well fertilised by fresh or previously-frozen sperm, and developed equally well to the D-stage. Frozen and thawed eggs, in contrast, were not fertilised by either category of sperm.

Future development therefore needs to concentrate on improvements in the process for freezing eggs, which clearly present greater difficulties than sperm by virtue of their greater size and water content.

It must be emphasised that work in this area has been carried out as and when opportunity has occurred, and has been neither intensive nor exhaustive. Successful cryopreservation and recovery of eggs has been reported (albeit on a limited number of occasions) and this level of success would need to be achieved for only a few pairings per year to produce sufficient material for several thousand tests. It is therefore important to determine the proportion of preservation attempts which will, for a given method, yield gametes of acceptable quality for test purposes. Preservation methods must not be 'hit or miss', but a certain level of failure can be tolerated since quality assurance for the test would be based on the documented performance of thawed, randomly-selected subsamples from each batch.

Future development in this area is dependent at present on the capacity of the collaborating company to generate material for evaluation. The potential benefits are considerable, and warrant continued interest.

SECTION C2 - ZOOPLANKTON TOXICITY TEST DEVELOPMENT

The cladoceran crustacean Daphnia has long been established as a standard freshwater toxicity test organism, and international guidelines for culture and test conduct are available. This allows the generation for freshwater systems of a large and uniform toxicity database. Toxicity testing for the marine and estuarine environments has, in contrast, no such established standard(s). As part of a continuing programme with the aim of meeting this need, effort has been devoted to establishing culture methods for marine and estuarine copepods. Copepods are, numerically, the largest group of marine metazoans, and form an important link in the food web in most pelagic systems. Culture and test methods have been published by a variety of organisations in several countries. In order to acquire experience in the maintenance and handling of copepods, culture techniques originally developed by the US EPA have been adopted in modified form at WRc.

Populations of Eurytemora affinis (Poppe) (an estuarine, euryhaline species) and Acartia tonsa (a marine species) have been successfully maintained in 10 l flow-through culture vessels for more than one year. The cultures are maintained in artificial seawater (Tropic Marin), enriched with marine algae produced by the batch method (Tetraselmis, Isochrysis, Phaeodactylum and Thalassiosira). These cultures have proven easy and cheap to maintain.

Existing protocols for planktonic copepods require tests to be conducted with uniformly-aged adults (approximately 20 days old), and as yet no practical evaluation has been attempted for any of these. Some, limited, work has been carried out to test the practicability of conducting tests on early developmental stages, since such tests would significantly reduce costs and may prove more sensitive than those using adults. The indications from this work are that a compact, rapid test may be practicable, although no conclusions can yet be drawn in respect of sensitivity.

Work has been conducted by a number of organisations on the development of test methods using small zooplankton (calanoid copepods), but these organisms are often fragile and difficult to handle. It is proposed to address this problem by investigating the possibility of developing tests using mysid species indigenous to the UK. Mysids are widely-distributed in UK coastal waters, and are known to be sensitive to many aquatic pollutants. However, the length of the generation time (about 6 months) makes the provision of test specimens of uniform age and sensitivity difficult and it will be necessary to investigate experimentally the practicability of laboratory culture.

The requirement for a readily-available, standardised and reproducible test could be met by the adoption of an existing test using the American species of mysid Mysidopsis bahia. This species has been widely-used, and an extensive toxicity database is available. Standard protocols have been published for both culture and test methods and the latter include acute (lethal) and chronic (lethal and developmental) responses.

Mysidopsis has a short generation time which makes provision of test organisms of standard age a practicable objective.

A test of this type would be appropriate for regular screening and compliance monitoring of surface waters and effluents, and could be easily applied by the water industry. The cultures of Mysidopsis bahia will be acquired in order to evaluate and characterise culture and test methods and sensitivity, with the aim of providing a rapid and widely-available screening tool.

The use of a non-indigenous species can best be justified if its responses are comprehensively intercalibrated with those of sensitive indigenous animals. Therefore, the sensitivity of Mysidopsis will be related to that of appropriate indigenous mysids in laboratory toxicity studies.

SECTION C3 - BIVALVE SHELL ACTIVITY MONITOR

Freshwater and marine environments may be subjected to continuous, periodic, or irregular discharges of contaminants. Whilst the former two categories are amenable to planned sampling and monitoring strategies, irregular discharges are not. Furthermore, irregular discharges include events such as chemical spills where attribution of the source may be legally important. In situations where concentrations of aquatic contaminants may increase markedly and unpredictably, regulators need some type of in-situ 'early-warning' system. Continuous chemical monitoring may be practicable where the hazard is restricted to a few specific contaminants, however biological methods are more appropriate where there is no such restriction, since aquatic biota will respond to a wide variety and combination of substances.

Over the past 10-15 years, a number of studies (mainly in the Netherlands) have investigated the possibility of using shell movement in bivalve molluscs as an indicator of stress response to aquatic pollution. Existing data suggest that this response is sufficiently

sensitive to be able to register episodic pollution events. Recent developments in microelectronics have facilitated the construction of systems which can continuously monitor the responses of up to eight bivalves during exposure to waters potentially subject to pollutant impact. Using an appropriate index of activity (such as percent of bivalves closed), an alarm response can be defined which can trigger, for instance, an automatic sampling device. In this way, unpredicted events can be registered and subsequent analysis of samples employed to attribute cause.

Bivalves are convenient and appropriate organisms to use in event monitoring, as they are easily deployed in receiving waters and have simple maintenance requirements. As valve movement is a clearly-defined and readily-measured behavioural response it is proposed to examine the feasibility of applying systems of the general type described above in monitoring episodic pollution events in the UK.

Initially, attention will be focused on identifying a set of practicable activity-monitoring techniques. If appropriate, an example of the existing system will be obtained, in order to evaluate its responsiveness, performance characteristics and practicality. The study aim will be to report on the range, costs, and reliability of existing and potential systems.

The systems developed in the Netherlands employ the bivalves Dreissena polymorpha (fresh waters) and Mytilus edulis (marine waters). The former would not be an appropriate choice for UK fresh waters due to its status as an immigrant and pest species in many regions and alternative species, such as Sphaerium sp., would need to be considered. WRc already possesses considerable experience in toxicity and monitoring studies with Mytilus edulis.

GENERAL CONCLUSIONS

In the validation studies of the Gammarus freshwater bioassay, feeding rate has been identified as the key component of the scope for growth technique and respiration rate measurements and the calculation of scope for growth were not found to be cost-effective test components. Feeding rate provided an indication of differences in water quality at sites above and below the discharges studied. However, pollution-induced depressions of feeding rate during field exposure did not persist in animals returned to the laboratory. On a general basis this would restrict the utility of the assay for assessing episodic pollution.

As variations in feeding rate responses were measured between populations at a given study site and in animals of the same population at different laboratories, additional characterisation of the bioassay is needed. Although laboratory-based differences were found, the pattern of feeding rate responses were similar and, in most cases, the two laboratories would have drawn similar conclusions.

The enzyme glutathione-s-transferase (GST) has been identified as a potentially useful biochemical index of physical or pollution stress, and following studies on the assay procedure, an accurate and reproducible test capable of measuring GST activity in individual tissue samples has been developed. Induction of GST activity has been measured in response to both organochlorine compounds and the heavy metal zinc. In addition the nature of GST induction is such that this technique appears to have the potential to indicate prior exposure to episodic events. A number of populations now need to be investigated in order to characterise the range of response patterns which might be encountered during routine application.

In future studies both the Gammarus feeding rate and Sphaerium GST activity tests will be refined, until each is a rapid, sensitive and cost effective bioassay tool that can be routinely applied to assess pollution events in streams and rivers.

The long-term maintenance of the copepods Eurytemora affinis (an estuarine species) and Acartia tonsa (a marine species) has been achieved, showing these organisms are readily cultured. In addition initial studies on the practicability of using early developmental stages in the toxicity tests has been conducted. There are encouraging prospects that further work will lead to the development of a compact and rapid copepod-based test for assessing estuarine contamination under environmentally realistic conditions.

In the oyster gamete study, effective cryopreservation and recovery of sperm has been demonstrated with successful fertilisation and development of fresh eggs by frozen and unfrozen sperm. However frozen eggs could not be used extensively in the oyster embryo test. There has been limited success in the cryopreservation and recovery of eggs and improvements in the freezing process are needed to achieve greater consistency in the provision of acceptable quality gametes.

The bioassays being studied are at different stages of development and the nature of future work planned for each study area have been described in detail in the relevant section. All the techniques described have potential for assessing general and site-specific water quality problems where traditional chemical monitoring methods would be inadequate.

The inherent limitations of new bioassays or established techniques have to be identified, addressed and understood prior to their routine application for monitoring aquatic pollution. The objective assessment of the bioassay would be improved by having an established protocol of development and standardisation guidelines against which present test method could be gauged. In such a protocol important criteria to consider include the replicability, inherent variability and sensitivity of the test, the interpretation of results derived and extrapolation between laboratory and field situations. It is recommended that the development of such a protocol should be considered an important component of future work.