The Development of the *Gammarus pulex* Feeding Rate Bioassay

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R&D Note 170

THE DEVELOPMENT OF THE *GAMMARUS PULEX* FEEDING RATE BIOASSAY

M Crane, L Maltby and I Johnson

Research Contractor:
WRc plc
Henley Road Medmenham
PO Box 16 Marlow
Buckinghamshire SL7 2HD

National Rivers Authority
Rivers House Waterside Drive
Almondsbury Bristol BS12 4UD

R&D Note 170

National Rivers Authority
Rivers House
Waterside Drive
Almondsbury
BRISTOL
BS12 4UD

Tel: 0454 624400
Fax: 0454 624409

 This report is the result of work jointly funded by the National Rivers Authority and the Scotland and Northern Ireland Forum for Environmental Research.

Dissemination Status
Internal: Released to Regions
External: Restricted

Statement of Use

This Note contains the salient features of the development of an ecotoxicological procedure for assessment of the toxicity of an effluent or sample of water. Draft Operating Procedures are also provided for evaluation by NRA Biologists. Comments on these Procedures and their utility would be appreciated by the Project Leader.

Research Contractor
This document was produced under R&D Contract 061 by:

WRc plc
Henley Road Medmenham
PO Box 16 Marlow
Buckinghamshire
SL7 2HD

Tel: 0491 571531
Fax: 0491 579094

WRc Reference: NR 3456/4233

NRA Project Leader
The NRA's Project Leader for R&D Contract 061:
R Sweeting, Thames Region

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EXECUTIVE SUMMARY

The use of ecotoxicological methods to complement traditional chemical and biological (macroinvertebrate) surveys can aid in making regulatory decisions regarding water quality. This relates particularly to:

1. examining discrepancies between chemically and biologically derived classes for river stretches;

2. providing evidence of cause and effect in pollution investigations and tracing the source of pollution events;

3. rapidly assessing the effects of effluent discharges on receiving waters.

This report describes the results of a study carried out to develop an *in situ* energetic bioassay using the freshwater amphipod *Gammarus pulex* (L) as part of National R&D Project 061 'Biological Methods for Assessing the Effects of Pollution' (see also R&D Notes 171, 172 and 173). The work described was carried out at WRc and under a subcontract at the University of Sheffield where the assay was initially developed.

The mortality, feeding and respiration rates, and the Scope for Growth of *G. pulex* have been used to assess the effects of pollutants in laboratory and field experiments. Feeding rate has emerged as the most sensitive and cost-effective sublethal measurement. It is also ecologically relevant because it is positively related to growth rate and reproductive fitness.

Interpopulation differences in feeding rate do not appear to be important if test animals are obtained from uncontaminated streams, although there is evidence that populations can differ significantly in response to acute lethal concentrations of pollutants. Differences occurred between operators and laboratories in the measurement of feeding rate, but these would not usually have affected the conclusions. Standard Operating Procedures for the laboratory and *in situ* (field deployed) versions of the technique are included in appendices.

KEYWORDS

Gammarus pulex, freshwater amphipod, feeding rate, respiration rate, Scope for Growth (SfG), *in situ* bioassay

1. INTRODUCTION

The assessment of riverine water quality has traditionally been achieved by the use of chemical and biological surveys. Ecotoxicological methods can provide regulators with an additional source of information with which to make decisions relating to:

1. the development of water and sediment quality standards;

2. the assessment of water and sediment quality;

3. the consenting and monitoring of effluent discharges;

4. the investigation of pollution incidents.

These methods can include:

1. toxicity tests, which measure the effects caused by exposure to a measured level of a substance or mixture of substances;

2. bioassays, which evaluate the relative effects of a substance or mixture of substances by comparing these effects with those of a standard preparation.

Most toxicity tests and bioassays are laboratory based and use field collected samples. However, field deployed or *in situ* bioassays have been used to assess both water quality (Widdows 1985) and sediment quality (Sasson-Brikson and Burton 1991) with both single species (Maltby and Calow 1989) and multispecies systems (Livingston 1988, Whaley *et al.* 1989). The advantage of *in situ* bioassays is that the uncertainty involved in extrapolating from laboratory results to the field is avoided because test organisms are directly exposed to ambient concentrations of contaminants in natural systems.

This R&D Note describes the important results and conclusions from a study to develop an *in situ* energetic bioassay with the freshwater amphipod *Gammarus pulex* (L.) as part of National R&D Project 061 'Biological Methods for Assessing the Effects of Pollution' (see also R&D Notes 171, 172 and 173). The work described was carried out at WRc and under a subcontract at the University of Sheffield where the assay was initially developed (Naylor *et al.* 1989, Maltby *et al.* 1990a,b). *G. pulex* was chosen for the assay since it is a widespread and abundant benthic amphipod found in many European streams.

The starting point for this study was the *G. pulex* Scope for Growth (SfG) bioassay (Naylor *et al.* 1989, Maltby *et al.* 1990a,b), which involves the measurement of feeding rate in caged adult animals held *in situ* for a period of six days. After this period they are returned to the laboratory for the measurement of respiration rate. Food consumption is measured by weight losses in leaf discs preconditioned with fungi, with a correction factor applied for weight losses due to leaching, or weight gains due to sedimentation. Weight losses in leaf discs are expressed in energy terms through conversion to Joule equivalents derived from bomb calorimetry. Energy absorbed is the difference between that consumed and that lost in faeces. Respiration rate is measured using conventional oxygen consumption techniques. The Scope for Growth of an individual is determined by subtracting the energy used in respiration from that absorbed.

Scope for Growth can either be:

1. positive, indicating that energy is available for growth and reproduction;

2. zero, when the energy adsorbed balances energy expenditure;

3. negative, when animals have to use their body reserves for essential metabolism.

Studies reported in this R&D Note describe the development of the bioassay in a series of laboratory and field experiments which considered the sensitivity, discrimination, variability, cost effectiveness and ecological relevance of the assay. The results, which are described in greater detail in a series of previous reports (Johnson *et al.* 1990a,b, 1991, 1993), were used to develop the laboratory and field procedures described in Standard Operating Procedures in appendices of this report.

2. INITIAL DEVELOPMENT OF THE BIOASSAY

2.1 Initial laboratory studies

2.1.1 Introduction

The feeding rate and Scope for Growth (SfG) bioassay could be applied to juvenile, adult male or adult female *Gammarus pulex*. Initial studies at the University of Sheffield assessed the effects of the common riverine pollutant ammonia on feeding rate and Scope for Growth in all test groups.

2.1.2 Experimental procedures

The studies were carried out using the procedures described in Appendix A. All animals were obtained from Crags Stream, Derbyshire (NGR SK 497745) and experiments were conducted at 15 °C using artificial pond water (APW) as the experimental medium and alder leaves (*Alnus glutinosa* L.) inoculated with *Cladosporium* as food.

The following un-ionized ammonia concentration ranges were prepared from a stock solution for each test group:

Test group	Un-ionized ammonia concentration range (mg NH_3 l^{-1})
Juveniles	0.002, 0.07, 0.109, 0.167, 0.185
Adult males	0.003, 0.093, 0.155, 0.212, 0.289
Brooding females	0.002, 0.093, 0.134, 0.143, 0.188

The experiments were conducted to determine for each group the highest ammonia concentration causing no effect on feeding rate and SfG (HNEC), the lowest ammonia concentration causing an observed effect on feeding rate and SfG (LOEC) and the ammonia concentration causing a 50% reduction in feeding rate and SfG (EC_{50}).

2.1.3 Conclusions

In the study the EC_{50}, HNEC and LOEC values for each test group exposed to un-ionized ammonia were the same for feeding rate and SfG, and are shown below:

Test group	EC_{50} (mg l^{-1})	HNEC (mg l^{-1})	LOEC (mg l^{-1})
Juveniles	0.169	0.109	0.167
Adult males	0.186	0.093	0.155
Brooding females	0.155	0.143	0.188

For all groups, there was a strong concentration-response relationship between feeding rate or SfG and un-ionized ammonia concentrations. The results showed that there were no marked differences in the sensitivity of different test groups to ammonia.

2.2 Initial field deployments

2.2.1 Introduction

In the initial stages of the assessment and development of the *G. pulex* feeding rate and scope for growth techniques, a series of field trials were carried out using draft protocols for laboratory and field studies. The objectives of the field trials were to:

1. assess the sensitivity and discrimination of the feeding rate and scope for growth techniques in response to a range of stressors;

2. determine the effect on sensitivity and discrimination of using different populations of *G. pulex*;

3. assess the extent of interlaboratory variability by measuring scope for growth in animals deployed by two laboratories (WRc and the University of Sheffield);

4. assess whether field-induced effects persist in the laboratory.

The results of these studies were used to assess the usefulness of the methods and identify areas requiring further attention.

2.2.2 Deployment procedure

The four 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 an uncontaminated 'reference' site in the same catchment, for comparison with the impacted sites. Table 2.1 outlines the characteristics of the four sites, while Table 2.2 summarizes the measurements taken during each deployment.

Table 2.1 Field sites used for testing the *G. pulex* Scope for Growth method

Trial No	Field site	National Grid reference	Composition of effluent discharged	Date of *G. pulex* SfG field trial (1989)
1	Colne Water, East Lancashire	SD86703930	Domestic sewage with metallic industrial waste	9 - 15 May
2	River Lea, Essex	TL12101800	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

In the field trials, *G. pulex* were obtained from Crags Stream, Derbyshire (NGR 49707450) and from Haseley Brook, Oxfordshire (NGR SU62409930). The pre-deployment holding conditions of animals used in the field trials are shown below:

Holding medium: Aerated ground water (pH=7.7-8.0, hardness = 270-290 mg $CaCO_3$ l^{-1})

Photoperiod: 12 h light : 12 h dark with 30 minutes artificial dawn

Feeding regime: Alder leaves inoculated with *Cladosporium* fungus supplied *ad libitum*

In each of the four trials, one hundred and sixty-eight adult male *G. pulex* were placed one each in individual field cages (see Figure B1) with 4 × 17 mm diameter discs cut from alder leaves and inoculated with the fungus *Cladosporium* to improve palatability.

Full protocols for the laboratory and field assessment of SfG, feeding rate and respiration rate are provided in Appendices A and B. The only two differences between the initial methods used in these deployments and the final methods described in Appendix B were:

1. the holding baskets that contained the field cages were orientated vertically and perpendicular to the flow of the streams, so that water flowed through the bore of the cages;

2. both sides of each holding basket were covered in 1 cm^2 mesh.

Table 2.2 Summary of the measurements made during each field deployment

Deployment	Populations	Feeding rate	Respiration rate	SfG	Body burdens		Persistence of effect
					Gammarus	Leaf discs	
Colne Water - East Lancashire	Crags Stream	Y	Y	Y	Y[1]	Y[1]	Y
	Haseley Brook	Y	Y	Y	Y[1]	Y[1]	Y
River Lea - Essex	Crags Stream	Y	Y	Y	N	N	Y
	Haseley Brook	Y	Y	Y	N	N	Y
West Okement - North Devon	Crags Stream	Y	Y	Y	Y[2]	Y[2]	Y
	Haseley Brook	Y	Y	Y	Y[2]	Y[2]	Y
River Erme - South Devon	Crags Stream	Y	Y	Y	N	N	Y
	Haseley Brook	Y	Y	Y	N	N	Y

[1] - Cadmium, chromium, copper, lead, nickel and zinc
[2] - Aluminium, chromium, iron, manganese and nickel

In all the trials a random number table was used to assign 56 field cages containing animals from Crags Stream (Population 1) and 56 cages containing Haseley Brook (Population 2) animals 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 is necessary because dried leaves are known to lose weight when immersed in water, by leaching of soluble substances (Barlocher 1991, 1992). Sedimentation of solids onto leaf material may also be a source of error in some stream systems. 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 2.1).

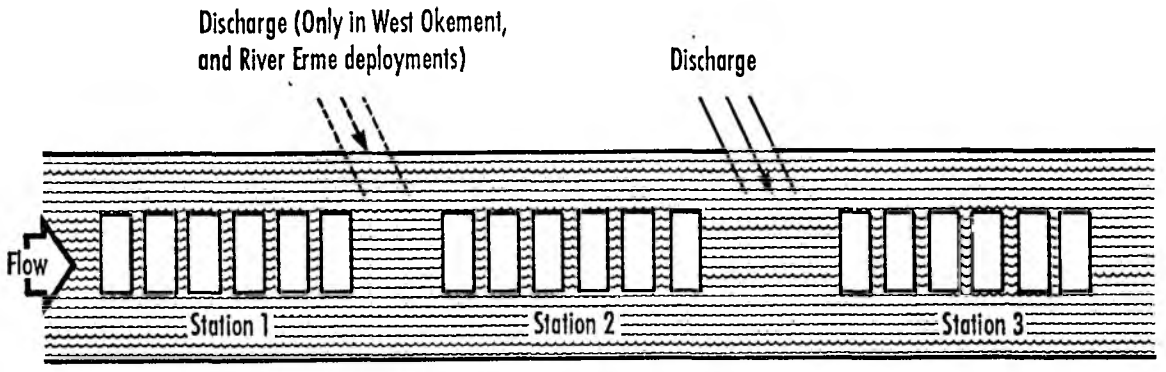

Figure 2.1 Holding basket positions during the initial field deployments

In the Colne Water and River Lea deployments, Station 1 was an uncontaminated reference site in the same catchment as Stations 2 and 3, which were located upstream and downstream respectively from the discharge point. In the West Okement and River Erme deployments, Station 1 was an uncontaminated 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 2.2 illustrates the relative positions of stations and discharges in each of the four field trials.

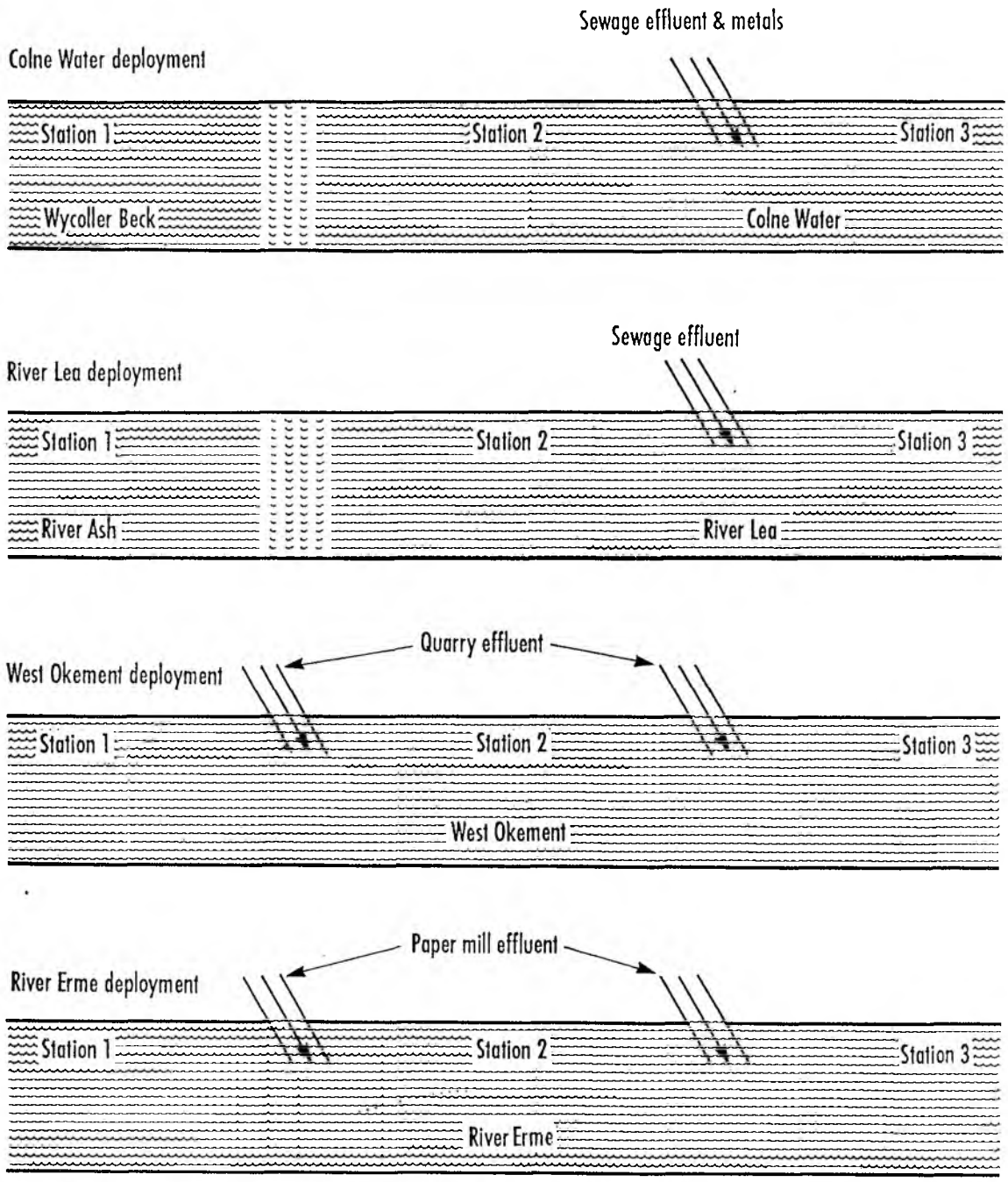

Figure 2.2 Relative postitons of stations and discharges during the initial field deployments

When the field cages had been distributed the holding baskets were secured to the substratum so that water could pass through the bore of the field cages. The following physical, chemical and biological parameters were taken at all stations:

Physical measures:	Channel width and depth, flow rate, substratum size, temperature, pH and conductivity;
Chemical measures: (One set per station)	Metals (Al, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Ni, Pb, Zn); Total organic carbon (TOC) as an indicator of organic contaminants; Alkalinity, total ammonia, chloride, nitrate, nitrite, soluble reactive phosphorus, sulphate, and suspended solids.
Biological measures:	Three-minute kick-samples with a standard 0.5 mm net at each station. Invertebrates were sorted, counted and identified to family or species level. A Biological Monitoring Working Party (BMWP) biotic score was then calculated (Armitage *et al.* 1983, Extence and Ferguson 1989) to provide an indication of macroinvertebrate species richness.

Phenol and organochlorine concentrations were also determined for samples taken from the River Erme.

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

2.2.3 Measurement of feeding rate, respiration rate and scope for growth

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 sacrifice and determination of dry weight.

The sub-sample of *G. pulex* selected for respirometry by each laboratory comprised eight animals from each population deployed at each station. A flow-through system was used with a Strathkelvin 78 1b oxygen meter and Radiometer Copenhagen E5046-0 electrode in a 15 °C constant temperature room.

Animals were acclimatised to the system for at least three 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. Measurements of respiration were made in water taken from the field station at which animals 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.

2.2.4 Persistence of effect

Laboratory 2 (WRc) alone undertook an investigation into the persistence of field effects. 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 in Appendix A. The chambers containing the *G. pulex* and ten 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.

2.2.5 Statistical analysis

The statistical procedures used to analyse the feeding rate, respiration rate and SfG data from the field deployments have been described in detail in Johnson *et al.* (1990a) and consisted of analysis of variance with either Tukey or Scheffé tests. The main effects analysed were Station (Station 1, Station 2 and Station 3), Population (Population 1 and Population 2) and Laboratory (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. After completing an ANOVA, the power of the test was calculated. Mortality was analysed using a Generalised Linear Model (GLM) with a logit link function and binomial distribution. Analyses were carried out using either the GENSTAT or MINITAB statistical packages. Manual calculations were performed according to Sokal and Rohlf (1981) and Zar (1984). In the analysis of the results from the persistence of effects experiments the factor 'laboratory' was omitted because the experiment was only performed by WRc (Laboratory 2).

2.2.6 Conclusions

Figures 2.3 to 2.6 show the results for feeding rate, respiration and scope for growth measured in each population at both laboratories during the field deployments. Table 2.3 shows the elevated levels of substances downstream of discharges in water and sediment samples, the mortality of *G. pulex* during the field deployments and the British Monitoring Working Party (BMWP) scores for all stations. Table 2.4 summarizes the results of the statistical analysis on the feeding rate, respiration and SfG data based on two stations (that is excluding the upstream reference) or three stations (including the upstream reference).

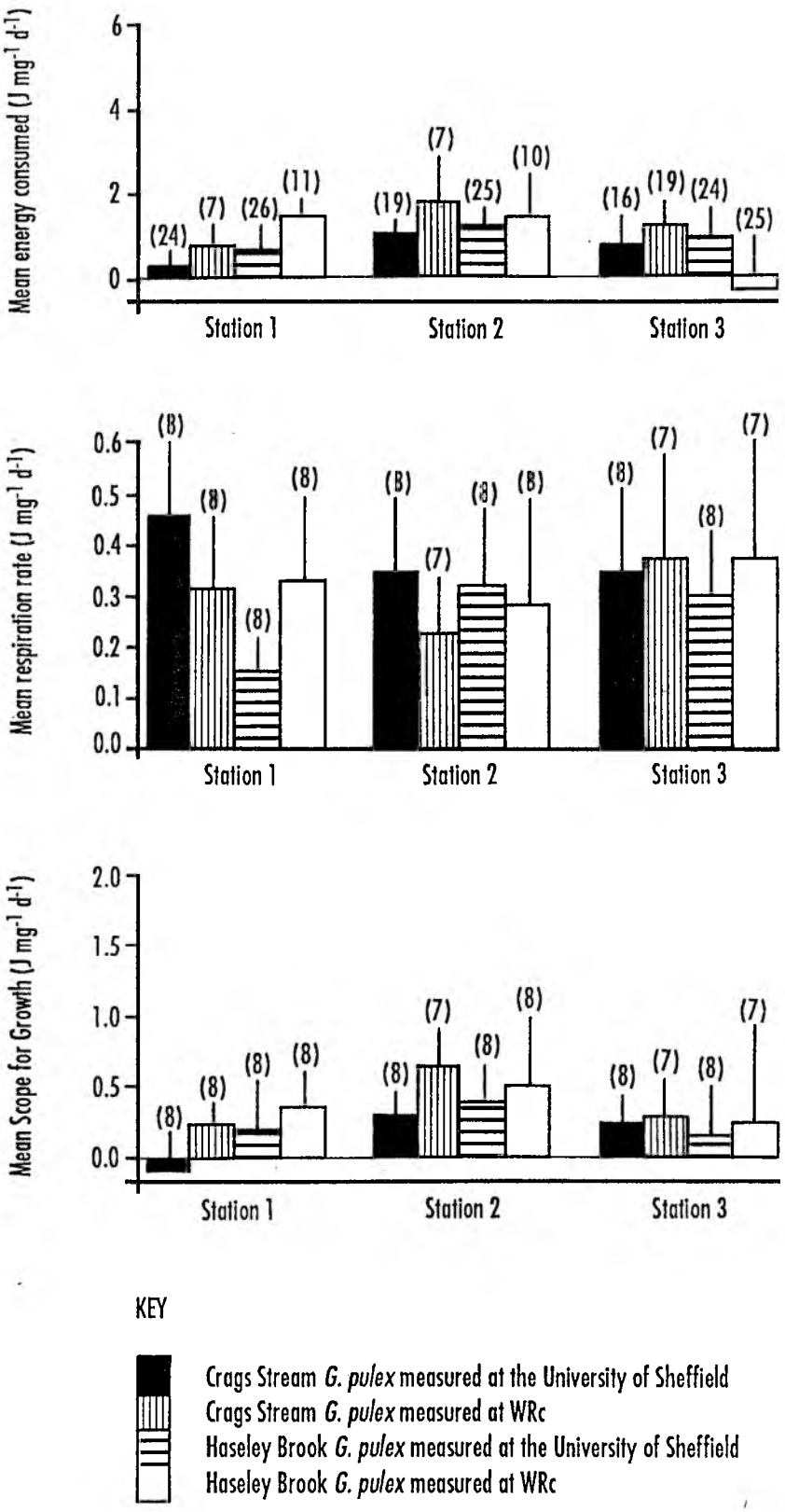

Figure 2.3 Mean energy consumed and respired and resulting Scope for Growth of *G. pulex* deployed in Colne Water. Numbers in parentheses refer to the number of individuals

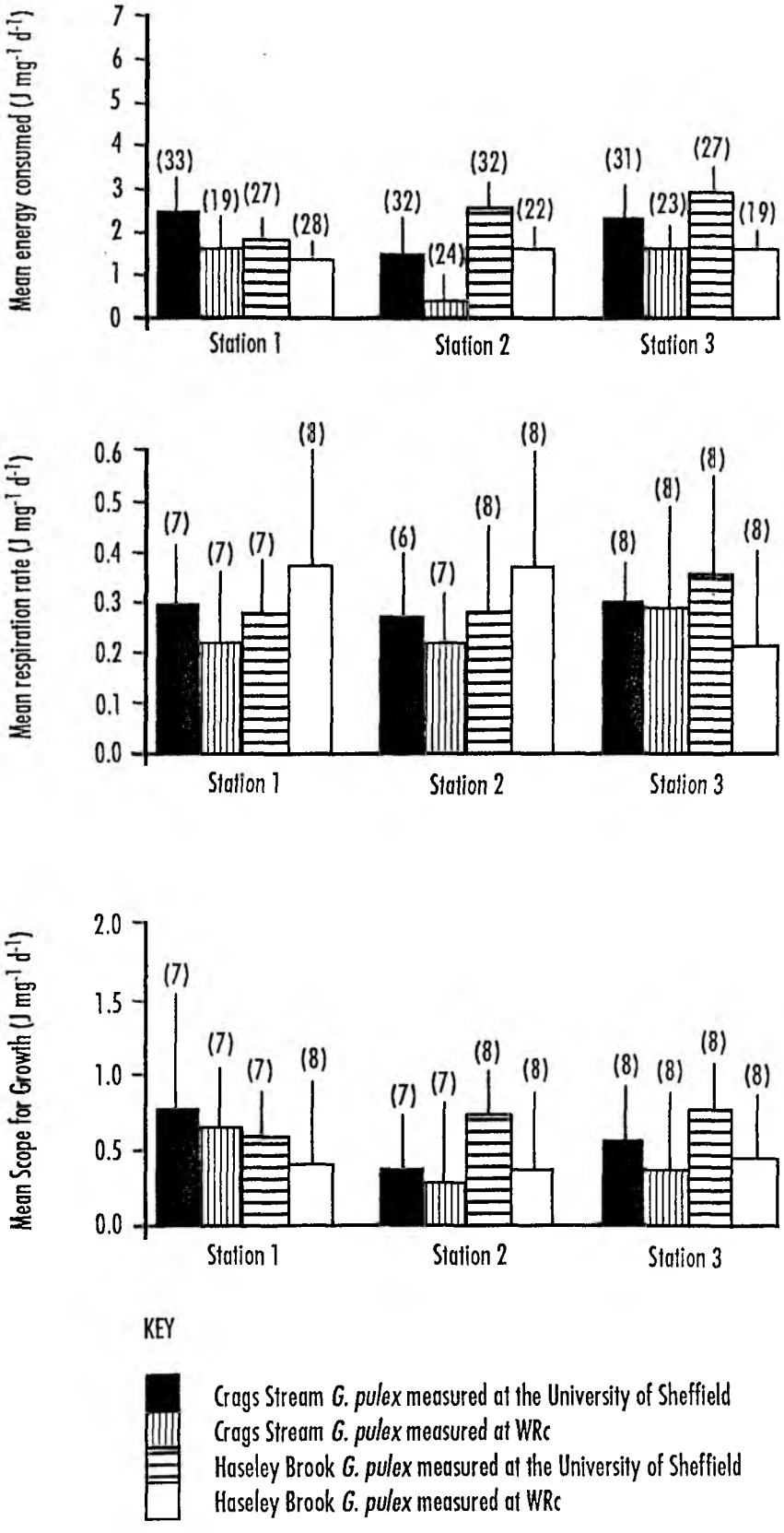

Figure 2.4 Mean energy consumed and respired and resulting Scope for Growth of *G. pulex* deployed in the River Lea. Numbers in parentheses refer to the number of individuals

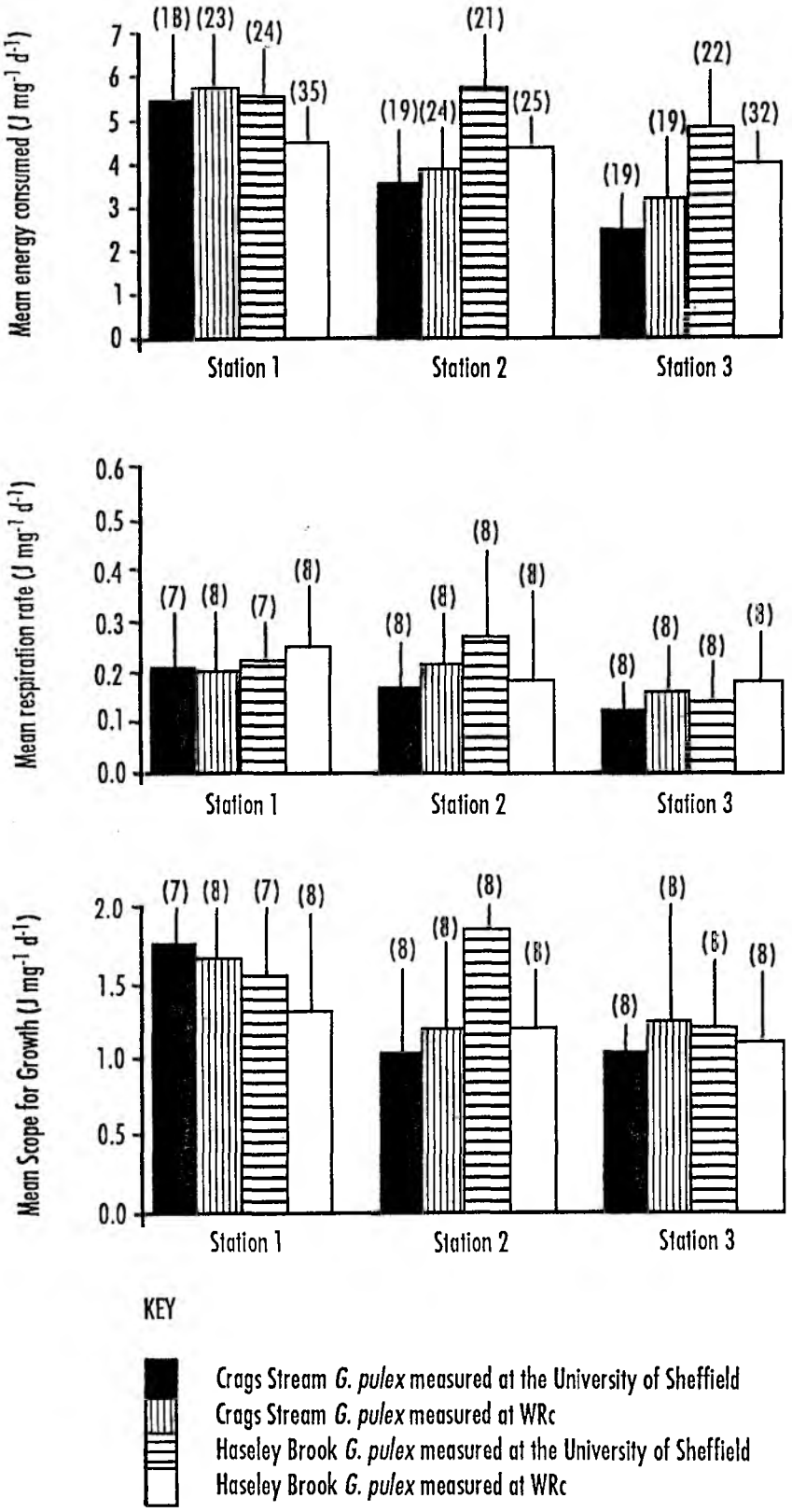

Figure 2.5 Mean energy consumed and respired and resulting Scope for Growth of *G. pulex* deployed in the West Okement. Numbers in parentheses refer to the number of individuals

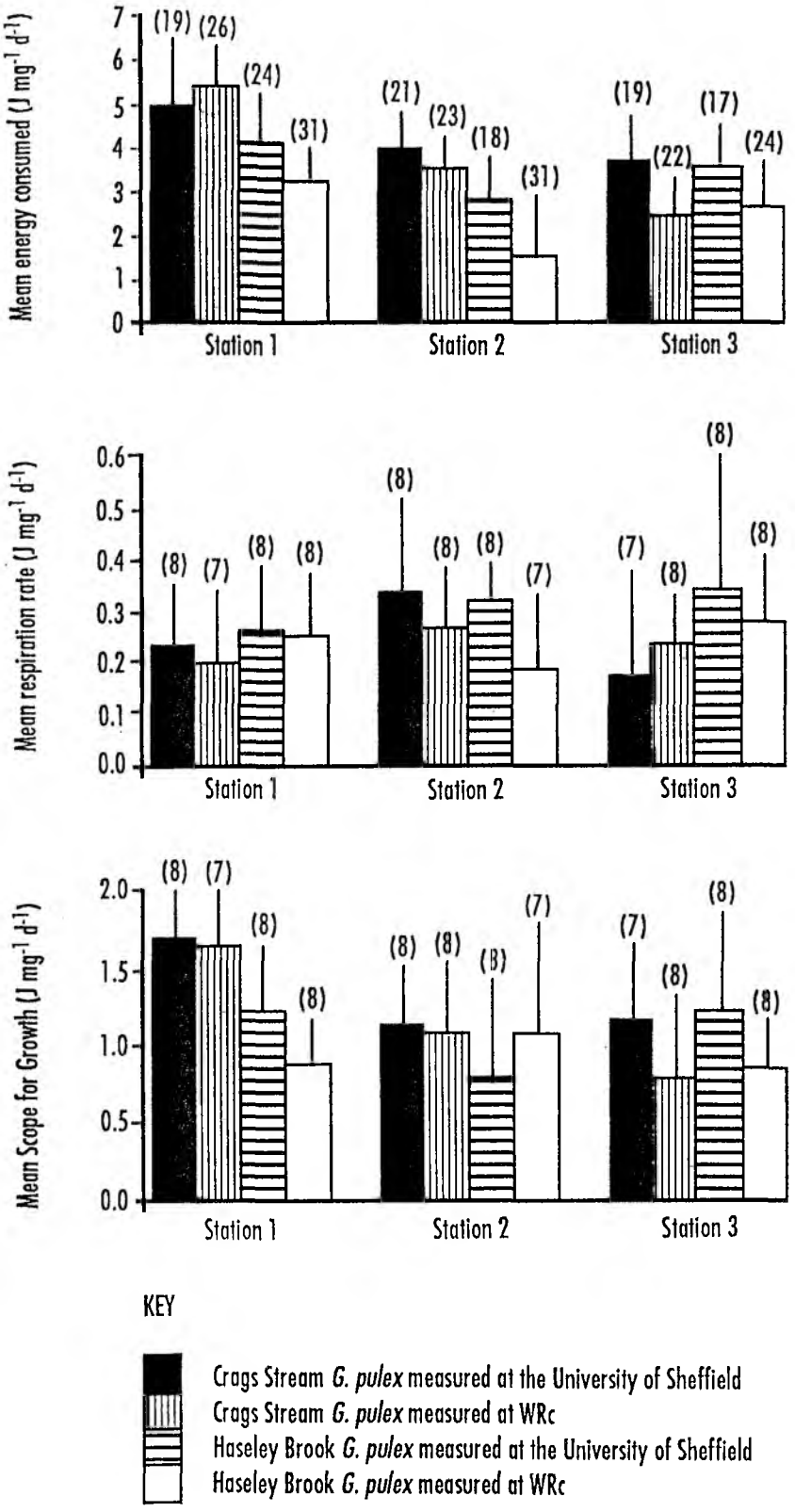

Figure 2.6 Mean energy consumed and respired and resulting Scope for Growth of *G. pulex* deployed in the River Erme. Numbers in parentheses refer to the number of individuals

Table 2.3 Elevated levels of substances downstream of discharges in water and tissue samples, the mortality of each *G. pulex* population during field deployments and British Monitoring Working Party (BMWP) scores for all stations

Deployment	Elevated levels of substances downstream of discharges		Mortality at each station for each population						BMWP score at each station		
			Crags Stream			Haseley Brook					
	Water samples	Tissue samples	1	2	3	1	2	3	1	2	3
Colne Water	NH_3, Al, Pb Zn, TOC	Pb	0	8	10	2	0	2	94	22	17
River Lea	Zn, TOC	NT	4	0	4	2	0	4	145	60	50
West Okement	Mn, Ni, Zn	Fe, Mn	10	12	25	0	2	0	48	32	27
River Erme	Mn	NT	8	11	23	2	2	11	67	41	40

TOC = Total organic carbon
NT = Not tested

Table 2.4 Summary of the statistical analyses of feeding rate, respiration rate and SfG data from field deployments

Deployment	Feeding rate		Respiration rate		Scope for Growth	
	2 Stations	3 Stations	2 Stations	3 Stations	2 Stations	3 Stations
Colne Water	S, P × L	S, S × P, S × L	NSE	NSE	S	S, L
River Lea	S, P, L, S × P	L, S × P	NSE	NSE	NSE	L
West Okement	N/A	S, P, S × P, P × L	N/A	S	N/A	S
River Erme	N/A	S, P, L, S × P	N/A	NSE	N/A	S, P

S = Station, L = Laboratory, P = Population, NSE = No significant effects, N/A = Not applicable

Colne Water deployment

Chemical and biological sampling during the Colne Water deployment suggested a marked decline in water quality between Stations 1 (the upstream reference) and 2 (above the discharge), and a further slight decline between Stations 2 and 3 (below the discharge) (Table 2.3). Mortality during the field deployment was only high in *G. pulex* from the Crags Steam (Population 1) animals located at Stations 2 and 3 (Table 2.3).

The interpretation of the feeding rate and scope for growth results partly depends on whether Station 1, the reference site, is included in the analysis (Figure 2.3 and Table 2.4). 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 in all population/laboratory combinations differed significantly between stations, with upstream animals feeding more than those deployed downstream. A similar trend was apparent in the estimation of SfG. No difference between stations could be detected when respiration rate was analysed, although the latter was 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.

A possible cause for the downstream reduction in feeding rate and SfG in the Colne Water deployment was lead, since levels in water and leaf disc samples from Station 3 were higher than that found to cause 100% mortality of *G. pulex* over a 28 day period under laboratory conditions (Zencirci 1980). Several other metals were found at relatively high concentrations at Station 3, especially iron and aluminium, but these were not considered to have been a major factor in the reduction of feeding.

River Lea deployment

Biological sampling during the River Lea deployment suggested a marked decline in water quality between Stations 1 (upstream reference) and 2 (above the discharge) and a further slight decline between Stations 2 and 3 (below the discharge) (Table 2.3). Mortality during deployment was low in both populations at all stations (Table 2.3).

Animals from Crags Stream fed significantly less at Station 2 than at the other two stations (Figure 2.4). The University of Sheffield (Laboratory 1) consistently measured a higher consumption rate than WRc (Laboratory 2). No significant differences could be detected between stations, populations or laboratories for respiration rate, but there was a significant interlaboratory difference in the measurement of SfG (Table 2.4).

These results suggest, surprisingly, that the water quality upstream from the sewage treatment works was lower than that downstream during the period of deployment. It is not clear which stressors were responsible for reducing the feeding rate of *G. pulex* in the River Lea deployments. The high ammonia levels at the Lea upstream station make ammonia a possible candidate, although levels of un-ionized ammonia were lower than those causing a reduction in *G. pulex* feeding rate in laboratory experiments (Maltby *et al.* 1990a). The results also suggest that, as in the Colne Water deployment, the choice of a remote reference station can confuse the interpretation of results.

West Okement deployment

Biological and chemical sampling during the West Okement deployment suggested a decline in water quality between Station 1, the upstream site, and Stations 2 and 3, both downstream from metallic quarry discharges (Table 2.3). Mortality at all three stations was low in Haseley Brook gammarids, but accounted for 10%, 12% and 25% of Crags Stream gammarids at Stations 1, 2 and 3 respectively (Table 2.3).

Crags Stream animals fed and respired less at one or both of the downstream stations and had a lower SfG than Haseley Brook animals, which did not respond in a similar fashion (Figure 2.5 and Table 2.4). These results suggest that the quality of the water at Stations 2 and 3 was lower than at Station 1.

In the West Okement deployment, metals were again the most likely stressors although only the Crags Stream population was affected. Metal accumulation data suggested that five metals, iron, manganese, zinc, nickel and aluminium could be the cause of the observed reduction in *G. pulex* feeding rate. However, correlation analyses performed with body loadings and feeding rate indicated that the only significant relationships were for iron and manganese with Crags Stream animals. As the body concentration of these two metals increased, feeding rate decreased. No significant relationships were apparent for Haseley Brook animals. In the water column, 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 (Table 2.3).

River Erme deployment

Biological sampling during the River Erme deployment suggested a decline in water quality between Station 1, the upstream site, and Stations 2 and 3, both downstream from paper mill discharges (Table 2.3). Chemical sampling did not produce a clear indication of any water quality problems (Table 2.3). Analysis of a pulse discharge that occurred upstream from Station 3 during the deployment of *G. pulex* revealed high levels of some heavy metals and a low pH. The mortality in Haseley Brook animals was low (2%) at Stations 1 and 2, rising to 11% at Station 3. Crags Stream gammarids had mortality levels of 8%, 11% and 23% respectively at Stations 1, 2 and 3.

The feeding rate of both populations was depressed at Station 2, downstream from the first effluent discharge (Figure 2.6). Only Crags Stream animals 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. The SfG of Crags Stream animals 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.

Persistence of effect

In the development of an *in situ* bioassay it is important to determine whether field effects persist because it determines the uses and limitations of that procedure. If it were possible to measure a persistent effect with the feeding rate bioassay 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 *G. pulex* 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.

However, no persistence of field effects could be detected in any of the deployments (Figure 2.7), which is consistent with the work of McCahon *et al.* (1989, 1991). There are two possible explanations for this lack of persistence:

1. the stressors responsible for reducing feeding in the field may have induced a behavioural response in the *G. pulex* rather than caused a physiological change;

2. the depuration rate of accumulated toxicants may have been too rapid to be detectable using a six day feeding rate bioassay.

If the latter was true then feeding may have been depressed only briefly, at the beginning of the persistence experiment. There is evidence from other work to support this idea (McCahon *et al.* 1989).

General

In the field deployments, feeding rate was consistently the most sensitive component of SfG to stressors. The results also confirm that feeding rate is more sensitive than mortality as an indicator of water quality, and that a relationship may or may not be apparent between these end points. Mortality may, however, influence the sensitivity of a sublethal measurement such as feeding rate. In the West Okement and River Erme deployments more than 20% of the Crags Stream population died at Station 3. This may have influenced the distribution of results in one of two opposing ways:

1. 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;

2. if 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. It, therefore, seems sensible to use both lethal and sublethal measurements as a combined tool for the investigation of water quality and to exercise caution when interpreting sublethal results from tests in which there is a high level of mortality.

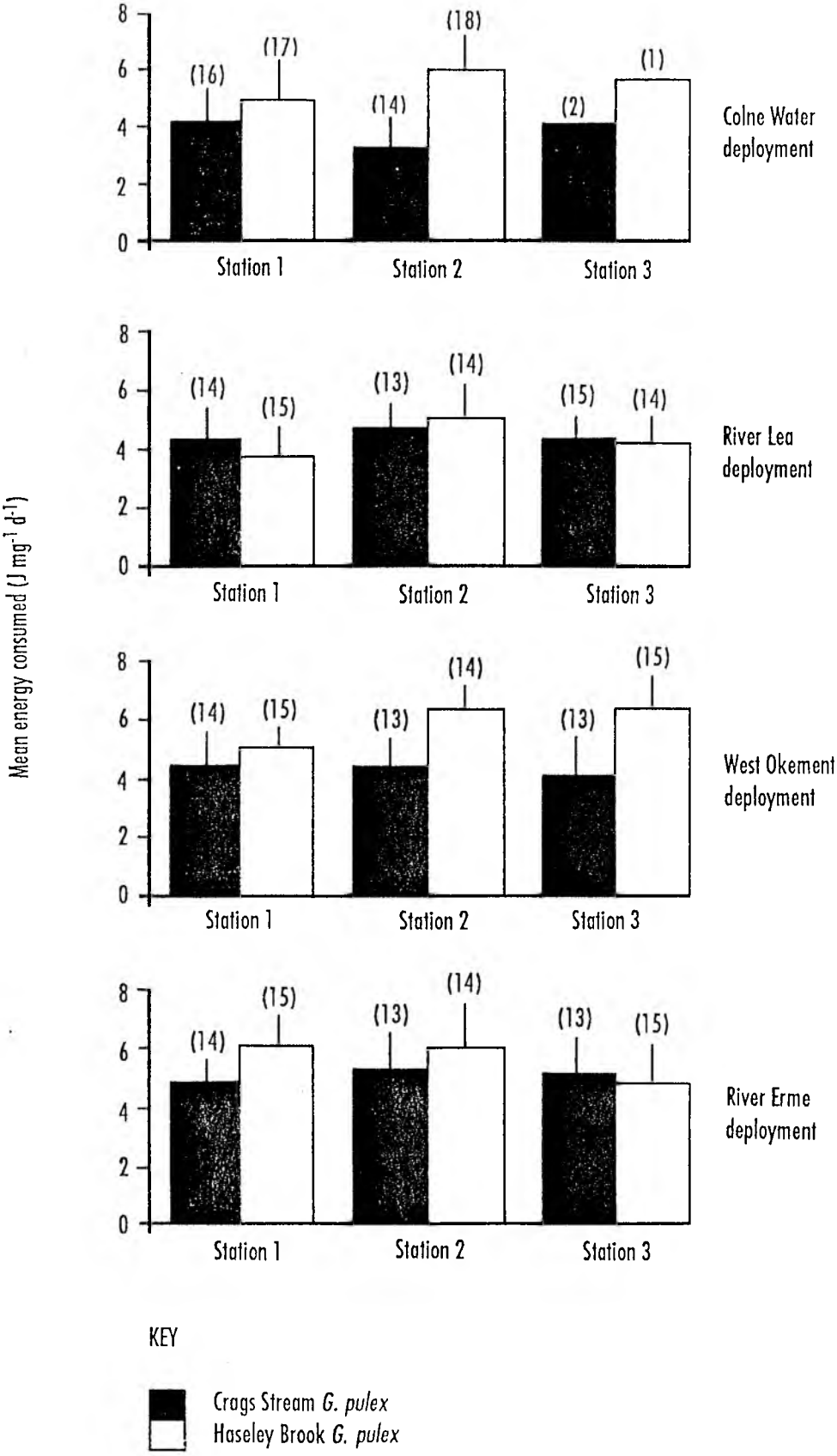

Figure 2.7 Mean energy consumed by *G. pulex* after six days in uncontaminated water following field deployments

A comparison of the feeding rate at reference stations between trials shows that this technique does not provide an absolute, replicable 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 there was no correlation between feeding rate and water temperature at the four reference stations. Similarly, there was no strong seasonal effect in animals from the clean stations used in the separate persistence of effect experiments. Such results suggest that the quality of the different stations was the most important factor influencing feeding rate, although more work would be 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 trial deployments, but compared reasonably well with similar, 'relevant' bioassays (for example Stay *et al.* 1989, Schimmel *et al.* 1989). 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 field deployments the lowest coefficients of variation achieved 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 75% (Sokal and Rohlf 1981) (Figure 2.8). 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 bioassay relies critically upon the level of variation within treatment groups and so it is important to look at the sources of variation and ways in which it could be minimized.

2.2.7 Summary

Several conclusions were drawn from the results of this study:

1. The measurement of components of Scope for Growth other than feeding rate was labour-intensive, technically difficult, subject to high variation and, therefore, insensitive. Feeding rate alone, which indicated differences in quality between stations above and below different types of effluent discharge, was considered to be a suitable end point for further investigation;

2. The feeding rate of *G. pulex* is a relative, rather than an absolute, measure of water quality. Environmental factors other than pollution are likely to vary between rivers and have some influence upon the response of test animals;

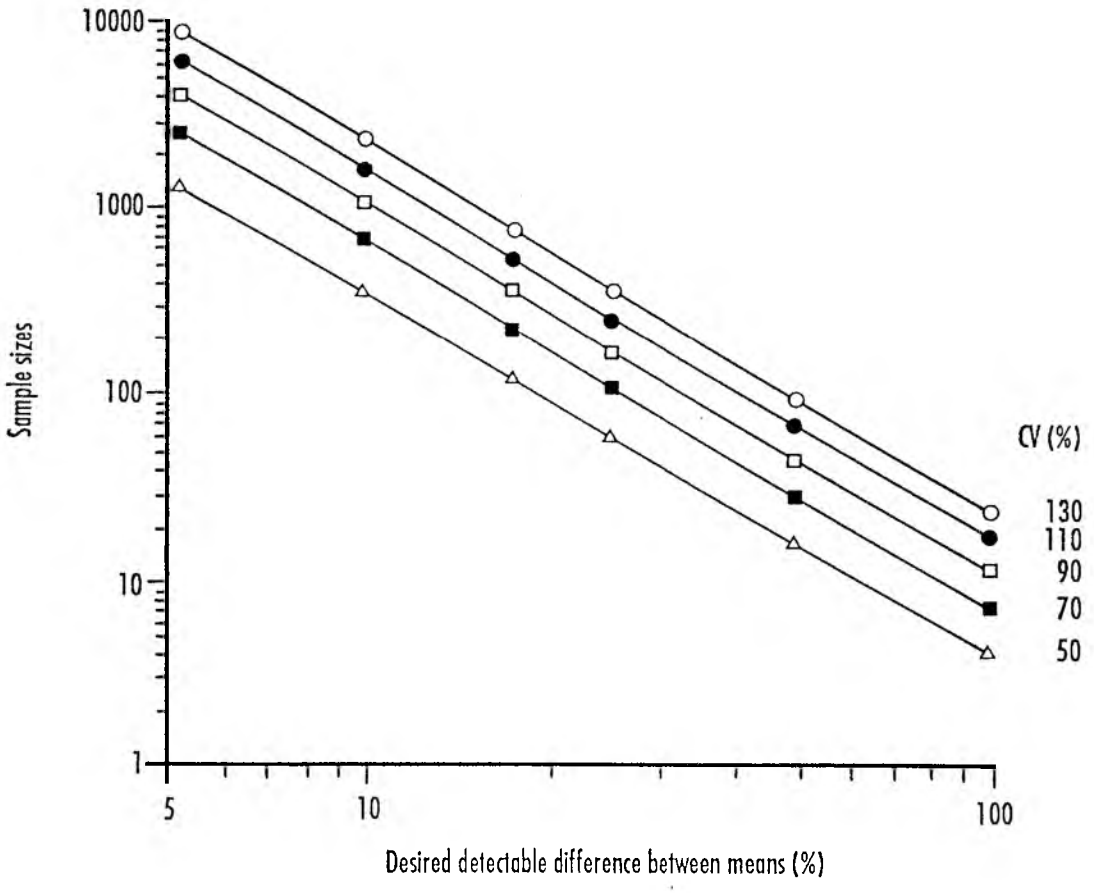

Figure 2.8 Sample sizes necessary to detect differences between treatment group means at five levels of variability (probability = 5%, power = 75%, treatments = 3)

1. The feeding rate depended upon the population of *G. pulex*, with the Haseley Brook population being more resistant to pollutants than the Crags Stream population, possibly because of prior exposure to metal contaminants;

2. The two laboratories (WRc and University of Sheffield) measured a statistically significant difference in the level of response, but this would not have led them to draw different conclusions about the presence or absence of an effect;

3. Within-treatment variation in response was high, but not higher than other bioassays, and there were indications that the level of variability could be reduced by minor methodological changes;

4. Effects on feeding rate observed during field deployment did not persist in animals returned to the laboratory. This suggests that the field response is behavioural, or that the depuration rates are too rapid to detect an effect over six days, depending on the nature of the pollutant.

The results from the deployments suggested that an *in situ* bioassay with *G. pulex*, using feeding rate, was feasible and could produce useful results. However, the trials also indicated that:

1. the feeding rate response depends upon the population of *G. pulex* used in the bioassay;

2. different laboratories may also measure a different level of response.

Further work was required to understand the causes of variability, and to minimise their effects. The following sections describe work carried out to:

1. improve the assay by changing the deployment procedure;

2. investigate the nature of variability due to using different populations.

3. OPTIMIZATION OF THE *G. PULEX* FEEDING RATE BIOASSAY

3.1 Introduction

The magnitude of feeding rate as well as the level of variability has a bearing upon its usefulness as a bioassay. If the feeding rate of *G. pulex* differs only slightly between unpolluted and polluted sites then relatively low levels of variation can make it difficult to detect significant differences between groups. It is, therefore, important to maximise the potential control response in order to increase the sensitivity of the method.

The field studies described in Section 2.2 showed that *G. pulex* feeding rate can be a useful indicator of water quality. However, these studies indicated several problems with the technique. Two seemingly methodological ones were high within-group variability and pseudoreplication.

A series of three experiments was, therefore, designed to:

1. determine the optimum orientation of the cages in a river;
2. overcome the impact of pseudoreplication in *in situ* deployments;
3. assess the impact of light on the feeding rate technique.

These studies aimed to improve the precision and sensitivity of the *G. pulex* feeding technique. Experiments 1 and 2 were performed in the field, with the primary objective of testing the effect on feeding rate variation of minor changes in cage orientation or experimental design. The third experiment was performed in the laboratory, and compared the feeding rate of *G. pulex* in clean water under light or dark conditions.

3.2 Optimum orientation of holding baskets

3.2.1 Introduction

An accurate assessment of the level of variation to be expected in a bioassay, and the effect that this may have on its sensitivity and replicability must form an important part of the development of any new test (Schaeffer *et al.* 1987). The variation in feeding rate reported in the series of field deployments described in Section 2.2 differed widely, depending on the trial in which the technique was used, as shown below:

Deployment	Coefficient of variation (%) Mean	Range	Suspended solids (mg l^{-1})
Colne Water	107.8	44.6 - 215.3	0.5 - 11.0
River Lea	107.5	41.2 - 417.8	2.0 - 9.0
West Okement	55.2	36.5 - 69.8	0.2 - 5.0
River Erme	68.5	40.1 - 223.7	1.0 - 2.5

The rivers with low levels of suspended solids, such as the West Okement and the River Erme produced results with lower CVs than the rivers in which suspended solids were relatively high. This finding suggested that variation in feeding rate might be affected by sedimentation inside cages and that it could be reduced by altering the deployment methodology.

In the initial field deployments, small cages containing *G. pulex* and food material were placed in larger holding baskets so that water flowed through the bore of the cages. In this experiment, the effect of changing the orientation of the baskets was assessed by comparing the feeding response and variation obtained from three different spatial orientations.

A 'worst case' site was chosen for this study so that a rigorous assessment of feeding rate variation could be made. Previous work on the River Lea below East Hyde Sewage Treatment Works (NGR TL 1210 1800) showed that the water at this site is high in suspended solids (8.2 - 9.0 mg l^{-1}), and that there was a high variability in *G. pulex* feeding rate (Section 2.2).

3.2.2 Deployment procedure

The deployments were carried out according to the procedure given in Appendix B using *G. pulex* collected from the River Darenth in North Kent (NGR TQ 5210 6230) six weeks before the start of the experiment. The animals were maintained under standard conditions of light (12 h light:12 h dark), temperature (15 °C) and food (alder leaves inoculated with *Cladosporium* fungus).

Thirty-six cages containing only leaf discs, to assess leaf weight gain or loss, were randomly distributed between nine holding baskets, each with the capacity to hold 22 cages. The remaining positions in each basket (a total of 162) were occupied by cages containing randomly allocated leaf discs and adult male *G. pulex*. The cages were secured within the holding baskets by netting and then transported to the study site in large plastic tubs containing groundwater.

Triplicate holding baskets were orientated in one of three ways:

1. vertically and perpendicular to the flow, so that water passed through the bore of each cage (deployment method used in Section 2.2);

2. vertically and parallel to the flow;

3. horizontally and perpendicular to the flow.

Baskets were randomly distributed in a 3 × 3 matrix in a 5 m^2 section of the river 20 m downstream from the sewage treatment works, and left *in situ* for 6 days. Animals were then returned to the laboratory where feeding rate was determined according to the procedures described in Appendix B. Statistical procedures used to analyse the data are described in Johnson *et al.* (1990a).

3.2.3 Conclusions

Mean feeding rates and coefficients of variation for each basket and mortalities in each deployment orientation are given in Table 3.1. *G. pulex* in baskets orientated vertically, and parallel to the flow of water in the river, consumed significantly less than those deployed in the other two orientations (ANOVA, $F=10.09$, $p<0.001$; Tukey $q>3.92$, $p<0.025$). There were no significant differences in the food consumed by animals in different baskets orientated in the same direction ($q>4.387$, $p>0.05$). Mortality was low in the deployments and only five *G. pulex* died in the field, one in each of the perpendicular deployments and three in the parallel deployment.

The leaf weight correction factors used played a major role in the significance of the levels reported above. Analysis of the experimental results without correcting for leaf weight loss or gain showed there were no significant differences between the types of orientation ($F=0.4$, $p=0.671$). The leaf weight correction factors calculated for each of the three orientations were significantly different from one another ($F=4.57$, $p=0.019$), as shown in Table 3.2. The factor for vertical, perpendicular baskets (1.089) was higher than that for vertical parallel baskets (0.952), ($q=4.181$, $p<0.025$). This indicates that there was a net loss of leaf disc mass in vertical, parallel orientated baskets, presumably due to leaching, and a net increase in leaf disc mass in vertical, perpendicular orientated baskets, presumably due to sedimentation. There were no significant differences in the factors calculated from different baskets within each type of orientation ($q>4.72$, $p>0.05$).

Table 3.1 Means and coefficients of variation for feeding rate data and mortalities for all cage orientations

Basket number	Cage orientation	Mean feeding rate (mg mg^{-1} d^{-1})	CV (%)	Number of dead in each deployment
1	Vertical and perpendicular	0.035	560.4	
3	"	0.132	78.1	
7	"	0.199	45.3	
1,3,7	"	0.122	123.5	1
2	Horizontal and perpendicular	0.068	162.3	
5	"	0.119	41.2	
8	"	0.092	118.1	
2,5,8	"	0.094	98.1	1
4	Vertical and parallel	0.043	220.5	
6	"	0.020	339.0	
9	"	0.044	167.2	
4,6,9	"	0.036	221.1	3

Table 3.2 Means and coefficients of variation for leaf weight control factors calculated for all cage orientations

Basket number	Orientation	Mean leaf weight control factor	CV (%)
1, 3 & 7	Vertical and perpendicular	1.089	13.71
4, 6 & 9	Vertical and parallel	0.952	10.06
2, 5 & 8	Horizontal and perpendicular	1.053	11.47

The orientation of the containers holding the test *G. pulex* had a significant effect on the feeding rate of the animals, but not in the way that was initially expected. A visual inspection of the cages upon retrieval suggested that those deployed vertically and parallel with the flow of water contained less sediment than either of the two groups orientated perpendicular to the flow of the water. This observation was supported by the leaf weight correction factors calculated from the cages containing only leaf discs.

The hypothesis formulated before the experiment was that high variability was associated with high sedimentation rates. However, the *G. pulex* deployed parallel to the flow fed less, and more variably, than those deployed perpendicularly (Table 3.1). This unexpected result may have been due to *G. pulex* consuming more of their own faeces in the parallel cages, and thus feeding less on the available leaf material. Coprophagy may have been lower in the perpendicular cages due to the removal of faeces by water flow in the vertical cages, or gravity in the horizontal cages. Different flow regimes through the individual cages may also have affected the *G. pulex* directly, but it seems unlikely that this would have been consistent enough in one orientation group to have produced statistically significant differences between treatment groups.

Coefficients of variation in individual baskets ranged from 41.2% to 560.4%, with both the lowest and the highest levels of variation found in baskets deployed vertically and perpendicular to the flow. The three highest CVs were found in the three baskets nearest to the sewage outfall. This response may have been coincidental, but the results suggest that the factors affecting animals in baskets deployed in the front row of the experiment were in some way different to those experienced by animals in the second and third rows of the deployment matrix.

The high feeding rate, low coefficients of variation and absence of sedimentation or leaching from leaf discs in the cages deployed horizontally and perpendicular to river flow indicated this would be the most appropriate means of orientating cages in field deployments.

The results from this initial experiment suggested further improvements in the design of the holding baskets. If holding baskets are deployed horizontally, a secondary cover constructed from fine mesh or transparent plastic placed on top of the holding basket could be the most effective way of excluding sediment. This method would also allow the expulsion of faeces, whilst still exposing test animals to ambient water quality. Therefore, three baskets were subsequently deployed horizontally and parallel to the flow of the River Lea at the same site. The only difference in the design of the baskets was that the netting on the upper side of the basket was replaced by transparent polythene.

The means and coefficients of variation for the feeding rate of *G. pulex* deployed in partially sealed baskets are presented in Table 3.3, along with data on mortality levels, which were higher in this experiment than during the previous deployment.

Table 3.3 Means and coefficients of variation for feeding rate data in holding baskets deployed horizontally and perpendicular to flow with a polythene barrier on the upper side

Basket number	Mean feeding rate (mg mg^{-1} d^{-1})	CV (%)	Number of deaths in the field
1	0.105	44.69	6
2	0.087	56.09	5
3	0.093	53.67	3
Mean	0.094	51.40	14

The results of the deployment with partially sealed baskets should not be compared directly with those from the earlier study, since this second deployment was an uncontrolled investigation designed to assess whether feeding rate variation could be reduced from the levels observed in the first deployment. However, it was interesting to note that exactly the same mean total feeding rate was found in horizontally-deployed baskets from both the first and second experiments.

The level of variation in the second experiment was considerably lower than for any of the treatments in the first deployment, and lower than that found in the 1989 deployment on the River Lea, suggesting that the horizontal deployment of holding baskets with an impermeable upper cover would be the most effective way of deploying the *G. pulex* feeding test in the field.

3.2.4 Summary

This optimized method of deploying the *G. pulex* feeding assay horizontally and perpendicular to flow with a polythene barrier on the upper side (see Appendix B) was used in subsequent studies of the development of the assay.

3.3 Assessment of a method for overcoming pseudoreplication in *in situ* studies

3.3.1 Introduction

Hurlbert (1984) has discussed the problems inherent in upstream-downstream studies of the type in which a bioassay, such as the *in situ G. pulex* feeding rate bioassay is likely to be used. 'Pseudoreplication' occurs in these experiments because it is impossible to allocate treatments randomly. Bioassays are placed either above or below the relevant discharge without the ability to control for other variables such as substrate type, flow rate or temperature. It is thus impossible to confidently ascribe the cause of any observed changes between upstream and downstream stations to the effects of the discharge.

Stewart-Oaten *et al.* (1986) have devised an experimental design that may overcome this problem in many environmental impact studies. They argue that the detection of the effect of a discharge may be achieved by testing whether the difference between underlying mean abundances or responses at control and impacted sites changes once the discharge begins. Samples replicated in time are taken **B**efore the discharge begins and **A**fter it has begun at both the **C**ontrol (upstream) and **I**mpacted (downstream) sites in the **BACI** approach. These data are then used to estimate the trends of the response before and after the impact commences and standard statistical techniques can be used to see if these trends differ significantly. This approach is similar to that recommended for sequential data in biological experiments (Roberts 1992).

Porthtowan Sewage Treatment Works (STW) in Cornwall was chosen for an assessment of the BACI approach. The works discharges domestic sewage effluent into a small stream, the Porthtowan River, which flows for a further 2 km through an abandoned copper mining area to the sea. South-West Water and NRA South West Region chose the STW for a pilot study on the efficacy and environmental effects of sewage disinfection with peracetic acid (PAA), thus providing the opportunity for a before and after study.

3.3.2 Deployment procedure

Field cages were prepared and filled with food material and with adult male *G. pulex* obtained from the River Darenth, as described in Appendix B. The deployment of the technique was staggered over a five-day period, both before and after the commencement of disinfection. The correct use of the BACI approach would normally require samples to be spaced randomly and separately in time to a far greater extent than in this study, in order to assess long-term effects. However, it was felt that the short-term approach described here would give a valuable indication of effects within a clearly circumscribed time frame.

On Day 1 of the 'Before' exposure period (4 November 1990), one holding basket, containing 18 *G. pulex* and four leaf weight control cages, was deployed at a station upstream from Porthtowan STW. A similarly filled holding basket was deployed simultaneously at a downstream station. The upstream station was located approximately 100 m above, and the downstream station approximately 100 m below the STW outfall. On each of days two, three, four and five of the Before study, a further pair of baskets was deployed at the stations above and below the STW, so that by Day 5 there was a total of five baskets at the upstream station, and five baskets at the downstream station.

Retrieval of the baskets was also staggered, with the pair deployed on Day 1 being removed on Day 6 (10 November), and the remaining pairs similarly being removed six days after deployment, on Days 7, 8, 9 and 10 respectively. After retrieval, live *G. pulex* and any fragments of leaf remaining in the cages were removed and rinsed in tap water until free of attached sediment. The weight of the leaf material and animals was then determined after drying at 60 °C to constant weight.

The same exercise was repeated after the commencement of disinfection on 28 November 1990. The first pair of holding baskets was deployed above and below the sewage treatment works on 29 November and retrieved six days later. The fifth pair was deployed on 3 December and retrieved on 9 December. The feeding rates were transformed to stabilise the variances using the function log(x+1). They were then analysed in two separate ways:

1. The five temporal replicates at each site were grouped and analysed using ANOVA for both Before and After deployments, a procedure that mimics the traditional method used for determining upstream and downstream differences in environmental impact assessments (Green 1979).

2. The BACI approach was adopted, with the mean response across cages calculated for each holding basket and the difference for each temporal replicate found between upstream and downstream. After testing for non-additivity with a Tukey test, the Before and After disinfection deployments were compared with a t-test, using for each data set the five replicates of difference between the upstream and downstream response as input parameters. All procedures were performed according to Stewart-Oaten *et al.* (1986) and Sokal and Rohlf (1981).

Mortality was analysed in a similar way to feeding rate. A Generalised Linear Model (GLM) was used to analyse differences in overall mortality between upstream and downstream sites on the Before and After deployments respectively. A t-test was then used to compare the differences in mortality after treating the data according to the BACI method.

3.3.3 Conclusions

Analysis of the mortality data (Table 3.4), revealed a significant difference in *G. pulex* mortality between the upstream and downstream sites before disinfection began ($G=34.06$, $p<0.001$), but not after it had commenced ($G=15.66$, $p>0.05$). The BACI approach also revealed a significant difference in the mortality rate between the before and after data sets ($t=3.78$, $p<0.01$). This consistent result from the two analytical approaches was primarily due to high upstream mortality during the Before trial.

There were no significant differences in the weight loss of the leaf controls between sites on either of the deployments, although there was a significant difference in weight loss between the before and after studies, due to a reduction in sedimentation during the latter. Because of this, the correction factors used were 0.90 and 0.95 respectively.

Table 3.4 Number of dead *G.pulex* following field exposure at upstream and downstream sites, before and after the commencement of PAA disinfection (n=18)

Deployment	Pair	Mortality	
		Upstream	Downstream
Before	1	5	2
	2	7	0
	3	10	1
	4	8	1
	5	3	4
After	1	1	0
	2	3	1
	3	3	4
	4	5	1
	5	4	5

The feeding rates of the deployed animals are shown in Figure 3.1. The negative feeding rates found for *G. pulex* deployed at the downstream site before disinfection began resulted from zero or near zero food consumption and an overcompensatory leaf weight correction factor.

An ANOVA performed on transformed results from the upstream and downstream sites, in which all the surviving animals in each of the baskets were treated as replicates, demonstrated a significant difference in the feeding rates of the *G. pulex* deployed upstream and downstream ($F=12.75$, $p<0.001$) before the commencement of disinfection. The surviving animals at the upstream site fed more than those deployed at the downstream site. After disinfection commenced, the mean feeding rate of the animals deployed below the STW increased, and there was no significant difference in this response between the two sites ($F=1.84$, $p=0.177$). The BACI analysis of feeding rate did not reveal a significant difference in response between the before and after studies ($t=1.71$, $p>0.05$), because the variation in the mean response was high in relation to the sample sizes.

The results from the *in situ* deployment of *G. pulex* suggest that the Porthtowan River is subject to variable water quality over short time periods. The significantly higher mortality of animals deployed at the upstream site during the Before trial indicates the presence of toxicity which can in no way be related to the Sewage Treatment Works. This toxicity did not persist, and *G. pulex* deployed at the same site one month later did not suffer the same level of mortality. The stressor or stressors responsible for this variable toxicity remain unidentified, although the nature of the catchment suggests that either heavy metals or farm waste could have been responsible.

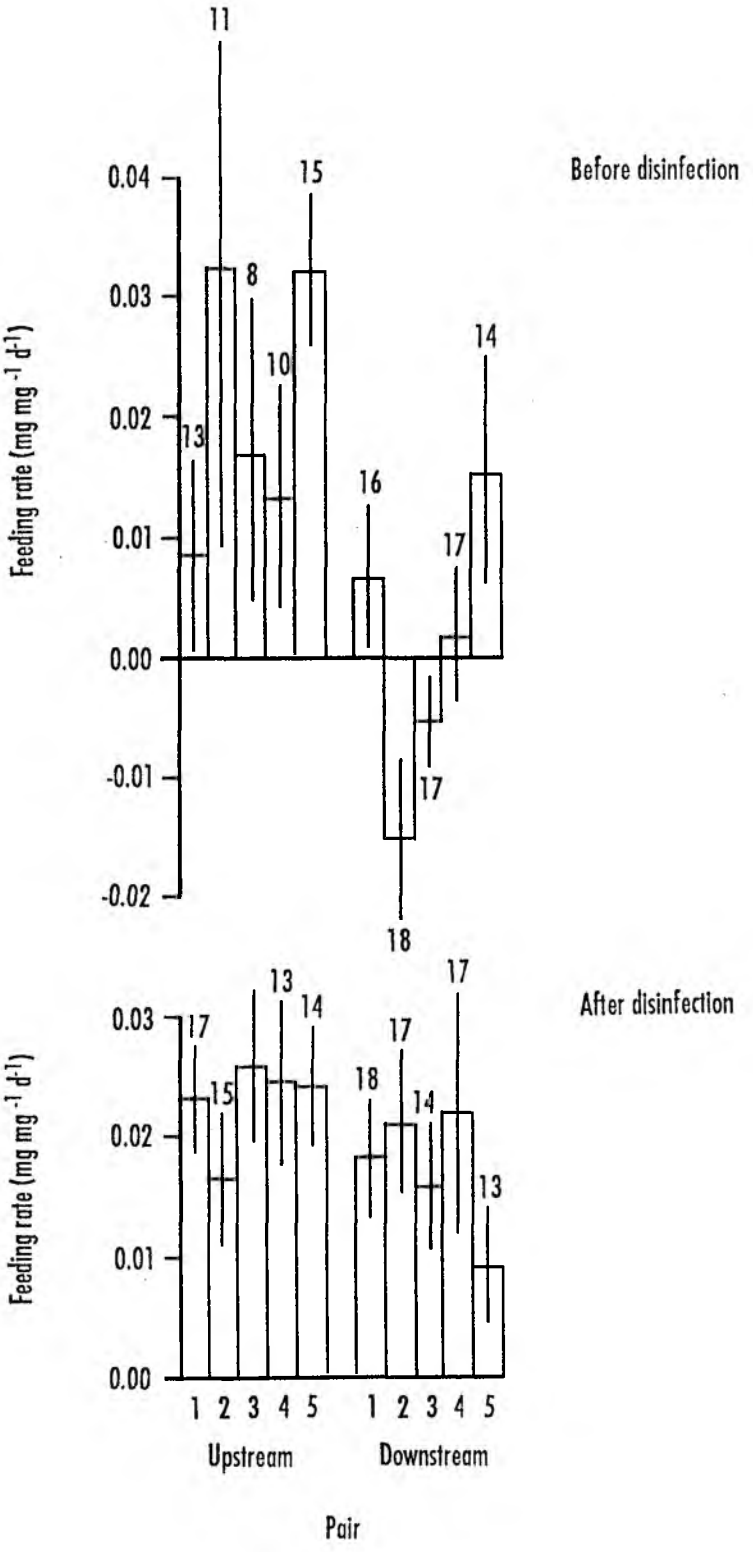

Figure 3.1 Feeding rate of *G. pulex* deployed in the Porthtowan River (error bars are one standard error of the mean)

The feeding rate of the *G. pulex* was low at both upstream and downstream sites, before and after disinfection. This low feeding rate may have been related to the low water temperature at this time of year, or to poor water quality at both sites, and affected the ability to detect a difference in response between the treatment groups. Interpretation of the feeding rate data was further confounded by the high mortality that occurred at the upstream site before disinfection. High mortality in a sublethal test is undesirable because the surviving individuals may be resistant and exhibit an atypical response (See Section 2.2.6).

The BACI approach to evaluating the impact of new effluent types can produce statistically and methodologically robust conclusions and recent theoretical efforts have improved the statistical rigour of this approach still further (Stewart-Oaten *et al.* 1992, Underwood 1992). In this study there was a clear indication from both the traditional and the BACI approach that upstream water quality could be lethal to *G. pulex*. However, the number of samples required when adopting the BACI approach may be high, especially when sublethal, and hence quite often variable, responses are measured. A pragmatic approach to *in situ* monitoring may therefore be the most cost-effective. For example, a simple initial upstream and downstream deployment of an appropriate *in situ* bioassay may indicate a potential problem that can subsequently be investigated under controlled laboratory conditions.

3.4 The effect of light on *G. pulex* feeding rate

3.4.1 Introduction

G. pulex normally lives in the darkness of interstitial spaces between pebbles on river or stream beds. In this laboratory experiment the effect of excluding light from *G. pulex* feeding chambers was examined, to assess whether the feeding rate could be enhanced by a low light regime.

3.4.2 Experimental procedure

Sixty polypropylene feeding chambers, consisting of two compartments divided by 1 mm nylon mesh, were prepared as described in Appendix A. Each chamber was filled with 250 ml of borehole water 24 hours before the start of the experiment. Alder leaf discs were prepared in the standard way and sets of four placed in each chamber. Fifty adult male *G. pulex* were caught from the River Darenth and kept under standard light, temperature and feeding conditions for one week before the experiment. Random number tables were used to position each chamber on a flat, uniformly lit bench in a 15 °C constant temperature room. One *G. pulex* was then randomly allocated to each of fifty chambers, leaving ten chambers containing only leaf discs to control for weight gain or loss. A further series of random numbers was then used to divide the chambers into two groups of 30 'light' and 30 'dark' (25 *G. pulex* and leaf discs, and 5 leaf controls in each group). Chambers in the light group were enveloped in transparent plastic sheeting, and those in the dark group in black plastic sheeting. After six days, the animals and any

remaining leaf material were removed from the chambers and the feeding rate of the animals determined.

3.4.3 Conclusions

The mean feeding rate of animals in light and dark chambers are shown below:

Exposure conditions	Feeding rate (mg mg^{-1} d^{-1}) Mean	SD	n
Light	0.194	0.086	24
Dark	0.172	0.078	22

No animals died in the experiment, but one moulted in the light treatment group and three moulted in the dark treatment group and were excluded from the analysis.

No significant differences in feeding rate were found between *G. pulex* in light or dark chambers (ANOVA F=0.82, p=0.371). It is therefore unlikely that the exclusion of light from field-deployed holding baskets would improve the performance of the *G. pulex* feeding test.

3.5 Overall Summary

The results from these studies suggest that the optimum way of deploying *G. pulex* in the field is in cages orientated horizontally and perpendicular to the flow of the stream, with the upper side sealed with a transparent cover. The experimental design should follow a pragmatic course, although the implications of pseudoreplication should be borne in mind when interpreting results.

4. ASSESSMENT OF THE USEFULNESS OF THE REVISED *G. PULEX* FEEDING RATE BIOASSAY IN THE RIVER AIRE

4.1 Introduction

The *G. pulex* feeding rate bioassay was developed to provide a simple and quantitative tool for assessing water quality. If the technique is to be of practical use it must assist regulators by identifying real water quality problems. The River Aire in Yorkshire has recently shown variable biological quality between stations in its upper reaches, while chemical data from the same stations suggest that water quality is constant (Matraves 1990). This discrepancy between biological and chemical data is a type of situation in which an *in situ* bioassay may be a useful explanatory tool.

The study reported in this section was designed to investigate:

1. the feeding rate of *G. pulex* deployed *in situ* at 11 stations in the River Aire;
2. the relationship between *G. pulex* feeding rate and biological and chemical quality at each station;
3. the transferability of the *G. pulex* feeding rate bioassay from WRc to NRA personnel.

A further objective was to examine the reproducibility of results obtained when using different deployment equipment: NRA Yorkshire Region had previously deployed the test using wire holding baskets and wished to study the efficacy of their baskets compared with those used by WRc.

4.2 Deployment procedure

Test animals were obtained from Crags Stream in Derbyshire one week before deployment of the test in the field. More than 750 precopula pairs were swept from the watercress beds at this site and transported to the NRA Yorkshire Region Laboratory (Leeds) in plastic buckets containing site water. These animals were kept at the laboratory in aerated groundwater at stocking densities of less than ten animals per litre and fed on food material similar to that supplied to them later in the field.

G. pulex leaf disc food material was prepared as described in Appendix B. Investigators from WRc and the NRA prepared 325 sets of weighed leaf discs for addition to cages with *G. pulex* and 98 sets of weighed leaf discs for addition to cages without *G. pulex*, as controls for leaf weight loss or gain during the experiment.

Male *G. pulex* were separated from females by WRc and NRA operators one day before field deployment, and placed in separate stock tanks without food. The leaf discs were also allocated to their respective numbered cages one day before deployment, according to a random number sequence.

On the day of deployment, *G. pulex* were added sequentially by the respective operators to the field cages arrayed in numerical order in plastic trays containing groundwater. After the *G. pulex* had been added to all the appropriate cages, these cages were placed in their respective holding baskets. The holding baskets were then sealed with a polythene top, except for those supplied by NRA Yorkshire Region. The holding baskets were transported to the field stations (see Table 4.1 for locations) in sealed buckets containing groundwater, and attached to the river bed with stakes and bricks.

Table 4.1 Location of eleven stations along the upper reaches of the River Aire, Yorkshire

Station	National Grid Reference	Description
1	SD 908550	Upstream reference
2	SD 917537	Upstream reference
3	SD 938539	Below Gargrave and above Johnson and Johnson works
4	SD 943539	Below Johnson and Johnson works (possible pharmaceutical discharge)
5	SD 946535	Below Gargrave Sewage Treatment Works
6	SD 961515	Above Broughton Beck
7	SD 963514	Below Broughton Beck (possible agricultural discharge)
8	SD 972505	Below Catlow Gill (possible agricultural discharge)
9	SD 977504	Below landfill (possible discharge of leachate)
10	SD 983501	Above Eller Beck
11	SD 985501	Below Eller Beck (possible urban runoff)

The following physical, chemical and biological parameters were taken at all stations:

Physical measures	Temperature, pH, and conductivity
Chemical measures	Metals (Al, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, S, Si, Sr, V, Zn) Total organic carbon as an indicator of organic contaminants Alkalinity, total ammonia, chloride, hardness, nitrate, nitrite, soluble reactive phosphorus, sulphate and suspended solids
Biological measures:	Three minute kick samples with a 0.5 mm mesh FBA net. Macroinvertebrates found in these samples were subsequently assessed using standard NRA techniques (Armitage *et al.* 1983).

Physical and chemical measurements were made both at the time of deployment and retrieval, whereas macro-invertebrate surveys were carried out on the day of retrieval.

The deployed baskets were retrieved after six days in the field and returned to the laboratory in sealed buckets containing groundwater to determine feeding rate according to the procedures in Appendix B.

For the determination of feeding rate, the field cages containing leaf discs prepared by WRc personnel were assigned to the WRc operator. The NRA Yorkshire region operator received cages containing material prepared by the NRA. Both sets of personnel then processed the contents of their respective cages in a similar way. The cages were placed in numerical order in trays containing groundwater, and opened in numerical sequence by each set of operators. Mortalities were recorded and live animals removed from their cages, rinsed twice in distilled water and placed in an appropriately numbered, pre-weighed foil boat. They were killed by gently squeezing the foil boat and then dried for four days at 60 °C before weighing. Leaf material remaining in each cage was removed and placed in a petri dish containing distilled water. A fine paint brush was used by each operator to remove any deposits on the surface of the leaf discs. Five strokes of the brush were applied to each side of each disc. If any material still obviously adhered to the disc after this procedure, it was removed by using forceps or further brushing. Cleaned leaf material was then dried for four days at 60 °C before weighing.

Mortality between sites was analysed using a Generalised Linear Model for binomial responses (Genstat 5 Committee 1987). Differences between treatment groups and operators were analysed, after plotting the residuals to ensure variance homogeneity, using a factorial analysis of variance (Genstat 5 Committee 1987). This analysis was then followed by a Tukey Multiple Comparison test (Zar 1984). Inter-operator comparisons were also made of measurements of *G. pulex* weight at the end, and leaf disc weights at the beginning and end of the experiment.

4.3 Conclusions

Numbers of dead *G. pulex* at each station across both laboratories after the six day deployment period are shown in Table 4.2. Mortality did not differ significantly between stations (Generalised Linear Model, deviance=5.61, $p>0.75$) and was generally 10% or less of the animals deployed. There was no evident pattern in moulting behaviour during deployment.

The leaf weight correction factors derived from leaf discs placed in cages without *G. pulex* differed significantly between stations (ANOVA, $F=7.06$, $p<0.001$) but not between laboratories ($F=0.26$, $p=0.612$). Separate correction factors were therefore applied to the results from each station, but not to those measured by each laboratory. The correction factors showed that leaf material was generally lost during the deployment, suggesting that sedimentation was not a problem.

Table 4.2 The mortality and feeding rates of *G. pulex* and macroinvertebrate survey data at 11 sites on the River Aire

Deployment site	Feeding rate (mg mg^{-1} d^{-1})		Number dead (% total)	Number moulted (% total)	Macroinvertebrate survey		
	WRc	NRA			BMWP score	ASPT score	No. scoring families
1	0.192	0.148	5 (10)	4 (8)	158	5.6	28
2	0.167	0.160	5 (10)	0 (0)	127	5.3	24
3	0.156	0.152	5 (10)	2 (4)	107	4.7	23
4	0.186	0.141	3 (6)	2 (4)	123	4.9	25
5	0.131	0.100	5 (10)	0 (0)	124	5.2	24
6	0.156	0.129	9 (18)	2 (4)	160	5.5	29
7	0.146	0.115	4 (8)	4 (8)	120	4.8	25
8	0.156	0.129	8 (16)	4 (8)	106	4.6	23
9	0.182	0.156	5 (10)	3 (6)	129	5.0	26
10	0.179	0.173	5 (10)	4 (8)	132	5.0	26
11	0.187	0.157	5 (10)	3 (6)	151	5.2	29

The general pattern of feeding rates measured by both WRc and the NRA was one of relatively high rates at the four upstream (1-4) and three downstream (9-11) stations compared with lower feeding rates in the central portion (Table 4.2). Coefficients of variation (CV) were in the range 25.03% - 54.54% (mean = 37.97%, SD = 7.95%), which are quite low.

There were significant differences in mean feeding rates between stations (ANOVA, $F=4.12$, $p<0.001$), due primarily to a reduction in feeding rate at Station 5. *G. pulex* at this station, below the sewage treatment works, fed at a significantly lower rate compared with the two reference stations (Tukey test $q=6.167$, $p<0.001$; and $q=5.272$, $p<0.025$ respectively), the station immediately upstream ($q=5.504$, $p<0.01$) and Stations 9, 10 and 11 ($q=5.921$, $p<0.005$; $q=6.748$, $p<0.001$; $q=6.337$, $p<0.001$). There was also an indication that the feeding rate at Station 7, below Broughton Beck, may have been affected during the deployment. *G. pulex* at this station fed significantly less than those at Stations 10 or 11 ($q=5.16$, $p<0.025$ and $q=4.733$, $p<0.05$).

A significant interlaboratory difference in the measurement of the feeding rate response was also evident ($F=41.38$, $p<0.001$) because the NRA operator consistently measured a lower feeding rate:

Operator	Feeding rate (mg mg^{-1} d^{-1}) Mean	SD
NRA	0.138	0.058
WRc	0.169	0.060

The most likely cause of this difference in feeding rate measurement was the significant difference in NRA and WRc leaf weights at the end of the experiment ($F=43.88$, $p<0.001$). Other differences in measurement between the two operators were also evident. There was a small, but significant difference in the weights of the leaf discs at the start of the experiment ($F=5.40$, $p=0.021$). Observation suggests that this was because the WRc operator tended to dislodge small amounts of leaf material when transferring the leaf discs from their drying container to the balance. However, this will not have affected the outcome of the experiment, and the protocol was designed to prevent further loss of material after this stage. A small, but significant difference in the measurement of *G. pulex* weights at the end of the experiment was also evident ($F=15.35$, $p<0.001$), although the *G. pulex* were removed from stock tanks at random by each operator. This suggests that the difference in measured weights occurred during field exposure.

Despite these inter-operator differences, there was no operator × station interaction effect ($F=1.19$, $p=0.284$), suggesting that the two operators measured a generally similar response trend.

The results of chemical analysis and the macroinvertebrate survey (Table 4.2) results produced by NRA personnel did not provide any clear evidence of a deterioration in water quality between any of the stations.

4.4 Summary

The results from this study suggest that water quality in this stretch of the River Aire was generally high during the period of investigation. Water chemistry and macroinvertebrate samples did not provide strong evidence for a deterioration in water quality at any of the eleven stations. This contrasts with previous information on macroinvertebrate richness in the River Aire gathered by the NRA (Matraves 1990). These data suggested that water quality at Stations 6 to 11 was lower than that at upstream stations, although no chemical cause could be found for the observed decline in macroinvertebrate richness.

The difference between the macroinvertebrate data collected in the present study and those collected in earlier surveys could be due to a number of factors. Non-persistent pollution events responsible for lower species richness at downstream stations may have ceased in the year between this study and the last detailed investigation of the River Aire. Alternatively, episodic events may be responsible for reduced species richness at certain times. There was no rainfall during this study, and hence no surface runoff, or other storm-related discharge, is likely to have entered the River Aire. Earlier surveys may have detected the effects of episodes, whereas this investigation happened long enough after the last event for invertebrate recolonisation to occur. The final possibility is that the inherent variability of macroinvertebrate results obtained by kick-sampling, and the difficulty in ascribing statistical significance to these results, led to a false interpretation of earlier surveys.

The *G. pulex* bioassay was the only one of the three survey approaches used (chemical, macroinvertebrate and bioassay) to provide a continuous and statistically robust assessment of environmental quality at the eleven River Aire stations during the six-day study period. The chemical data obtained from two spot samples at each station were difficult to interpret because their relevance to the river fauna is largely unknown, and because they are only 'snapshots' taken at either end of a time period during which an unknown event may have occurred.

The macroinvertebrate data are difficult to interpret because their method of collection does not allow us to undertake a rigorous statistical analysis. RIVPACS can be used to provide a baseline 'expected' value for comparison with observed data. However, the chance of misclassification can be high using this technique, especially if data are used from only one seasonal set of samples (Wright *et al.* 1984). The problem is compounded in this study because the macroinvertebrate data are not very different between stations.

In contrast, the bioassay approach was able to discriminate unequivocally between stations. *G. pulex* placed at Station 5, below Gargrave Sewage Treatment Works, fed less than those at control stations. This does not necessarily mean that water quality at this station was poorer; other factors may have influenced the results. However, the significant reduction in *G. pulex* feeding rate at Station 5 does allow water quality managers to focus their attention on the central part of this stretch of the River Aire.

The consistent difference in the results measured by the WRc and NRA operators requires further investigation. A similar problem was encountered in the work reported in Section 2.2, and efforts were made in the present study to reduce sources of variation as far as possible. Hence, both operators worked in the same laboratory and used the same pieces of equipment during all the critical phases of the investigation. No significant differences were found between the leaf weight correction factors calculated by the two operators, suggesting that they both cleaned the leaf material returned from the field to a similar extent. This operation was identified as the most prone to subjective differences, but the evidence from the weight control leaf discs shows that the operators achieved similar results. The significant differences between *G. pulex* and leaf disc weights at the end of the experiment suggest that the test animals loaded into cages by the WRc operator really did eat more than the NRA-loaded animals. Although the operators appeared to handle the *G. pulex* in a similar way, the NRA operator may unknowingly have used a consistently more stressful technique when separating males from females, or when loading males into cages. Future protocols should seek to minimise such possible sources of stress. However, it is important to note that both operators achieved results in the same direction, and would both have identified Station 5 as a potential problem site.

In conclusion, this study has shown that bioassays can be a useful complement to traditional biological survey and chemical-based approaches. Such a quantitative and statistically based approach to problem solving would help to increase the confidence with which regulators assess water quality.

5. AN ASSESSMENT OF THE CAUSES OF VARIABILITY IN THE FEEDING RATE BIOASSAY

5.1 Introduction

Variability in the results of toxicity assessment has been found at every level of biological organisation, from subcellular (Rattner and Fairbrother 1991) to community (Taub *et al.* 1989). The level and source of variability in toxicity tests and bioassays should be understood if fair regulatory decisions are to be made on the basis of results obtained from them (Warren-Hicks and Parkhurst 1992). Precision is a general term for the variability between repeated tests, and comprises repeatability (intralaboratory variability) and reproducibility (interlaboratory variability) (ISO 1986). A test is repeatable if similar results can be obtained from a number of tests performed by the same operator, in the same laboratory and with the same pieces of equipment. A test is reproducible if similar results can be obtained by different test operators in different laboratories using different equipment.

Intralaboratory variability in a toxicity test or bioassay can be affected by changes in the sensitivity of the source population over time, while interpopulation variability may result from the use of different populations with different inherent sensitivities in different laboratories. A series of studies have been conducted at WRc and the University of Sheffield to assess the extent to which these factors affect the feeding rate bioassay, and these are discussed in the following sections.

5.2 An assessment of the temporal variability of the feeding rate test

5.2.1 Introduction

A potential source of intralaboratory variability is changes in the sensitivity of organisms over time and any assessment of the variability of a technique should consider temporal repeatability. Work at the University of Sheffield has identified temporal variation in the feeding rate of *G. pulex* under control conditions (Johnson *et al.* 1993). It is important to establish whether such variability influences the sensitivity and repeatability of the feeding rate bioassay. If feeding rate is to be used as a comparative measure (for example for upstream/downstream comparisons) then such variability may be unimportant, as long as the sensitivity of the response criterion remains unchanged. However, if sensitivity is dependent upon total consumption, then temporal variation in feeding rate has obvious implications for the application of the technique.

The specific objectives of this study were to:

1. quantify the extent of temporal variation in the feeding rate of a single population of *G. pulex*;

2. assess the implications of temporal variation in feeding rate for the sensitivity of the technique as a measure of stress.

5.2.2 Experimental procedure

Temporal variation in the feeding rate of *G. pulex* was investigated during the period May 1990 to March 1991. Animals were collected from Crags Stream populations at monthly intervals and screened against a single toxicant, zinc, under standard laboratory conditions. Male *G. pulex* (7-11 mg dry weight) were acclimated to test conditions (temperature = 15 °C food = conditioned alder leaves, medium = artificial pond water (APW), photoperiod = 12 h light: 12 h dark) for seven days prior to use.

The effect of zinc on the feeding rate of *G. pulex* was assessed each month. Five concentrations of zinc were used: 0.3, 0.4, 0.5, 0.6 and 0.7 mg Zn l^{-1}, and artificial pond water (APW) alone as a control. Solutions were prepared by dissolving $ZnSO_4.7H_2O$ in APW. Test solutions were changed daily and old and new solutions were analysed by atomic absorption spectrophotometry.

The studies were carried out according to the procedures described in Appendix A and used 20 animals per treatment, each in individual experimental chambers containing a known amount of food (conditioned alder leaves). Five control chambers contained food but no animals. Ten animals per treatment were analysed for total body zinc content at the end of the study.

5.2.3 Conclusions

The energy consumption of unstressed animals varied significantly ($F=7.63$, $df=10,204$, $P<0.001$) over the period May 1990 to March 1991. Consumption rates ranged from >8 J mg^{-1} d^{-1} (August) to <4 J mg^{-1} d^{-1} (January and March); the average ($\pm$ SE) being 5.47 ($\pm$ 0.16) J mg^{-1} d^{-1}.

Feeding rate may be affected by a number of factors both intrinsic (for example body size and reproductive state) and extrinsic (for example food quality and temperature). In these experiments, intrinsic variables such as sex and reproductive condition were controlled for in the choice of test animals. However, body size did vary significantly over the study period ($F=4.9$, $df=10,204$, $p<0.001$) and could, therefore, contribute to the observed temporal variability in feeding rate. This possibility was explored by plotting feeding rate against body weight. However, there was no significant correlation between body weight and feeding rate irrespective of whether the analysis was performed on individual data points ($r=-0.05$, $df=213$, $p>0.05$) or monthly means ($r=-0.45$, $df=9$, $p>0.05$).

Of the extrinsic factors which may affect feeding rate, food quality and quantity and experimental media were standardized throughout the study. Temperature is known to be positively correlated with the feeding rate of *G. pulex* (Johnson *et al.* 1993) and, although laboratory temperatures were controlled, stream temperatures would have varied. A significant positive correlation between mean monthly air temperature and feeding rate was found ($r=0.7$, $df=9$,$p<0.05$), suggesting that seasonal changes in stream temperature may at least partly explain temporal variation in feeding rate.

Exposure to zinc resulted in a significant depression in feeding rate ($F>4.4$, $df=5,90$, $p<0.01$) in all months. However, there was significant temporal variation in the lowest concentration causing a significant reduction in feeding rate (LOEC) (Figure 5.1) or the concentrations causing a 50% reduction in feeding rate, relative to controls (EC_{50}) (Figure 5.2).

The EC_{50} and LOEC ranges recorded are shown below:

Parameter	Range (mg l^{-1})
EC_{50}	0.35 (January) - 0.71 (October)
LOEC	0.25 (March) - 0.58 (October)

However, neither LOEC nor EC_{50} values were significantly correlated with temporal changes in the feeding rate of control animals (LOEC: $r=0.41$, $df=9$, $p>0.05$; EC50: $r=0.55$, $df=9$, $p>0.05$). Therefore, although the feeding rate of *G. pulex* does exhibit temporal variation, this does not directly influence the sensitivity of feeding rate as a measure of zinc stress.

There was a significant negative relationship between feeding rate and tissue concentrations of zinc (ANCOVA: $F=14.81$, $df=11,630$, $p<0.001$). The form of the relationship did not differ significantly between months (ANCOVA: $F=1.67$, $df=10,630$, $p>0.05$) having a common regression coefficient of -0.007. However, there was no significant relationship between the mean monthly zinc content and feeding rate of control animals. Therefore, natural variations in zinc body concentrations were not responsible for the observed temporal variation in the feeding rate of control animals.

5.2.4 Summary

The feeding rate of Crags Stream *G. pulex* varied significantly over the period May 1990 to March 1991. Feeding rate was positively correlated with field temperatures, being highest in summer and lowest in winter. The sensitivity of feeding rate as a measure of zinc stress also varied over the study period, being most sensitive in spring (LOEC = 0.25 mg l^{-1}) and least sensitive in autumn (LOEC = 0.58 mg l^{-1}). However, sensitivity was not correlated with the feeding rate of control animals, therefore *G. pulex* feeding rate can be used throughout the year to provide a comparative measure of stress.

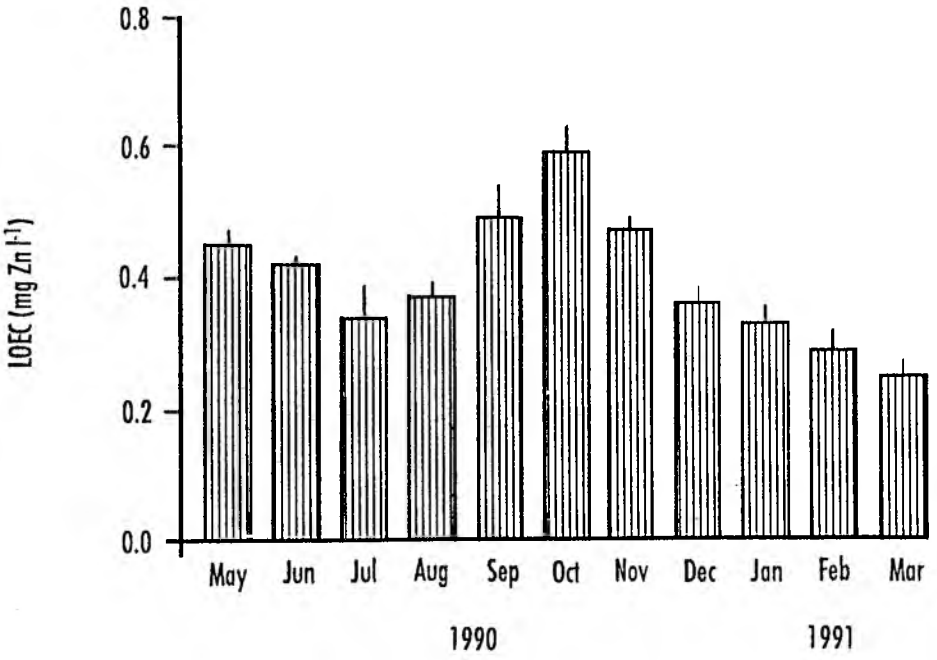

Figure 5.1 Temporal changes in the mean (+1 S. E.) LOEC for feeding rate of *G. pulex* exposed to zinc

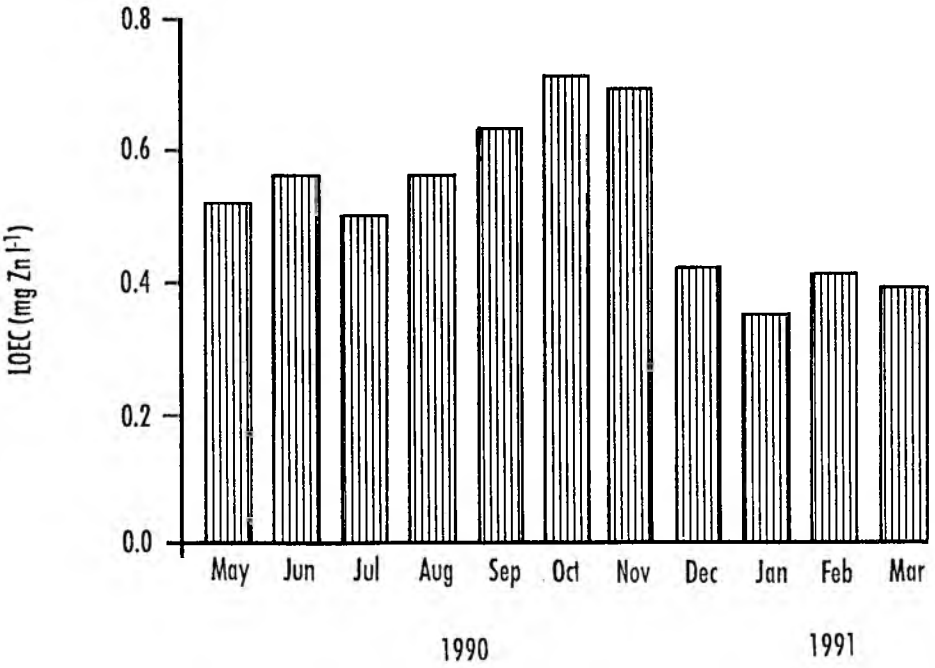

Figure 5.2 Temporal changes in the EC_{50} for feeding rate of *G. pulex* exposed to zinc

5.3 Assessment of effect of interpopulation differences on the *G. pulex* feeding rate technique

5.3.1 Introduction

Two studies have been carried out at WRc to assess the effects that different populations have on the sensitivity and discrimination of the *G. pulex* feeding rate bioassay. The first study considered the differences in response to iron and manganese of 'clean' and impacted populations. The second study considered the effect of zinc on four populations from 'clean' sites.

5.3.2 An assessment of interpopulation variability in the feeding response to iron and manganese

Introduction

A field study carried out in 1989 (see Section 2.2) on the West Okement River showed that the feeding rate of *G. pulex* from Crags Stream was reduced below two quarry effluent discharges. The reduction was positively correlated with the presence of iron and manganese in the water column. The feeding rate of Haseley Brook gammarids was not significantly affected, possibly because these animals had apparently been exposed to metals in their native streams. To ascertain whether previous exposure to pollutants had an effect on the feeding rate response of differing populations of *G.pulex*, a laboratory based study exposing Crags Stream and Haseley Brook gammarids to iron and manganese was carried out.

Experimental procedure

Three hundred and fifty precopula pairs of *G. pulex* were collected from the Crags Stream and Haseley Brook populations. Water samples were taken from each site and subsequently analysed for total and dissolved metals, alkalinity, ammonia, chloride, hardness, nitrate, nitrite, soluble reactive phosphorus, suspended solids and total organic carbon.

The *G. pulex* from each site were transported to the laboratory in plastic containers containing site water and immediately transferred to aerated 15-litre glass tanks containing reverse osmosis water and groundwater in a ratio of 12:1. This mixture mimicked the water quality found in the West Okement River. Alder leaf material inoculated with *Cladosporium* fungus was supplied as food *ad libitum* during a two week acclimation period.

Adult male *G. pulex* from each population were separated from the other captured animals one day before the exposure phase of the experiment by netting precopula pairs from each tank and prising the male from the female. Males were then placed in a separate tank containing water, but no food material.

The test apparatus consisted of 15-litre glass aquaria in which twelve 250 ml polythene tubes were suspended. The lower ends of these tubes were sealed with 1 mm nylon mesh.

The following dosing regime was applied to each gammarid population in duplicate aquaria:

Iron: 0, 1.0, 2.0 and 3.0 mg l^{-1} as Analar $FeSO_4.7H_20$
Manganese: 0, 0.1, 0.3 and 0.5 mg l^{-1} as Analar $MnSO_4.4H_20$

These concentrations were chosen to span the range measured in the previous field deployment (see Section 2.2).

Test solutions were changed daily during the six day exposure period after measuring pH, temperature, dissolved oxygen and conductivity. Composite samples from each test concentration were also taken daily for total and dissolved iron or manganese analysis using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES).

Weighed sets of alder leaf discs were prepared as described in Appendix A, placed randomly in the experimental tubes and allowed to rehydrate in the respective test solutions for four days. After this period of rehydration, the test animals were placed in the tubes in a randomized block design. Certain tubes were left without a test animal to provide an assessment of leaf weight gain or loss due to factors other than *G. pulex*. The animals were monitored daily, and dead animals recorded and removed from the experiment.

On day six of the exposure period, the remaining leaf disc sets were removed and treated as described in Appendix A to determine feeding rate. Five of the leaf disc sets from each treatment group were digested and analysed for iron or manganese content.

After the leaf disc sets were removed on day six, the test vessels containing the gammarids were taken from the test solutions and placed in 15-litre glass tanks containing clean test water, so that feeding rates could be determined during the depuration phase. This water was replaced after four hours so that contaminated faeces and precipitate were removed from the systems. The depuration phase leaf disc sets, which had been rehydrated in clean water for four days, were then added to the test vessels in the same way as the exposure sets. Test vessels were monitored each day for moulting and death and water quality measurements were taken at the beginning and end of the exposure period. At the end of the depuration phase all the surviving *G. pulex* were removed from the test vessels, killed and weighed as described in Appendix A.

Iron and manganese were measured in animals (5-10) at the time of capture (14 days before the start of the experiment) to assess background levels, at the beginning of the study, on days three and six of the uptake phase and on days three and six of the depuration phase. Gammarids taken for analysis of body burdens were rinsed twice in distilled water, killed by squeezing in individual pre-weighed foil boats and dried at 60 °C for four days. They were then weighed and acid digested before measuring metal levels using ICP-AES.

Conclusions

Mortality data showed that there was no significant difference in mortality rates associated with the two different *G.pulex* populations, but that the mortality rates increased significantly with increased concentrations of iron.

Table 5.1 The mortality of *G. pulex* during six days exposure to iron or manganese

Substance	Concentration (mg l^{-1})	Number in treatment	Number dead after 6 days		Percentage dead	
			Crags	Haseley	Crags	Haseley
Control	0	50	0	2	0	4
Iron	1.0	36	1	2	2.8	5.6
	2.0	36	1	2	2.8	5.6
	3.0	36	5	4	13.9	11.1
Manganese	0.1	36	0	1	0	2.8
	0.3	36	0	0	0	0
	0.5	36	0	1	0	2.8

The feeding rates of both populations declined with increasing concentrations of iron (Figure 5.3). There was a highly significant effect of iron concentration, and a significant population by concentration effect (ANOVA, $F=25.12$, $p<0.001$ and $F=2.68$, $p=0.047$ respectively). Significant reductions in feeding rates compared with control values were found at > 2.0 mg Fe l^{-1} for both populations (Tukey test, $q=4.77$, $p<0.025$ and $q=6.64$, $p<0.001$).

No significant differences were apparent between the two populations at the different concentrations. However, the shape of the response to iron did differ between the populations. The feeding rate of *G. pulex* from Crags Stream declined steadily as the concentration of iron increased, with no significant differences in feeding rate between consecutive concentrations. In contrast, *G. pulex* from Haseley Brook showed a significant stepped decrease in feeding rate between 1.0 and 2.0 mg Fe l^{-1} ($q=6.58$, $p<0.001$). No significant pollutant-induced differences in feeding rates were apparent when *G. pulex* exposed to iron were removed to uncontaminated water. Manganese had no significant effect on feeding rates at levels dosed in the experiment either during or after exposure (Figure 5.4). However, the feeding rates in control animals during the depuration phase were considerably lower than those measured in control gammarids during the exposure period.

In this study, the background iron levels measured in the two populations were lower than those recorded in the animals before the previous field deployment.

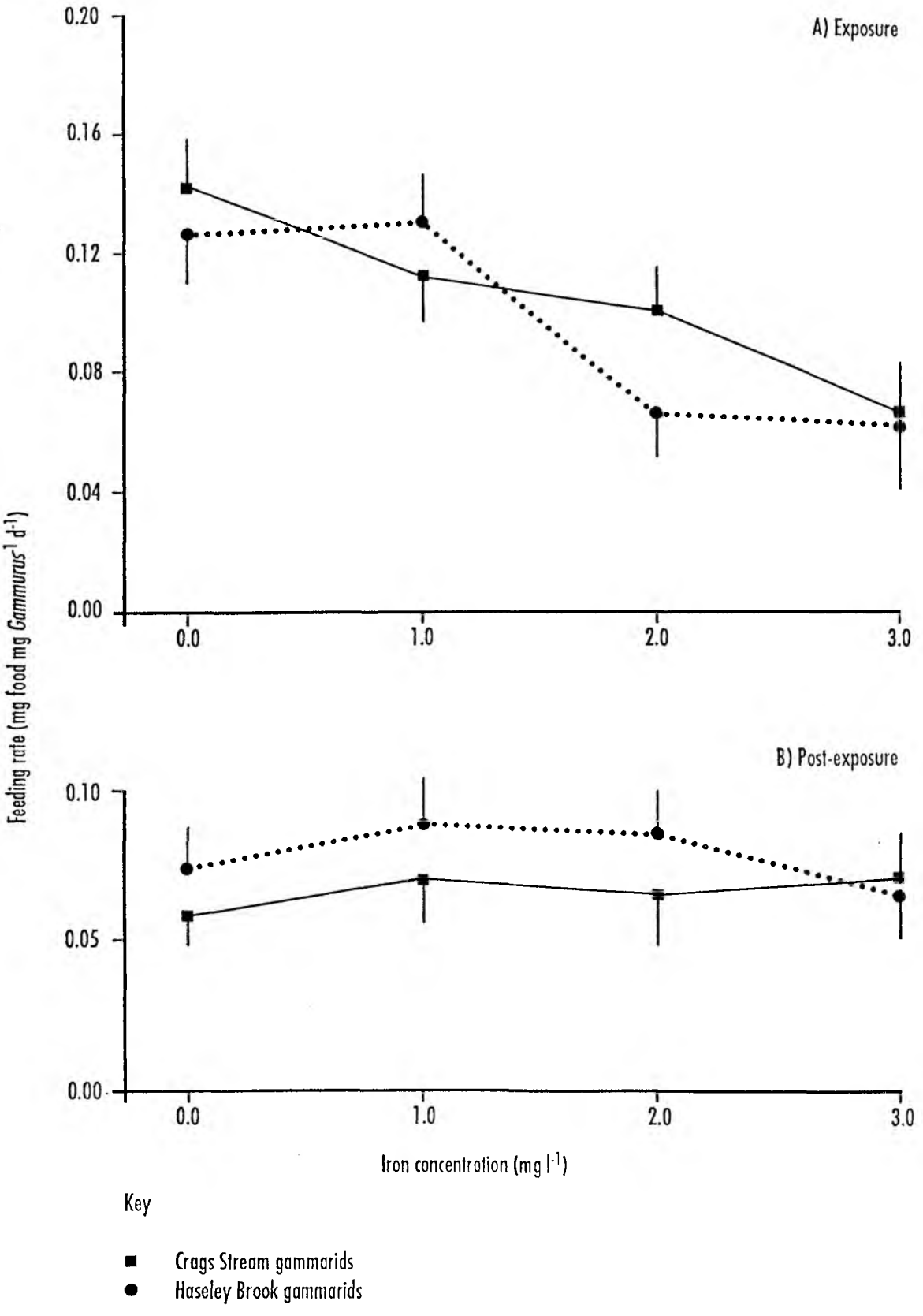

Figure 5.3 Effect of iron on the feeding rate ot two populations of *G. pulex* during A) exposure and B) post exposure. Values given are means ±95% confidence limits

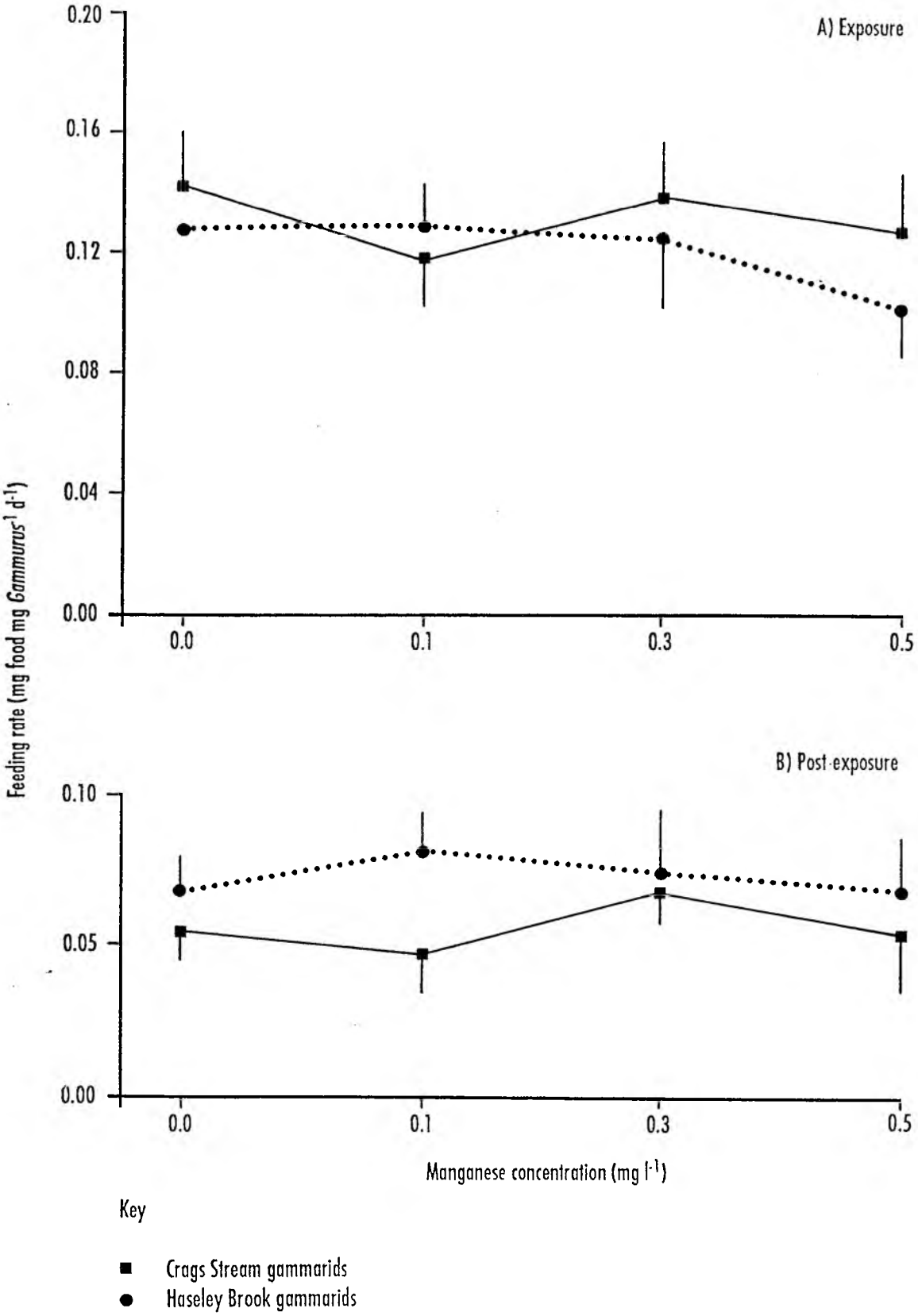

Figure 5.4 Effect of manganese on the feeding rate of two populations of *G. pulex* during A) exposure and B) post exposure. Values given are means ±95% confidence limits

Population	Tissue iron concentration (µg g^{-1}) 1989	1992
Crags Stream	215	88 ± 79
Heaseley Brook	1525	213 ± 263

Iron body burdens were highly variable and no significant differences could be detected between Crags Stream and Haseley Brook gammarids, unlike similar measurements taken during the previous study. In this study, manganese body burdens in *G. pulex* from Haseley Brook (24.8 ± 24.8 µg g^{-1}) were higher than those in Crags Stream animals (13.5 ± 4.5 µg g^{-1}).

A similar pattern of iron accumulation and depuration was apparent for both populations of *G. pulex*, with a general increase in iron burdens with increasing time and concentration. Accumulation of manganese by *G. pulex* was variable at the highest concentration (0.5 mg Mn l^{-1}), with no particular trend apparent for either population. The uptake of both iron and manganese by the food material supplied to the *G. pulex* during the experiment was considerable and increased linearly with water concentration. The only significant relationship between metal body burden and feeding rates was for iron in Crags Stream gammarids ($t=2.68$, $p=0.015$, $r=0.534$).

The similarity of the response of the two populations in the laboratory feeding rate experiment compared to the previous field deployment data (see Section 2.2), support the view that the difference in resistance between Crags Stream (sensitive) and Haseley Brook (resistant) populations of *G. pulex* in the earlier field experiments was due to physiological or behavioural acclimation rather than genetic adaptation. This acclimation may have been achieved by the induction of metallothionein-like metal-binding proteins (Stuhlbacher and Maltby 1992) or by the sequestration of metals into compartments of the hepatopancreas (Brown 1978, Dixon and Sprague 1981, Hopkin and Martin 1982a,b, Winner and Gauss 1986) although there is, as yet, no evidence that either of these processes occur with iron in *G. pulex*.

Summary

The source of test animals in this experiment did not greatly affect the final result, but there is evidence for a difference in the type of response of *G. pulex* from different environments. The implications for regulators using the technique are that they should establish that test animals caught from wild populations are obtained from streams with a low level of anthropogenic or natural contamination. This should be assessed by measuring both the water quality of the source stream and the body burdens of relevant contaminants in the test animals.

5.3.3 Effect of zinc on feeding rate and mortality in four populations of *G.pulex* from uncontaminated sites

Introduction

Since experiments with *G. pulex* normally use individuals collected from the wild, selection pressures or environmental conditions are likely to vary between test populations before capture to a greater extent than for cultured organisms such as the freshwater cladoceran *Daphnia magna* (Straus). This may increase the variability of results. To determine the extent of interpopulation differences in the response of *G. pulex* to pollution, the effects of zinc on the feeding rate and lethality of four populations of *G. pulex* from streams regarded as uncontaminated by the NRA were assessed.

Experimental procedure

Four hundred precopula pairs of *G. pulex* were collected during June 1991 by kick-sampling from:

1. River Darenth, Kent (NGR TQ 52106230);
2. Crags Stream, Derbyshire (NGR SK 49707450);
3. River Og, Wiltshire (NGR SU 19506960);
4. Pickering Beck, North Yorkshire (NGR SE 79708460).

The captured *G. pulex* were transported to WRc in plastic containers filled with site water and immediately transferred to flow-through 20-litre glass tanks containing groundwater. Alder leaf material conditioned with *Cladosporium* fungus was supplied as food *ad libitum*.

The feeding rate of groups of adult males from each population exposed to a range of zinc concentrations was measured according to the procedures in Appendix A. The test apparatus consisted of 15-litre glass aquaria in which twelve 250-ml polythene tubes were suspended. The lower ends of these tubes were sealed with 1-mm nylon mesh. Zinc was dosed to each population of *G. pulex* in duplicate aquaria at 0, 0.3, 0.4, 0.5, 0.6, 0.7, 1.3, 1.5, 2.0, 2.5 and 4.0 mg Zn l^{-1} as Analar $ZnSO_4.7H_2O$. The solutions were changed daily after measuring pH, temperature, dissolved oxygen and conductivity. Samples were also taken daily for total zinc analysis by ICP-AES.

The mortality data were used to calculate population-specific LC_{50} values after 24, 48, 72, 96, 120 and 144 hours of exposure (Finney 1971). These LC_{50} values were then used to construct toxicity curves for each population. The mean feeding rates of each population at each concentration during and after zinc exposure were calculated as described in Appendix A. All statistical analyses other than probit analysis were performed according to Zar (1984) using Genstat software (Genstat 5 Committee 1987).

Conclusions

Physico-chemical measurements taken from each of the population source streams confirmed that few differences were apparent in the water quality of the different streams. In the experimental studies concentrations of zinc in all of the test vessels remained within 20% of nominal concentrations.

The lethal toxicity of zinc to *G. pulex* differed depending upon the source of the animals (Figure 5.5). *G. pulex* from Crags Stream were considerably less sensitive to zinc at the beginning of the experiment (<96 h) than the other three populations. *G. pulex* from the River Darenth were of intermediate sensitivity, while those from Pickering Beck and the River Og showed a similar, relatively high sensitivity to zinc. These differences became less apparent over time, until by the end of the six-day experiment the LC_{50} values for all four populations were broadly similar at an asymptotic LC_{50} of near 0.75 mg Zn l^{-1}. Naylor *et al.* (1990) reported a 24 hr LC_{50} for *G. pulex* from Crags Stream of 7.57 mg Zn l^{-1} in water with a low hardness. The higher hardness used in the present experiment could explain the higher 24 hr LC_{50} value reported here. The feeding rates of all the *G. pulex* populations declined with increasing concentrations of zinc ($t > -6.25$, $p<0.001$, Figure 5.6). The intercepts of the feeding rate regression lines also differed significantly between populations ($F=34.42$, $p<0.001$), but the slopes of the responses were not statistically different ($F=0.46$, $p>0.05$). The estimated feeding rate EC_{50} for all four populations was, therefore, near 0.5 mg Zn l^{-1}. The value for the Crags Stream gammarids was consistent with the range of feeding rate EC_{50} values of 0.35-0.71 mg l^{-1} reported for this population between May 1990 and March 1991 (see Section 5.2).

Summary

Interpopulation differences were apparent in acute lethality among the *G. pulex* exposed to zinc in this study, particularly for animals from Crags Stream. However, sublethal effects of zinc on feeding rate measured after six days of exposure were similar for all four populations, suggesting that the chronic effects of zinc are not population dependent and that only short-term mechanisms of tolerance are available to this species.

5.4 General summary

The United States Environmental Protection Agency (EPA) has investigated variability within toxicity test methods and concluded that if test protocols are followed properly then the range of variability will be within acceptable limits (US EPA 1991), with the reproducibility of a test using the same organism and chemical lying within one order of magnitude (Roop and Hunsekar 1985). If populations from 'clean' sites, which are confirmed as not having recently experienced pollutant exposure, are used for field deployments and the standard operating procedures are followed by appropriately trained staff, variability should be reduced to an acceptable minimum which will allow real differences in water quality between sites to be detected.

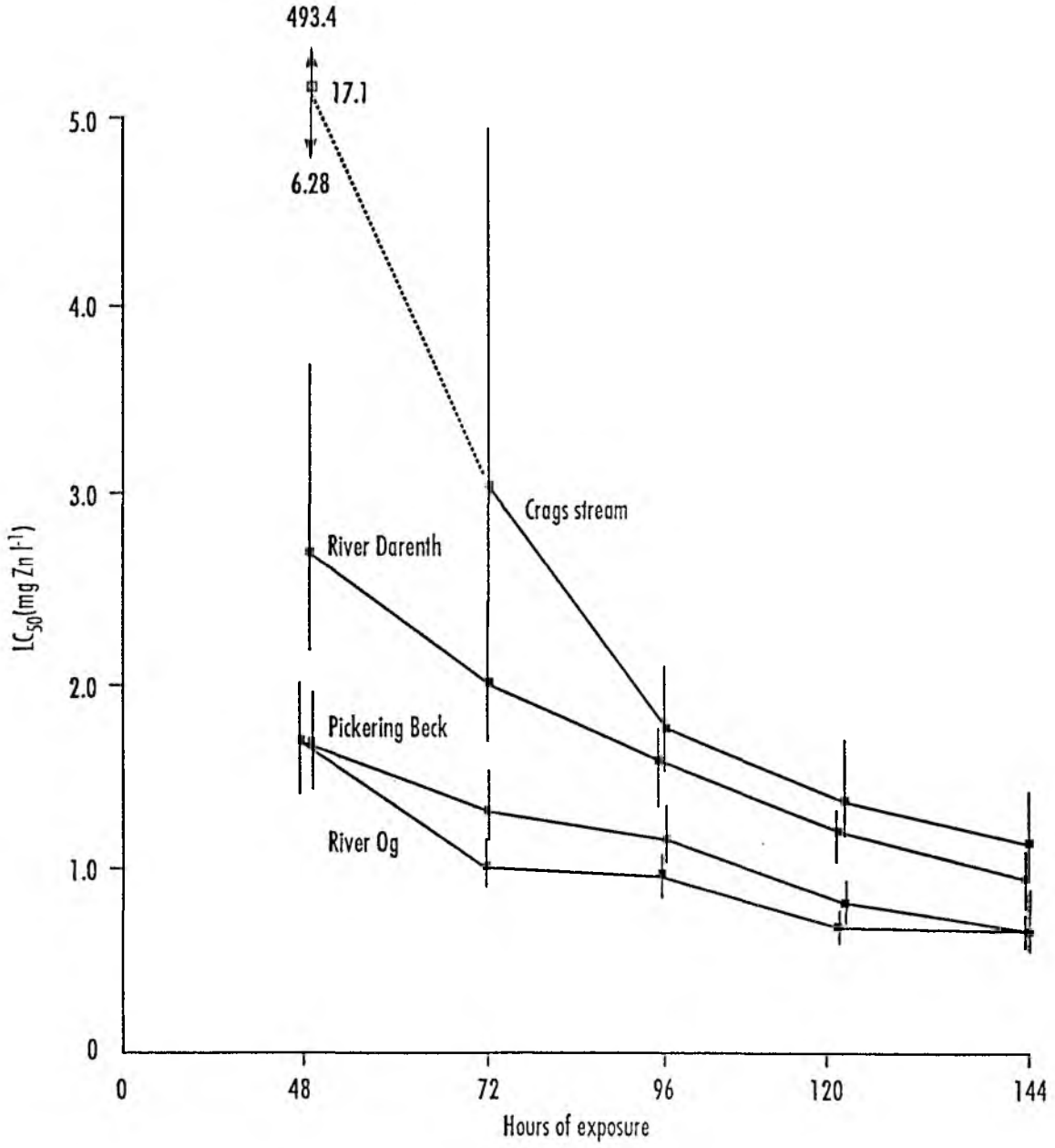

Figure 5.5 Toxicity curves for four populations of *G. pulex* exposed to zinc. Values are LC_{50}s and 95% confidence limits

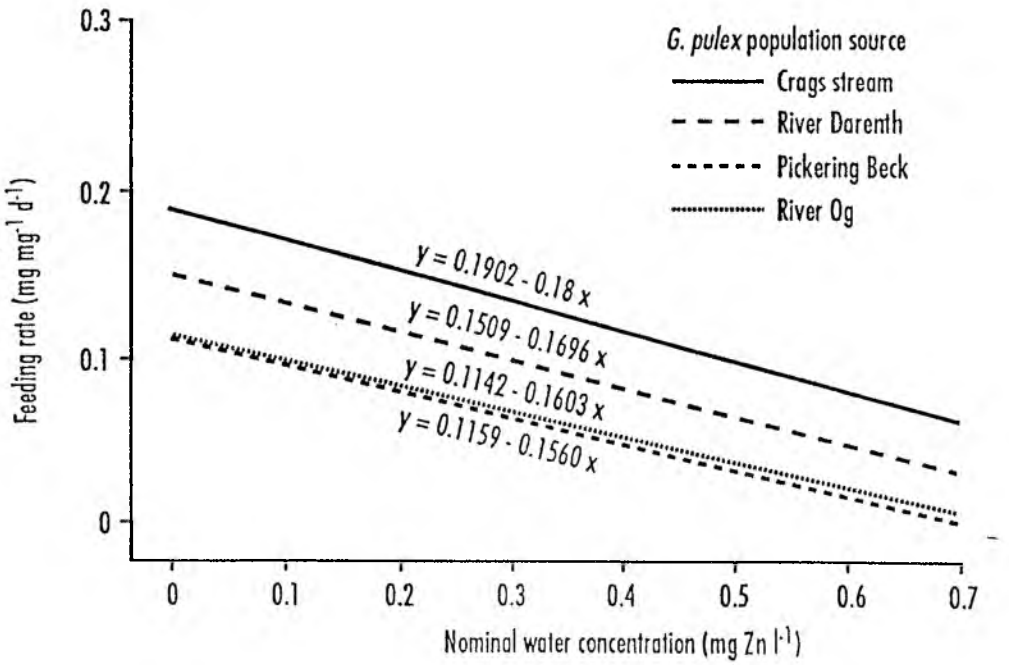

Figure 5.6 Feeding rates for four populations of *G. pulex* exposed to zinc.

6. RELATIONSHIPS BETWEEN PHYSIOLOGICAL INDICES (FEEDING RATE AND SCOPE FOR GROWTH) AND GROWTH AND REPRODUCTION IN *G. PULEX*

6.1 Relationship between Feeding Rate and Scope for Growth (SfG) and the parameters of Growth and Reproduction in laboratory studies with *G. pulex*

6.1.1 Introduction

Researchers at the University of Sheffield have investigated whether short-term physiological measures such as feeding rate and Scope for Growth can indicate longer term whole-organism effects. Studies with juveniles, males and brooding females compared the effects of ammonia on feeding rate and scope for growth with those on growth (juveniles) and reproduction (brooding females). These are described in greater detail in Johnson *et al.* (1991, 1993).

6.1.2 Experimental procedures

Juvenile and brooding female *G. pulex* were collected from Crags Stream. Juveniles were blotted dry and their fresh weight measured (mean fresh weight=4.69 mg, SE=0.12, n=414) before they were randomly allocated to experimental chambers. Brooding females were directly allocated to the chambers.

Chambers consisted of plastic tanks (25 × 8.5 × 9 cm) containing 0.75 litres of test solution which was continuously pumped through the chamber at a rate of 125 ml h^{-1}. Animals were provided with food (alder leaves colonized with *Cladosporium*) *ad libitum*. Test solutions were prepared by dissolving NH_4Cl in APW (see Table 6.1). The pH of test solutions was monitored daily and total ammonia concentrations were determined twice weekly using a Russell ammonia electrode (ISE 95-5129120). All experiments were conducted in a temperature controlled room (15 °C) which had a 12 h light : 12 h dark photoperiod.

In the juvenile growth experiment, juveniles were reweighed at two-weekly intervals. The specific growth rate (G_s, % wet weight d^{-1}) was calculated as:

$$G_s = 100 \quad \frac{(\ln W_t - \ln W_o)}{t}$$

where W_o is the fresh weight (mg) of animals at time 0 and W_t their corresponding weight at time t (=24-28 days).

Table 6.1 Experimental details and results from laboratory studies assessing the effects of un-ionized ammonia on feeding rate, scope for growth, growth and reproduction in *G. pulex*

Endpoint	Life stage	Ammonia concentration range (mg l^{-1})	Duration (days)	EC_{50} (mg l^{-1})	HNEC (mg l^{-1})	LOEC (mg l^{-1})
Feeding rate/ SfG	Juveniles	0.002, 0.07, 0.109, 0.167, 0.185	6	0.169	0.109	0.167
	Adult males	0.003, 0.093, 0.155, 0.212, 0.289	"	0.186	0.093	0.155
	Brooding females	0.002, 0.093, 0.134, 0.143, 0.188	"	0.155	0.143	0.188
Growth	Juveniles	0.002, 0.059, 0.095	24-28	0.060	0.059	0.095
Reproduction: Offspring size and number	Females	0.002, 0.051, 0.091	-	-	0.091	-
Aborted broods	"	0.002, 0.094, 0.11, 0.14, 0.171, 0.188	-	0.103	-	-

In the experiments with brooding females, the animals were removed from the experimental chambers just prior to brood release and placed into individual pots containing APW. Once the brood had been released, the number and size (length, mm) of offspring were recorded. The female was then either killed and her dry weight determined (='current brood') or the animal was remated and allowed to incubate a subsequent brood (='subsequent brood') under control conditions. The number and size of offspring released from these broods were also determined. The effect of ammonia on number of broods aborted was also investigated for a range of concentrations.

6.1.3 Conclusions

Table 6.1 summarizes the experimental details and the main results from these studies. For all the test groups in the Scope for Growth studies (see Section 2.1), the proportion of the energy consumed and absorbed across the gut wall decreased and the percentage metabolized increased as the ammonia concentration increased. This resulted in a reduction in the proportion of the consumed energy allocated to production until ultimately all the energy absorbed was required for maintenance and no production (as growth or reproduction) was evident.

In ammonia-exposed gammarids it was evident from feeding rate and Scope for Growth studies (see Section 2.1) that concentrations greater than or equal to 0.165 mg NH_3 l^{-1} would result in zero growth or reproduction (Table 6.1).

The studies on the effects of un-ionized ammonia on juvenile growth and reproduction identified the following relationships:

Specific growth rate =	- (13.1 x Ammonia concentration) + 1.67	(r^2=0.96)
Broods aborted (%) =	(521 × Ammonia concentration) - 3.61	(r^2=0.97)

Subsequent studies showed zero growth in juveniles at ammonia concentrations greater than 0.13 mg l^{-1} and 100% abortion of broods at 0.188 mg l^{-1}. Therefore, toxicant-induced changes in feeding rate and Scope for Growth in juveniles and brooding females were found to be indicative of longer term changes in growth and reproduction respectively.

6.2 The Relationship between Feeding Rate and Growth in field deployed *G. pulex*

6.2.1 Introduction

Feeding rate is the most important and pollution-sensitive parameter in the calculation of *G. pulex* SfG (see Section 2.2), and both feeding rate and SfG are positively correlated with the growth of juvenile *G. pulex* in the laboratory (see Section 6.1). The correlation of physiological condition and growth is more difficult for adult *G. pulex* because growth during this life-history stage is not exponential, hence changes are harder to detect. However, an examination of the relationship between feeding rate and growth was

attempted in this study, with the aim of showing that feeding rate measured in the field can be a useful short-term indicator of longer-term changes in growth rate.

The River West Okement in Devon receives waste from Meldon Quarry containing a mixture of heavy metals, and a previous study (see Section 2.2) at stations above and below the two quarry discharge points showed lower feeding rates and SfG at the downstream sites. These results suggested that this stretch of the West Okement was a good site for further evaluation of the relationship between *G. pulex* feeding rate and growth.

6.2.2 Deployment procedure

G. pulex in precopula pairs were obtained from the River Darenth, Kent by kick-sampling, and transported to the laboratory in a sealed plastic bucket containing site water. They were then transferred to a 20-litre flow-through tank containing groundwater, and fed *Cladosporium*-conditioned leaf material. Male *G. pulex* in precopula pairs were gently separated from their female partners two days before field deployment and placed in a separate stock tank without food. These males were removed from the stock tank, blotted twice on laboratory tissue and weighed before addition to individual, numbered field cages. Alder leaf food discs of known weight were prepared as described in Appendix B and added to the field cages which were then sealed and placed in holding baskets according to a random number sequence. Each holding basket contained at least one field cage without *G. pulex* for use as a control for leaf disc weight changes due to sedimentation, leaching, or any other factor unassociated with *G. pulex* feeding. The holding baskets were then sealed with a transparent polythene top and transported to the field in trays containing groundwater.

One hundred and fifty *G. pulex* contained in nine holding baskets were attached to the river bed at the upstream reference station used in 1989 (Section 2.1). Another one hundred and fifty *G. pulex* were placed in a similar fashion at a station 100 m below one of the quarry discharges. Temperature, pH, conductivity and dissolved oxygen were measured *in situ* at both stations and water samples were taken for analysis at intervals during the study.

Fifty *G. pulex* cages with their associated leaf weight controls were recovered from the upstream and downstream stations after 6, 14 or 28 days field exposure and returned to the laboratory in trays containing site water. The field cages were opened and the number of surviving *G. pulex* recorded before transferral to vessels filled with groundwater, where they were left for 24 hours to allow defaecation to occur. They were then blotted twice on laboratory tissue, weighed, killed by gently squeezing them in foil boats, dried at 60 °C for four days and reweighed. Any food material remaining in the cages was removed, brushed in distilled water to remove any attached sediment, dried for four days at 60 °C and weighed.

The feeding rate of deployed animals was calculated and differences in the feeding rate of *G. pulex* deployed upstream and downstream were analysed using a one-way analysis of variance (ANOVA) after log transformation of the data. The percentage change in the

fresh weight of the *G. pulex* after field deployment was also calculated and compared between upstream and downstream stations using ANOVA after an arcsine transformation (Zar 1984, Genstat 5 Committee 1987).

6.2.3 Conclusions

A storm during the first week of deployment led to the loss of several baskets. As a result of this event it was decided to reduce the number of *G. pulex* retrieved on day 14, rather than spread the loss evenly across the three retrieval dates. The number of *G. pulex* mortalities, and the number missing from their cage (Table 6.2) did not differ significantly between the upstream and downstream stations, but did rise significantly at both stations over the course of the experiment (GLM, increment in deviance 42.2, $p<0.001$). *G. pulex* decompose rapidly after death and it is likely that the majority of missing animals died, rather than escaped.

Table 6.2 Number (and percentage) of *G. pulex* dead or missing during deployment in the West Okement

Day	Mortality		Missing		Total dead or missing	
	U/S	D/S	U/S	D/S	U/S	D/S
6	4 (8%)	0	2 (4%)	2 (4%)	6 (12%)	2 (4%)
14	2 (4%)	0	4 (8%)	5 (10%)	6 (12%)	5 (10%)
28	9 (18%)	2 (4%)	14 (28%)	22 (44%)	23 (46%)	24 (48%)

There were no significant differences in the feeding rates of *G. pulex* deployed above and below the quarry discharge (Table 6.3). The *G. pulex* tended to lose weight at both stations, with no significant differences in the percentage weight loss between stations (Table 6.4) and a highly variable but positive relationship was found between feeding rate and 'growth' (or reduced loss of weight) (Table 6.5).

Table 6.3 Feeding rates of *G. pulex* (mg mg^{-1} day^{-1}) deployed above and below a quarry discharge for 6, 14 and 28 days. Values are mean, standard deviation (SD), sample size (n) and coefficient of variation (CV)

Day	Feeding rates of *G. pulex* (mg mg^{-1} d^{-1})									
	Upstream (US)				Downstream (DS)				Anova US vs DS	
	Mean	STD	n	CV(%)	Mean	STD	n	CV(%)	F	p
6	0.094	0.081	43	86	0.088	0.122	44	139	0.07	0.786
14	0.071	0.055	22	78	0.098	0.040	21	41	3.23	0.080
28	0.083	0.046	26	55	0.087	0.041	39	47	0.19	0.665

Table 6.4 Percentage change in the fresh weight of *G. pulex* deployed above and below a quarry discharge for 6, 14 and 28 days. Values are mean, standard deviation (SD) and coefficient of variation (CV). F and p values are for arcsine-transformed data

Day	Percentage change in fresh weight							
	Upstream (US)			Downstream(DS)			Anova US vs DS	
	Mean	SD	CV(%)	Mean	SD	CV(%)	F	p
6	-5.89	5.93	101	-4.88	4.49	92	0.85	0.358
14	-5.49	3.84	70	-5.82	5.46	94	0.06	0.812
28	-7.50	4.33	58	-6.51	5.06	78	0.67	0.417

Table 6.5 Regression equations of growth (y) on feeding rate (x) for *G. pulex* returned from both upstream and downstream stations after 6, 14 or 28 days in the field

Day	Equation	r	t	df	p
6	y = -6.263 + 9.74x	0.158	1.80	85	<0.05
14	y = -9.500 + 45.70x	0.470	3.59	41	<0.0005
28	y = -8.830 + 22.50x	0.158	1.63	63	<0.1
Total	y = -7.218 + 14.73x	0.212	3.18	193	<0.001

The similarity in the feeding rates measured at the upstream and downstream stations in this study suggests that the level of stress experienced by the *G. pulex* at both stations was similar. This contrasts with the results from this site in 1989 and reported in Section 2.2. The discharge into the West Okement from Meldon Quarry contained high levels of metals in 1989, but these levels had declined by the time of this experiment in 1991 due to a number of toxicity reduction measures taken by the quarry owners (B Milford, NRA South West Region, pers. comm.). The lack of toxicity to *G. pulex* observed in this experiment suggests that these measures were effective.

Feeding rates remained constant over the course of the experiment despite the storm and an increase in temperature during the later stages of the exercise. In an earlier study, both flow rate and temperature affected *G. pulex* feeding rates, with higher flows or lower temperatures associated with reduced feeding (Johnson *et al.* 1993). In this experiment the feeding rate technique provided highly replicable results, although the storm may have induced the greater variation in feeding up to day 6.

The increase in mortality over time at both stations was probably due to the low water hardness and pH of the West Okement. These are not ideal conditions for *G. pulex* and no examples of this species were found in benthic samples taken in 1989 (see Section 2.2). Adult *G. pulex* moult frequently during the summer months, losing a considerable proportion of their calcium content during this process (Wright 1980). The low levels of calcium in West Okement water may have been insufficient to replenish that lost during moulting, contributing to the high mortality observed at the end of the study. The loss of weight observed during the experiment may also have been due to low environmental calcium levels. Alternatively, the handling stress induced by initial drying and weighing may have contributed to weight loss. However, although the fresh weight data were highly variable, they were still significantly related to feeding rates. This suggests that the measurement of feeding rate over a few days is a useful indicator of longer-term effects on growth, an important ecological parameter. In the study, those *G. pulex* that fed most tended to lose least weight.

6.3 Summary

The usefulness of an *in situ* bioassay is clearly increased if the responses can be interpreted in terms of effects on ecologically relevant parameters such as growth and reproduction. Laboratory studies and field deployments have indicated that the short-term measurement of feeding rate is indicative of longer-term effects on both juvenile and adult male growth and reproduction in females (as broods aborted).

7. CONCLUSIONS ON THE SENSITIVITY, DISCRIMINATION, VARIABILITY AND ECOLOGICAL RELEVANCE OF THE *IN SITU G. PULEX* FEEDING RATE BIOASSAY

7.1 Sensitivity and discrimination

The responses of *G. pulex* from the initial field deployments described in Section 2 indicated that water quality has a significant effect upon feeding rate and SfG. A reduction in SfG was apparent in only two of the deployments, Colne Water and the West Okement River. A reduction in respiration rate could be detected only in the West Okement deployment. 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 uphold or reject the null hypothesis that there was no difference in the response of treatment groups (Hayes 1987).

However, it is difficult to see how the power of the analysis could be increased without a very large increase in sample size. This would involve a concomitant increase in the number of animals used to measure respiration, which would be an expensive, labour-intensive and technically difficult operation. Furthermore respiration rate makes a small contribution to the overall calculation of a single *G. pulex* energy budget, compared to the correspondingly large contribution made by the feeding rate measurement. Consequently feeding rate represents the most sensitive and cost-effective measure of a gammarid's energetic response to stress.

Effects observed in the field tended not to persist when test animals were placed in uncontaminated water, suggesting that changes in feeding rate are behaviourally based, or that detoxification mechanisms are rapid in *G. pulex*, depending on the nature of the pollutant.

Although the reduction in feeding rate of control animals with decreasing water temperature may tend to reduce the discrimination of the assay in winter, the Ministry of Agriculture, Fisheries and Food (MAFF) have been able to detect pollutant impact at survey sites throughout the year (Matthiessen, pers. comm.)

7.2 Sources of variability

The simple and inexpensive deployment equipment has proved to be robust under various conditions of field exposure. Under stable conditions the within-site coefficient of variation is normally less than 50%. Minor changes in the equipment used appeared to reduce the level of variability to a consistent level, so that during the study reported in Section 4 a CV near 40% was achieved at all 11 stations in the River Aire. This allows the detection of differences between treatment groups of 25-30% or more using analysis of variance (Conquest 1983). Such differences appear to be meaningful since decreases in longer-term growth and fecundity can be measured in *G. pulex* whose feeding rate has been reduced by about 25% (see Section 6.1).

Variability of the feeding rate assay between laboratories, operators and populations has been identified in these studies. In all four field trials described in Section 2, differences in the measurement of feeding rate between the two laboratories occurred either as a main effect (that is 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 these deployments 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. The results from the study in the River Aire reported in Section 4 suggest that the way the test animals are handled may be an important factor contributing to interlaboratory differences. This hypothesis would bear closer examination with the ultimate aim of refining the standard operating procedure and testing it in an interlaboratory exercise involving several organisations.

The investigations conducted into interpopulation differences in response have shown that prior exposure to metal contamination in *G. pulex* seems to confer resistance to subsequent heavy metal exposure (Klerks and Weiss 1987). The evidence suggests that this resistance is due to acclimation rather than genetic adaptation. *G. pulex* from Haseley Brook appeared to have lost their resistance over a period of eighteen months during which levels of heavy metals in the brook declined. Metallothionein-like proteins have been found in *G. pulex* (Stuhlbacher and Maltby 1992) and the induction of these may have conferred initial resistance upon the Haseley Brook population. Other non-genetic factors may also have been responsible for interpopulation differences in the response to toxic stress.

The results obtained from the exposure of four 'clean' populations of *G. pulex* to zinc (see Section 5.3.3) suggest that some toxicological end points may be more susceptible to interpopulation differences than others. Acute mortality differed considerably between the parental populations, but mortality and effects on feeding rate over six days were similar. If this is a general trend, and not just specific to zinc or heavy metal contamination, the implication would be that toxicity studies lasting for two days or less should be replaced, where possible, with longer-term experiments. This hypothesis should be tested not only with *G. pulex,* but also with other toxicity test species.

Although the causes of interpopulation differences are unclear, these results are further confirmation of the intrinsic differences that exist between populations in their response to the environment and show that care must be exercised in the selection of appropriate populations of toxicity test or bioassay organisms.

7.3 Ecological relevance

The ecological relevance of the response measured in the *G. pulex* feeding rate bioassay depends on whether a reduction in feeding rate or SfG automatically leads to a lower population level, or the elimination of *G. pulex* entirely. It is difficult to determine whether effects at lower levels of biological organisation will impinge upon higher levels (Luoma and Carter 1991). 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 *G. pulex* 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 *G. pulex* are subjected to stress in the laboratory they produce fewer, smaller offspring (Maltby and Naylor 1990). If this was 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 *G. pulex* 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 *G. pulex* comprising males, females and juveniles.

7.4 Areas of uncertainty

Although the research carried out to date has resulted in a robust and reliable assay, there remain areas of uncertainty where further work could enhance the usefulness of the technique. These include:

1. Information on the sensitivity of *G. pulex* feeding rate to a wider range of chemical classes, particularly organic contaminants. These data would be of considerable use in interpreting the effects of field deployments and would complement that available for heavy metals and ammonia.

2. An assessment of whether *G. pulex* feeding rate provides a reasonable assessment of river quality. The evidence accumulated throughout this work shows that *G. pulex* feeding rate is a relative and not an absolute measurement of stress. However, it would be useful to determine how far feeding rate measurements reflect the established quality of stretches of river by deploying the technique at several sites in each of the NRA river classes.

8. THE PRACTICAL USE OF THE *G. PULEX* FEEDING RATE BIOASSAY

Although there are areas of the *G. pulex* feeding rate bioassay that require further work the development of the assay has reached the stage where it can be used routinely by regulators. This technique, like other bioassays, is a research tool that can provide reliable and statistically robust answers to specific questions. As such, its use should be limited to those situations in which standard chemical and macroinvertebrate survey techniques are inadequate for identifying either the source or extent of river pollution.

G. pulex populations are often excluded, or diminished in abundance in river systems exposed to heavy metal or pesticide pollution. Feeding rate assays would be useful for tracing the precise source or sources of the pollutants causing these population effects. Similarly, *in situ* assays such as *G. pulex* feeding rate may be useful where macroinvertebrate richness is poor both above and below a discharge of interest. Bioassays may be used to detect the additional effects of the discharge on a sensitive species such as *G. pulex,* where the absence of sensitive species in the macroinvertebrate fauna does not allow such a comparison to be made.

In situ bioassays will also be of use in other circumstances when macroinvertebrate sampling is logistically difficult, or provides only unclear evidence of effects. The patchy distribution of many invertebrate species in space and time makes quantitative sampling difficult, which is one reason for the popularity of qualitative, or semi-quantitative, assessment procedures such as the BMWP system. Such survey techniques can provide a highly cost-effective indication of river quality problems, but their noise-to-signal ratio may obscure subtle or intermittent problems. Under these circumstances, replicated bioassays with sensitive species like *G. pulex* and a sensitive sublethal endpoint like feeding rate may be more powerful tools for confidently ascribing significance to apparent differences in water quality.

The methodological problem of pseudoreplication (Hurlbert 1984) remains, however, and can probably only be resolved by pragmatic means. The investigations into an alternative method of deploying bioassays and analysing the results (Stewart-Oaten *et al.* 1986) was not particularly successful, partly because the study site conditions were poor. However, sufficient information was obtained to suggest that the best approach to bioassay assessments is one in which there is an iterative cycle between field and laboratory. Field bioassays are useful because relevant organisms are exposed to real environmental concentrations of contaminants under realistic environmental conditions. Laboratory bioassays lack this realism but can be used to confirm cause and effect relationships in randomised, replicated and controlled experiments. This necessarily involves an element of induction (Popper 1980), but that is true of most risk or hazard assessments.

The use of tissue or body burdens in a tripartite approach involving the measurement of environmental concentrations and toxic responses can provide a clearer picture of exposure conditions, providing physiological factors, such as moulting, are taken into account. The use of body burdens, in addition to external concentrations of contaminant, has been recommended by several authors (Connolly 1985, Lynch *et al.* 1988, Johnson and Crane 1991,Shutes *et al.* 1991). This is because it allows the integration of all sources

of contaminant uptake and provides a firmer basis for either the prediction or interpretation of effects in natural systems.

G. pulex may be a particularly useful species for field biomonitoring programmes because of its widespread distribution and functionally important niche (Zauke 1982). The deployment of *G. pulex* in a tripartite approach would also have the advantage of providing an opportunity for assessing contaminant uptake via both food and water. This may be important when attempting to predict invertebrate environmental exposure patterns, especially for some metal contaminants (Timmermans *et al.* 1992).

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APPENDIX A PROTOCOL FOR *G. PULEX* FEEDING RATE AND SCOPE FOR GROWTH TESTS IN THE LABORATORY

A1 INTRODUCTION

This Standard Operating Procedure (SOP) describes how to measure the feeding rate of *G. pulex*, a freshwater amphipod crustacean, in the laboratory. By measuring respiration, the Scope for Growth (SfG) of *G. pulex* can also be determined. This is the energy that is available for growth and reproduction after an organism's basic metabolic needs have been met. Population-level changes may occur if individuals suffer a reduction in feeding rate or SfG due to external stress. Testing differences in the feeding rate or SfG of *G. pulex* exposed to different concentrations of contaminant is thus a useful, sublethal measure of the potential environmental impact of that contaminant.

A2 SAFETY

All test and reference substances or environmental samples (including sediments and effluents) must have a Control of Substances Hazardous to Health (COSHH) assessment sheet prepared before any work can commence.

Experimental procedures may require substances to be handled in ways which deviate considerably from the manufacturer's intended use, and for which the hazard was assessed. If there is any doubt about the safe handling of the material, in relation to the procedures to be employed, then the advice of a chemical safety officer should be sought. The test apparatus and procedures employed should be designed taking into account the hazard involved. For example, it may be necessary to carry out the procedure under a fume hood, or to use completely enclosed test vessels to minimise operator contact with the test material.

Personal safety precautions to be taken will include wearing adequate protective clothing. Lab coats and gloves are a minimum requirement. Eye protection, and in special cases dust masks, may be required. Any person involved in a study who considers that inadequate information or precautions have been provided or taken is at liberty to refuse to carry out the procedure. Experimenters must also ensure that the correct procedure in the event of spillage of test material is known. Any spillage must be dealt with immediately.

A3 STUDY OBJECTIVES

1. To measure the feeding rate of *G. pulex* exposed to three concentrations of a test substance plus a control;
2. To measure the faecal production of the animals in (1) above;
3. To measure the respiration rate of *G. pulex* from (1) above;
4. To calculate the SfG of *G. pulex* in the four treatment groups.

A4 MATERIALS

A4.1 Feeding rate of *G. pulex*

(a) Two hundred tri-pour polypropylene beakers (250 ml), such as those supplied by Phillip Harris Scientific Ltd;

(b) One hundred pieces of 1 mm diameter nylon weld mesh measuring 15 cm × 15 cm such as that supplied by Lockertex Ltd;

(c) Seventeen litres of each concentration of the test substance, the concentrations of which will be specified in a Study Plan;

(d) Four hundred 'conditioned' alder (*Alnus glutinosa*) leaf discs;

(e) Eighty male *G. pulex* measuring not less than 7 mm in length, normally collected from the field. An acclimatization period of at least one week must be observed immediately before the test. During this acclimatization period the test animals must be kept under similar temperature, lighting and feeding conditions to those during the period of experimentation;

(f) One hundred and eighty aluminium foil boats with a diameter of 1 cm, contained in 8 × 25-chamber plastic containers such as those supplied by Sterilin Ltd;

(g) One top-loading balance accurate to 0.01 mg, such as the Sartorius 1712 MP 8;

(h) One oven capable of maintaining a constant temperature of 60 °C ± 5 °C;

(i) One set of random number tables.

A4.2 Assimilation rate of *G. pulex*

(a) One hundred polycarbonate membrane filters with a diameter of 49 mm and a pore size of 1 µm such as those supplied by Nuclepore Corp;

(b) One Quickfit filtering flask (1 litre) with an electric vacuum pump attached.

A4.3 Respiration rate of *G. pulex*

(a) Eighty male *G. pulex* as described above;

(b) Four 20-litre nalgene aspirators such as those supplied by Jencons (Scientific) Ltd;

(c) Eight 60 cm^3 plastic syringe barrels capped with silicone rubber bungs;

(d) Eight 50 cm lengths of 3 mm diameter silicone rubber tubing;

(e) Eight 20-litre glass waterbaths;

(f) Sixty-four Terumo syringe needles, 50 mm in length with a bore of 1.1 mm, from which the plastic luer fitting has been removed with a hacksaw;

(g) Thirty-two Terumo syringe needles, 50 mm in length with a bore diameter of 1.1 mm and a 19G × 2" plastic luer fitting;

(h) Thirty-two 2-ml Segma glass syringe barrels capped with silicone rubber bungs. The syringe barrels are arranged in sequentially numbered holders constructed from 2 cm lengths of rubber tubing affixed to a plastic rack;

(i) Thirty-two 7-cm lengths of 0.38 mm diameter silicone tubing such as that supplied by Watson-Marlow Ltd;

(j) Two Strathkelvin oxygen meters model 78 lb and electrodes;

(k) Two Radiometer Copenhagen electrode water jackets, each attached to a retort stand;

(l) Four 250 µl Hamilton glass microsyringes;

(m) Two peristaltic pumps with 16-channel pumpheads such as those supplied by Watson Marlow model 502S;

(n) A timer.

A5 METHODS (ESTIMATED TIME TAKEN TO PERFORM TASK)

A5.2 Preparation of feeding material

(a) Collect alder leaves in autumn between abscission and shedding and store in cardboard boxes at room temperature. Under these conditions the leaves will become dry and brittle; **(0.1 days)**

(b) When required, rehydrate the leaves in uncontaminated water contained in a large, clean vessel for one hour; **(0.1 days)**

(c) Using a 17 mm diameter cork borer cut discs from between the main veins of the rehydrated leaves. Cut the discs over a cardboard base and use a clean glass rod to remove them from the borer; **(0.2 days)**

(d) Place sets of 120 discs in 500-ml Pyrex conical flasks. Add 400 ml of uncontaminated water to each flask and then seal with cotton wool, tin foil and autoclave tape and autoclave for 15 minutes at 1.1 kg cm^{-2}; **(0.2 days)**

(e) *Cladosporium* fungus is cultured from an initial sample provided by Dr Lorraine Maltby from Sheffield University. The culture method is as follows:

- Cut a 17 mm disc from a fungal culture using a 17 mm cork borer;
- Plate a malt extract broth plate (Oxoid CM59) with four drops of 10% lactic acid to prevent bacterial contamination;
- Place the fungal disc on the broth plate using aseptic technique and incubate at room temperature in the light;
- Inspect the plate after two weeks and discard if contaminated by bacteria or fungi other than *Cladosporium*. Store successful cultures at 4 °C ± 2 °C.

(f) When cool, inoculate the autoclaved leaf discs with *Cladosporium* fungus as follows:

- Use a cork borer to cut six 17 mm diameter fungal discs from the broth plate on which they have been cultured. Add the fungal discs to the autoclaved leaf discs in the 500 ml flask;
- Re-seal the flask and leave at room temperature for 10 days. Shake the contents of the flask daily;
- After 10 days tip the contents of the flask into a sieve with a mesh size of 1-5 mm. Tip the contents of the sieve into a large vessel containing uncontaminated water;
- Prepare the conditioned leaf discs for the test by removing them from the vessel, placing them in individual Sterilin tray compartments in sets of four and drying them in an oven for four days at 60 °C ± 5 °C. **(0.2 days)**

A5.3 Measuring the feeding and assimilation rates of *G. pulex* exposed to three concentrations of a test substance

Construct 100 *G. pulex* feeding chambers as shown in Figure A1; **(0.2 days)**

(a)Number 100 adhesive labels from 1 to 100 and stick them in 10 rows and 10 columns to a flat, uniformly lit surface in a room at a constant temperature of 15 °C ± 2 °C. Use a random number table to assign 25 chambers to each treatment group (three concentrations and a control). The first 20 chambers in each treatment group will contain animals. The remaining five chambers will contain only leaf discs and will act as leaf disc weight controls; **(0.1 days)**

(b)Weigh each set of four leaf discs to an accuracy of 0.01 mg and record the weight on the relevant data sheet. Rehydrate each set of four leaves in the appropriate test solution for four days; **(0.3 days)**

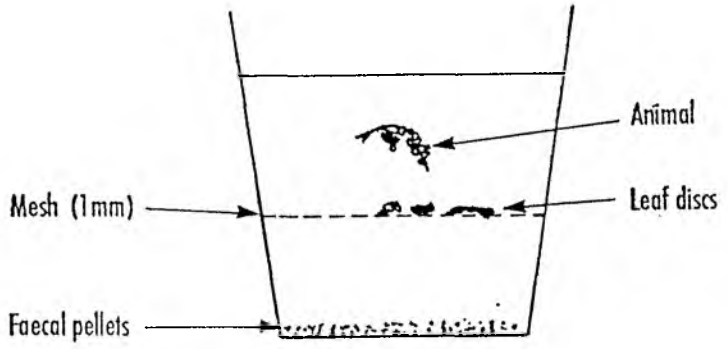

Figure A1 Laboratory test apparatus for *G. pulex* feeding rate

(d) On day 1 of the experiment measure 250 ml of the appropriate solution into the feeding chambers and add one set of four conditioned leaf discs to each chamber; **(0.3 days)**

(e) After placing the leaf discs in the chambers add one male *G. pulex* to each chamber, except those designated as leaf weight controls. Monitor the chambers daily and record the date of any moult or mortality on the relevant data sheet; **(0.3 days)**

(f) After 6 days use a random number table to select 16 surviving animals that have not moulted from each treatment group to measure their respiration rate, if a full Scope for Growth is to be calculated. Kill excess animals by squeezing in a pre-dried, pre-weighed foil boat. Place them in an oven for four days at 60°C ± 5°C. Weigh to a precision of 0.01 mg, taking care that rehydration does not occur. Record the dry weight of the *G. pulex* on the relevant data sheet; **(0.3 days)**

(g) Remove leaf remains from the upper section of the feeding chamber and place in numbered foil dishes. **(0.2 days)**

(h) Filter the contents of the lower section of the feeding chamber through pre-weighed polycarbonate membrane filters using the Quickfit filter apparatus and vacuum pump if absorption rates are to be calculated; **(0.5 days)**

(i) After filtration, a visual inspection will identify any leaf fragments on the filter. Remove these leaf fragments from the filter using forceps and place in the appropriate foil boat with the leaf fragments from the upper section of the feeding chamber; **(0.1 days)**

(j) After removing the leaf fragments, only *G. pulex* faeces should be present on the cellulose filter. Fold the filter over to contain the faeces and place in a numbered chamber in a Sterilin plastic tray; **(0.1 days)**

(k) Place the filters containing the faeces and the foil boats containing the leaf fragments in an oven and dry for four days at 60°C ± 5°C. Weigh to a precision of 0.01 mg taking care not to allow rehydration to occur. **(0.3 days)**

A5.4 Measuring the respiration rate of *G.pulex* exposed to three concentrations of a test substance

(a) Fill the aspirators with the appropriate solution and the water baths with deionized water at least two days before the start of respirometry to ensure that they have reached a stable temperature; **(0.3 days)**

(b) Assemble the respirometry equipment as illustrated in Figure A2. The distribution of each treatment must be fully randomized between the respiration chambers; **(0.5 days)**

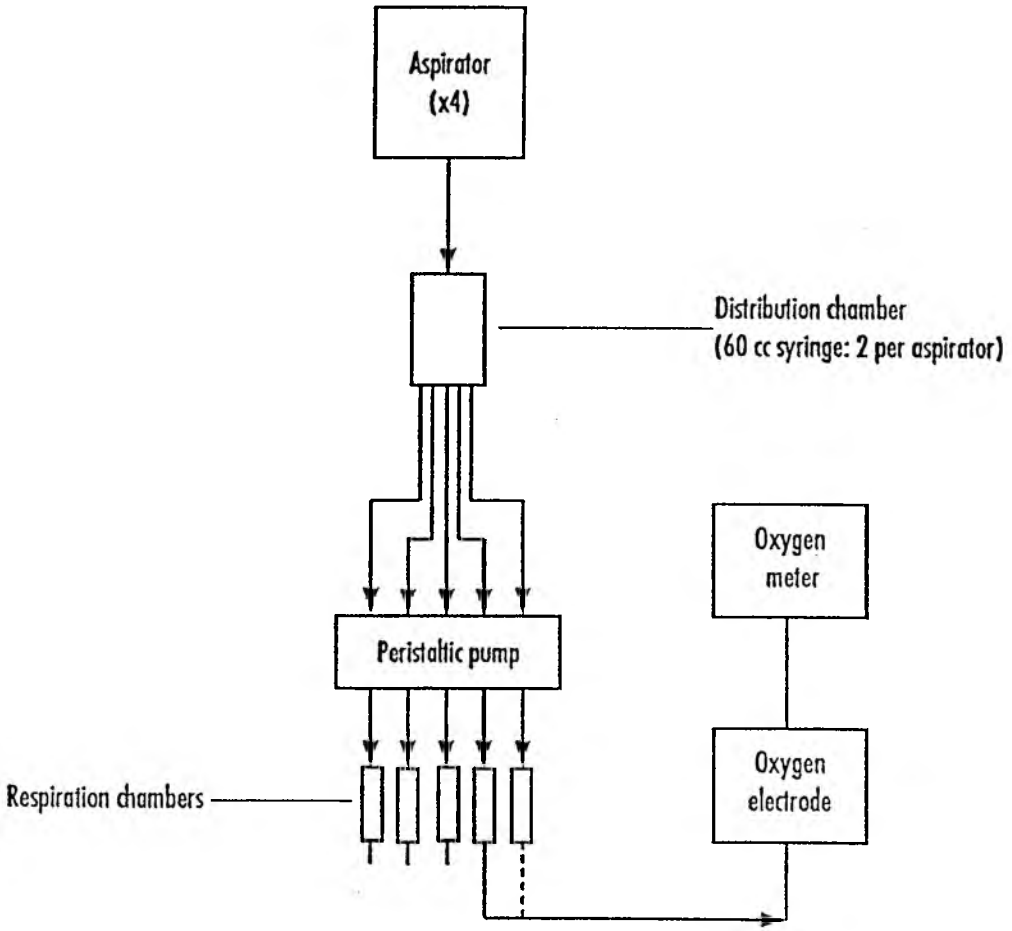

Figure A2 Apparatus for measuring *G. pulex* respiration rate

(c) Start the peristaltic pumps to circulate water through the electrode jacket and the respiratory chambers at least 3 hours before the start of respirometry; **(0.1 days)**

(d) Calibrate the oxygen electrode initially using sodium sulphite and air-saturated water one hour before the start of respirometry; **(0.1 days)**

(e) The selection of animals for this part of the study was described above;

(f) Two operators are required to measure the respiration rate of *G. pulex*, each responsible for a separate rack of animals. Leave blank chambers at four randomly selected positions on the respirometry rack used by Operator 1 and four positions on the rack used by Operator 2. Attach each blank to a different aspirator, so that all four test concentrations are represented. The remaining positions are occupied by the animals whose respiration is to be measured; **(0.3 days)**

(g) Position the animals in the following way:

- Use a random number table to randomize the position of test animals in the following blocks:

Morning:	Operator one : 3 animals × 4 treatments
	Operator two : 3 animals × 4 treatments
Afternoon:	Operator one : 3 animals × 4 treatments
	Operator two : 3 animals × 4 treatments

- Carry out the respirometry in 2 runs; in the morning and afternoon of day 7 of the experiment. Each run will involve 24 animals.

- Once the position of each animal on the respirometry rack has been decided attach the appropriate tubing to the respiration chamber so that the respiration of the animal is measured in the correct concentration of test substance. Place the animals in their respective chambers and allow to acclimatise for 3 hours; **(0.2 days)**

- This section describes the procedure followed to take oxygen readings. For the sake of simplicity only one run and the actions of only one operator are described. Exactly the same procedure is used by the first operator for run 2 and by the second operator for both runs 1 and 2. The first operator measures the dissolved oxygen and flow rate of positions 1 to 16. The second operator takes measurements from positions 17 to 32;

- Take and record two oxygen readings from each animal and each blank chamber, unless there is a two torr or greater difference between the first and second readings, in which case take and record a third reading;

- Take samples from the respirometry chambers by allowing the water that is leaving the chamber to flow into the barrel of a 250 µl microsyringe attached to the end of the chamber via a 7 cm length of marprene tubing;

- When the syringe barrel is full carefully depress the syringe plunger. Remove the syringe from the marprene tubing and rapidly inject the sample from the respirometry chamber into the electrode jacket. After 2 minutes record the reading on the oxygen meter, withdraw the sample from the electrode jacket and inject the next sample. Thus each sample is injected in a numerical sequence from 1 to 16;

- Recalibrate the oxygen meter between every reading by pumping water-saturated air in and out of the jacket with a 2 ml syringe. If after two minutes the meter does not show a reading of 160 torr, reset it to this figure. When the calibration of the meter has been checked, and if necessary altered, the next sample can be injected; **(2.5 days)**

- Calculate the flow rate of the water passing through the respirometry chambers by recording the time taken in seconds for each microsyringe to fill to the 250 µm mark;

- When all the animals in the rack have been respired place them in pre-weighed, pre-numbered foil boats, kill them by squeezing and dry in an oven for four days at 60 °C ± 5 °C. Weigh the dried animals to a precision of 0.01 mg, taking care that rehydration does not occur. **(0.5 days)**

The methods used to measure *G. pulex* scope for growth are shown in Figure A3.

A6 CALCULATIONS

A6.1 Calculation of feeding rate, assimilation rate, respiration rate and Scope for Growth

The Feeding rate (C) Assimilation rate (A), Respiration rate (R) and Scope for Growth (SfG) of each individual *G. pulex* are calculated in the following way:

(a) Calculate the mean dissolved oxygen leaving each respirometry chamber. If three readings were taken from any chamber, calculate the mean of the two most similar readings; **(0.1 days)**

(b) Estimate the mean dissolved oxygen entering each respirometry chamber by calculating the means of the blank chamber readings for each run and each concentration of test substance and control. If three readings were taken from any blank chamber, calculate the mean of the two most similar readings; **(0.1 days)**

(c) Calculate the mean time in seconds that each respirometry and blank chamber took to fill a 250 µm syringe. If three readings were taken from any chamber, calculate the mean of the two associated with the two most similar oxygen readings; **(0.1 days)**

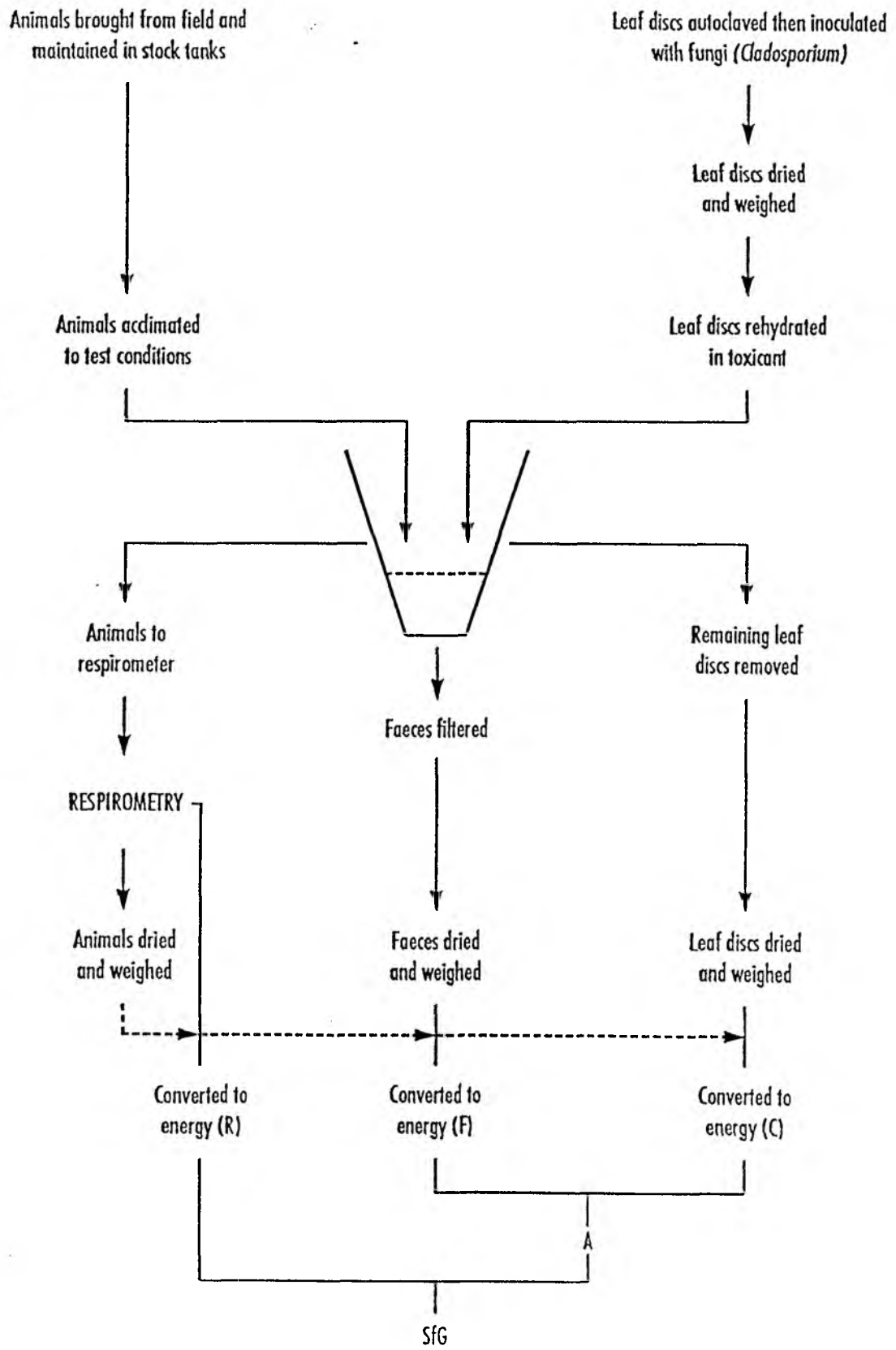

Figure A3 Summary of laboratory methods for measuring *G. pulex* scope for growth

(d) Calculate the leaf weight correction factor for each treatment by dividing the final dry weight of each set of leaf weight control discs by its initial weight. Calculate a mean correction factor for each treatment; **(0.1 days)**

(e) Use a spreadsheet to calculate A, R and SfG for each *G. pulex* from the data on the relevant data sheet. Enter raw data onto the spreadsheet in the following way: **(0.2 days)**

COLUMN	DATA
A	FEEDING CHAMBER NUMBER
B	TREATMENT TYPE
C	RUN (AM/PM)
D	OPERATOR (1/2)
E	DRY WEIGHT OF *G. PULEX* (MG)
F	APPROPRIATE MEAN LEAF LEACH CORRECTION FACTOR
G	DRY WEIGHT OF LEAF DISCS PLACED IN CHAMBER
H	FINAL WEIGHT OF LEAF DISCS PLACED IN FEEDING CHAMBER
I	DRY WEIGHT OF FAECES
J	MEAN DISSOLVED OXYGEN LEAVING RESPIROMETRY CHAMBER (TORR)
K	MEAN DISSOLVED OXYGEN ENTERING RESPIRO-METRY CHAMBER (TORR)
L	MEAN TIME TO FILL 250 µM SYRINGE (SECS)

Calculations are performed on the raw data in the following way:

COLUMN	FUNCTION	
M	$\frac{21.552((G \times F)-H)}{(E \times 6)}$	= amount of energy consumed (C) ($J\ mg^{-1}\ day^{-1}$)

Feeding rate may be expressed as mg leaf consumed per mg animal per day by omitting the energy constant (21.552) in the above calculation.

N	$\frac{18.737 \times I}{(E \times 6)}$	= amount of energy lost in faeces ($J\ mg^{-1}\ day^{-1}$)
O	$\frac{20.432(K-J)}{(L \times E)}$	= energy lost in respiration (R) ($J\ mg^{-1}\ day^{-1}$)
P	M-N	= energy assimilated (A) ($J\ mg^{-1}\ day^{-1}$)
Q	P-O	= Scope for Growth (SfG) ($J\ mg^{-1}\ day^{-1}$)

A6.2 Statistical analysis of experimental results

Analysis of variance (ANOVA), or analysis of covariance (ANCOVA) with body weight as a covariate, can be performed on the feeding rate, respiration rate and SfG of the different treatment groups. If a significance level of $P<0.05$ is found, a Dunnett's test can be performed to compare treatment group means with the control group mean. A Tukey test can be used if all treatment means are to be compared (Zar 1984). **(0.1 days)**

A7 ESTIMATED TIME RESOURCING REQUIREMENTS (FULL STAFF DAYS)

1.	Feeding rate	3 days
2.	Assimilation rate	4 days
3.	Respiration rate	5 days
4.	Scope for Growth	9 days

APPENDIX B PROTOCOL FOR THE *G. PULEX* FEEDING RATE BIOASSAY IN THE FIELD

B1 INTRODUCTION

This Standard Operating Procedure (SOP) describes how to measure the feeding rate of *G pulex*, a freshwater amphipod crustacean, during field deployment. The feeding rate of *G. pulex* is related to its growth rate and can be measured over a shorter period of time. It can therefore be used as a relatively rapid and ecologically relevant index of pollution.

B2 SAFETY

See information in Appendix A2

B3 STUDY OBJECTIVES

To measure and compare the feeding rate of *G. pulex* exposed at an impacted site and a reference site.

B4 MATERIALS

(a) One hundred and thirty-two field cages constructed as shown in Figure B1;

(b) Six holding baskets constructed as shown in Figure B2;

(c) Five hundred and twenty-eight 'conditioned' alder (*Alnus glutinosa*) leaf discs;

(d) One hundred male *G. pulex* measuring not less than 7 mm in length, normally collected from the field. An acclimatization period of at least one week must be observed immediately before the test. During this acclimatization period the test animals must either be kept under similar temperature, lighting and feeding conditions to those during the period of exposure (that is acclimatized to field conditions) or held under standard conditions in the laboratory;

(e) Two hundred and sixty-four aluminium foil boats with a diameter of 1 cm, contained in 8 × 25-chamber plastic containers such as those supplied by Sterilin Ltd;

(f) One top-loading balance accurate to 0.01 mg, such as the Sartorius 1712 MP 8;

(g) One oven capable of maintaining a constant temperature of 60°C ± 5 °C;

(h) One set of random number tables;

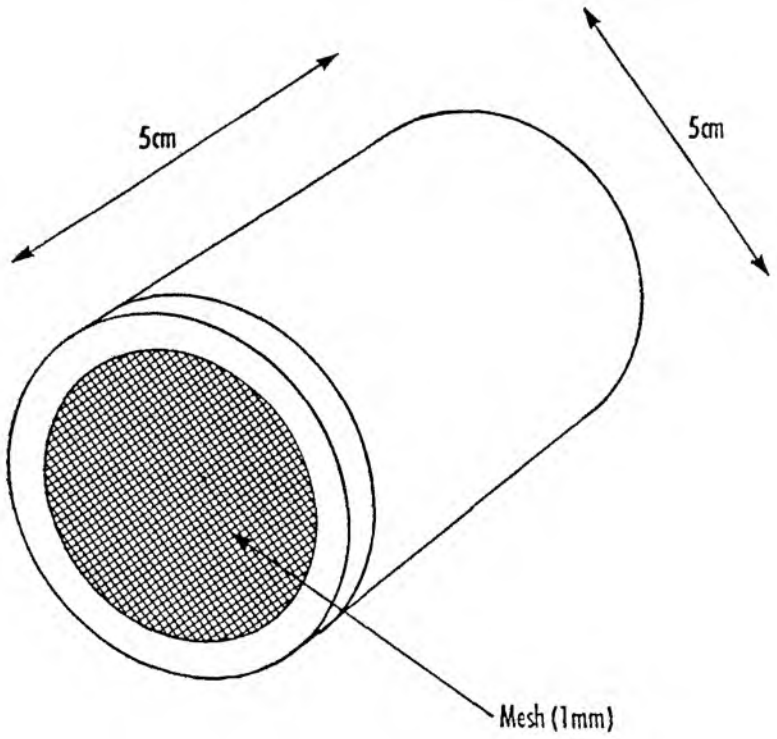

Figure B1 Field cage

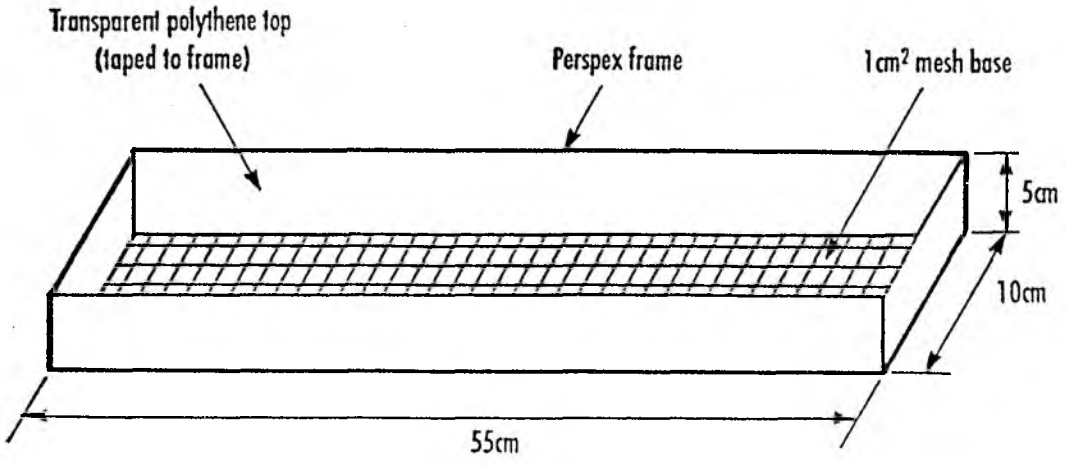

Figure B2 Holding Basket

B5 METHODS (ESTIMATED TIME TAKEN TO PERFORM TASK)

B5.1 Preparation of feeding material

(a) Collect alder leaves in autumn between abscission and shedding and store in cardboard boxes at room temperature. Under these conditions the leaves will become dry and brittle; **(0.1 days)**

(b) When required, rehydrate the leaves in uncontaminated water contained in a large, clean vessel for one hour; **(0.1 days)**

(c) Using a 17 mm diameter cork borer cut discs from between the main veins of the rehydrated leaves. Cut the discs over a cardboard base and use a clean glass rod to remove them from the borer; **(0.2 days)**

(d) Place sets of 120 discs in 500 ml Pyrex conical flasks. Add 400 ml of uncontaminated water to each flask and then seal with cotton wool, tin foil and autoclave tape and autoclave for 15 minutes at 1.1 kg cm^{-2}; **(0.2 days)**

(e) *Cladosporium* fungus is cultured from an initial sample provided by Dr Lorraine Maltby from Sheffield University. The culture method is as follows:

- Cut a 17 mm disc from a fungal culture using a 17 mm cork borer;
- Plate a malt extract broth plate (Oxoid CM59) with 4 drops of 10 % lactic acid to prevent bacterial contamination;
- Place the fungal disc on the broth plate using aseptic technique and incubate at room temperature in the light;
- Inspect the plate after two weeks and discard if contaminated by bacteria or fungi other than *Cladosporium*. Store successful cultures at 4 °C ± 2 °C;

(f) When cool, inoculate the autoclaved leaf discs with *Cladosporium* fungus as follows:

- Use a cork borer to cut six 17 mm diameter fungal discs from the broth plate on which they have been cultured. Add the fungal discs to the autoclaved leaf discs in the 500 ml flask;
- Re-seal the flask and leave at room temperature for 10 days. Shake the contents of the flask daily;
- After 10 days tip the contents of the flask into a sieve with a mesh size of 1-5 mm. Tip the contents of the sieve into a large vessel containing uncontaminated water;

- Prepare the conditioned leaf discs for the test by removing them from the vessel, placing them in individual Sterilin tray compartments in sets of four and drying them in an oven for four days at 60 °C ± 5 °C. **(0.2 days).**

B5.2 Measuring the feeding rates of *G. pulex* in the field

Fifty male *G. pulex* should be deployed at each field station with sixteen leaf weight control cages. This means that a total of 66 field cages should be used at each station, contained in three holding baskets. The test system should be prepared as follows:

(a) Weigh the leaf disc sets and rehydrate them in uncontaminated water in numbered Sterilin tray compartments for four days prior to field deployment; **(0.3 days)**

(b) On the day of deployment, place the weighed and rehydrated leaf discs in their respective, numbered field cages arranged in numerical order in plastic trays containing uncontaminated water. Add the *G. pulex* sequentially to the field cages, by removing them gently from their stock tank with a sieve, and gently brushing them into their field cage with a fine paint brush. Fix the field cage lids in place; **(0.2 days)**

(c) Place the field cages containing *G. pulex* plus leaf weight control cages in holding baskets according to a pre-recorded random number distribution. Fasten the polythene top onto the holding baskets to retain the field cages; **(0.2 days)**

(d) Transport the holding baskets to the field stations in sealed buckets containing uncontaminated groundwater. Fasten them to the river bed at the field station with metal stakes, or weight them down with bricks. The holding baskets should be orientated so that the polythene cover is uppermost and the long axis is perpendicular to the flow of the river. Ensure that flow rates past the holding baskets are similar within and between stations; **(0.5 days)**

(e) Retrieve the holding baskets after they have been deployed for six days. *G. pulex* may be deployed for longer periods if basic water quality characteristics such as alkalinity are favourable and if more than four food discs are provided; **(0.5 days)**

(f) Remove the lids from the field cages and record the number of mortalities. Remove live *G. pulex* from their cages and place them in numbered and preweighed foil dishes. Kill them by squeezing the dishes. Place in an oven for four days at 60 °C ± 5 °C. Weigh to a precision of 0.01 mg, taking care that dehydration does not occur. Record the dry weight of the *G. pulex;* **(0.4 days)**

(g) Remove leaf remains from the field cages and place in another set of numbered, but unweighed, foil dishes. Place in an oven for four days at 60 °C ± 5 °C. Weigh to a precision of 0.01 mg, taking care that rehydration does not occur. Record the dry weight of the discs taken from each cage; **(0.4 days)**

B6 CALCULATIONS

B6.1 Calculation of feeding rate

The feeding rate of field deployed *G. pulex* can be calculated from the following formula:

$$C = \frac{((D_1 \times L) - D_2)}{N(W_1 - W_2)}$$

where C = feeding rate (mg mg^{-1} day^{-1})
D_1 = dry weight of food initially supplied (mg)
D_2 = dry weight of food remaining after N days (mg)
W = dry weight of *G. pulex* (mg)
L = leaf weight change correction factor

L is calculated from the change in leaf weight in cages that did not contain a test animal, that is

L = the mean of $\frac{\text{final control leaf weight}}{\text{initial control leaf weight}}$

for each deployment station **(0.3 days)**

B6.2 Statistical analysis of experimental results

Analysis of variance (ANOVA), or analysis of covariance (ANCOVA) with body weight as a covariate, can be performed on the feeding rate, of the different treatment groups. If a significance level of $P<0.05$ is found, a Dunnett's test can be performed to compare treatment group means with the control group mean. A Tukey test can be used if all treatment means are to be compared (Zar 1984). **(0.1 days)**

B7 ESTIMATED TIME RESOURCING REQUIREMENTS (FULL STAFF DAYS)

Feeding rate 4 days