

The use of a biomarker to assess the effect of xenobiotic exposure on the freshwater invertebrate *Gammarus pulex*.

Oliver William Tindle Warwick

Department of Animal and Plant Sciences
University of Sheffield

Submitted for the degree of Doctor of Philosophy

June 1997

CONTENTS

Acknowledgements	i
Summary	ii
Chapter One.	
The use of biochemical biomarkers in the assessment of xenobiotic contamination of aquatic environments.	1
1.1 Monitoring water quality	1
1.1.1 Chemical monitoring	2
1.1.2 Biological monitoring	3
1.2 Biomarkers	10
1.2.1 Biochemical biomarkers	15
1.2.2 Biomarkers of exposure and effect	21
1.3 Linking levels of organisation	24
1.4 Aim of this study	27
1.4.1 Hypotheses and approach	29
Chapter Two.	
Measuring the activity of the detoxification enzyme glutathione s-transferase in <i>Gammarus pulex</i>.	33
2.1 Introduction	33
2.1.1 Glutathione s-transferase activity in crustaceans	33
2.1.2 Isoenzymes of glutathione s-transferase	34
2.1.3 In vitro determination of GST activity	37
2.1.4 'Baseline' GST activity and sources of variability	39
2.1.5 Kinetics of glutathione s-transferase	41
2.1.6 Aims and objectives	43
2.2 Materials and methods	44

2.2.1 Reagents	44
2.2.2 Collection and holding of animals	44
2.2.3 Preparation of animal material for assay	45
2.2.4 Spectrophotometer assay	46
2.2.5 Multi-cell spectrophotometer	47
2.2.6 Multi-well plate reader method	47
2.2.7 Protein measurement	48
2.2.8 Calculation of enzyme activity	50
2.2.9 Experiments	51
2.2.10 Effect of extrinsic and intrinsic factors on GST activity	55
2.2.11 Statistical analysis	58
2.3 Results	60
2.3.1 Maximum absorbance wavelength and extinction coefficient	60
2.3.2 Detection and linearity in pure and <i>G. pulex</i> GST	61
2.3.3 Spectrophotometric methods for the assay of GST	62
2.3.4 Kinetics of pure and <i>G. pulex</i> homogenate GST	63
2.3.5 Optimum assay conditions for <i>G. pulex</i> and pure (Equine) GST	64
2.3.6 Enzyme linked immuno-sorbent assay detection of GST in <i>G. pulex</i>	65
2.3.7 Effects of intrinsic and extrinsic factors on GST activity	66
2.4 Discussion	75
2.4.1 Measurement of GST activity in crude extracts of <i>G. pulex</i>	75
2.4.2 Effects of intrinsic and extrinsic factors on GST activity	81
2.4.3 Baseline activity	84
Chapter Three	
The effect of xenobiotic exposure on glutathione s-transferase activity in <i>Gammarus pulex</i>.	86
3.1 Introduction	86

3.1.1 Test chemicals	88
3.1.2 Objectives	92
3.2 Materials and methods	93
3.2.1 Reagents	93
3.2.2 Experimental animals	93
3.2.3 Preparation of test solutions	93
3.2.4 Short-term lethality tests	93
3.2.5 Induction of GST activity	95
3.2.6 Chemical analysis of test solutions	98
3.2.7 Statistical analysis	100
3.3 Results	102
3.3.1 Short-term lethal tests	102
3.3.2 Sublethal exposure	103
3.3.3 Exposure of <i>G. pulex</i> to alcohol ethoxylate in semi-natural conditions	105
3.4 Discussion	107
3.4.1 Laboratory exposures	107
3.4.2 Exposure under semi-natural conditions	110
3.4.3 Sensitivity of the GST response	112
3.4.4 Conclusion	114
Chapter Four	
Glutathione s-transferase activity in contaminated and non-contaminated populations of <i>Gammarus pulex</i>	115
4.1 Introduction	115
4.1.1 GST activity in contaminated and non-contaminated populations	115
4.1.2 Lindane contamination of freshwaters	116
4.1.3 Objectives	118

4.2 Materials and Methods	119
4.2.1 <i>Gammarus pulex</i> populations	119
4.2.2 Sampling of <i>Gammarus pulex</i> populations	123
4.2.3 Interpopulation variation in response to short-term lindane exposure	124
4.2.4 Effect of pre-exposure on the GST response to short-term exposure	124
4.2.5 Transportation of frozen samples	125
4.2.6 Lindane analysis	125
4.2.7 Statistical analysis	125
4.3 Results	126
4.4 Discussion	131
4.4.1 Interpopulation differences in GST activity	132
4.4.2 Effect on GST activity of pre-exposure to lindane	134
4.4.3 Conclusions	136
Chapter Five	
Physiological responses of <i>Gammarus pulex</i> to lindane exposure	138
5.1 Introduction	138
5.1.1 Levels of xenobiotic effect	138
5.1.2 Scope for growth	141
5.1.3 Stored energy	142
5.1.4 Effects of short-term and long-term exposure	143
5.1.5 Objectives	143
5.2 Materials and methods	145
5.2.1 Short-term lindane exposure	145
5.2.2 Long-term lindane exposure experiments	150
5.2.3 Effect of lindane exposure on glycogen concentration	151
5.2.4 Statistical analysis	155

5.3 Results	156
5.3.1 Short-term lindane exposure	156
5.3.2 Long-term exposure to lindane	160
5.4 Discussion	164
5.4.1 Effect of short-term lindane exposure on energy absorption and loss	164
5.4.2 Long-term lindane exposure	167
5.4.3 Conclusions	170
Chapter Six	
Glutathione s-transferase as a biomarker of organic xenobiotic exposure and effects	172
6.1 Measurement and change of GST activity in <i>G. pulex</i>	173
6.2 The use of GST as a marker of organic xenobiotic exposure	179
6.3 Population differences	180
6.4 GST as a marker of effect	183
6.5 Conclusions	185
References	188
Appendix 1	232
Appendix 2	235
Appendix 3	237

Acknowledgements

This study was supported by a Natural Environment Research Council CASE studentship with Shell Research Ltd.

I would like to thank Dr Lorraine Maltby for supervision, Professor John Lee and the Department of Animal and Plant Sciences, University of Sheffield for provision of facilities, Dr Lisa Tattersfield and Dr Robert Lyne (Shell Research Ltd) for provision of support and facilities at Shell Research Ltd. I would also like to thank Uwe Kirchberger, Dr Ralf Schulz and Dr Mathias Liess at the Institute of Zoology, Technical University of Braunschweig, for the provision of facilities and information and for their kind hospitality during my visit there.

Thanks also to (ex)members of the freshwater ecotoxicology group, Danielle Ashton, Dr Sue Bermingham, Dr Rachel Grant, Dr David Forrow and Dr Deborah Roberts for their advice and support. Finally, but by no means least, I would like to thank Natasha Callow and Penelope Warwick for their constant love and support.

The use of a biomarker to assess the effect of xenobiotic exposure on the freshwater invertebrate *Gammarus pulex*.

Oliver William Tindle Warwick.

Contamination of freshwaters is of concern because of its effect on ecosystem health. The effect of contamination can be assessed at all levels of biological organisation, from the ecosystem level to the molecular level. At the biochemical level, enzymes that are involved in the detoxification of organic chemical contaminants are useful as markers of contaminant exposure as they are often one of the first systems to respond to chemical exposure. Furthermore, changes in the activity of these enzymes may be indicative of effects at higher levels of biological organisation. In this study, the use of the detoxification enzyme glutathione s-transferase (E.C. 2.5.1.18) (GST) in the freshwater invertebrate *Gammarus pulex* (Crustacea: Amphipoda), as a biomarker of organic xenobiotic exposure and effects was assessed. Toxicant induced changes in GST activity were related to changes in energy status and physiological energetics.

A GST assay was developed that allowed the rapid analysis of GST activity in up to ninety individual animal samples simultaneously. Optimum sample handling and assay conditions were determined for the assay of GST in *G. pulex*, and extrinsic factors (e.g. environmental temperature, feeding and holding conditions) and intrinsic factors (e.g. body size), affecting variability of GST activity in *G. pulex* were assessed. The effect of exposure to the organic xenobiotics lindane (an organochlorine insecticide) and alcohol ethoxylate (a non-ionic surfactant detergent), on GST activity in *G. pulex* was described. The magnitude and duration of the response of GST activity to exposure to both chemicals was assessed under laboratory conditions and with alcohol ethoxylate, using outdoor artificial streams.

The use of GST as a marker of pesticide exposure in field populations of *G. pulex* that were subject to pesticide contamination was studied in comparison with animals from non-contaminated, reference sites. The differences in GST activity between animals from a contaminated site and clean site were investigated by short-term and long-term exposure to lindane in the laboratory. Changes in energy status and physiological energetics were investigated in *G. pulex* on exposure to lindane by measuring glycogen concentration and scope for growth. Measuring these parameters on short-term and long-term exposure to lindane allowed the sensitivity of these responses to be related to the GST response and allowed the energetic cost of exposure to be assessed.

It was concluded that increase in GST activity may provide a rapid and sensitive biomarker of xenobiotic exposure in the short-term and in populations subject to pesticide contamination. The affect on GST activity may be indicative of effects at higher levels of biological organisation, such as scope for growth and glycogen concentration. However, GST activity is affected by a variety of intrinsic and extrinsic factors and should therefore be used only as part of a comparative study. Because of the transient nature of the GST response, *in situ* assessments should be based on 24-hour exposure periods.

CHAPTER ONE

The use of biochemical biomarkers in the assessment of xenobiotic contamination of aquatic environments.

This study is concerned with the use of a biochemical biomarker the detoxification enzyme glutathione s-transferase, in the assessment of organic xenobiotic contamination of freshwater streams. Contamination of freshwaters is of concern because of its effect on ecosystem and human health, and thus the monitoring and assessment of water quality has been a concern for many years (Fish, 1992). A number of methods have been used to monitor water quality, chemical and biological methods of varying degrees of sophistication have been used. The use of biological monitoring has enabled the integration of the deleterious effect of xenobiotic chemical input to freshwater environments with time and biotic affect. These methods have included multi-species assessments through to *in situ* single-species bioassays. The biomarker approach is used to combine the sensitivity that is expected at the sub-organism level at sub-lethal exposure concentrations with a mechanistic link to the actual toxicity of contaminants, thus providing a useful and relevant tool in the assessment of water quality.

1.1 MONITORING WATER QUALITY.

There has been an increasing use of anthropogenically produced and derived chemicals (xenobiotics) in the twentieth century (Nagel, 1993). The acquisition, production and use of these chemicals has led to the release of many of them into ecosystems where they were not previously found or dramatically increased their concentration. The ultimate sink for many xenobiotics is the aquatic environment and therefore aquatic organisms have become increasingly exposed to these chemicals (George, 1993). Xenobiotics that have a deleterious effect on organisms have been defined as pollutants (Moriarty, 1991). It is because of their suspected deleterious effect on the health of the ecosystem and on human health

that it is desirable to monitor the quantity and effect of pollutants entering freshwater environments in order to assess and predict the extent of damage to individuals, populations and communities. This is done so that further deleterious effects can be limited or avoided.

Pollution of water courses with xenobiotics can be monitored both chemically and biologically. Chemical analysis provides precise data on the composition and quantity of pollutants in a water body at a point in time. However, not all chemicals present will be potentially toxic. A proportion of the chemical may be bound to particulate material or present in complexes and thus not taken up by organisms, i.e. not bioavailable. Biological monitoring provides information on the bioavailable fraction of xenobiotics. Furthermore, the toxic effect of chemicals, on organisms and interactions between organisms can be assessed.

1.1.1 Chemical monitoring.

The measurement of chemicals in freshwater environments has reached a high degree of sophistication and current analytical techniques enable estimates of contaminants to very low levels (i.e. to the nanogram level). Using techniques to extract, separate and concentrate chemicals from water and sediment samples combined with sensitive detection methods (e.g. inductively coupled plasma or gas chromatography coupled with mass spectrometry) allows the identification and precise quantification of compounds (Holme and Peck, 1993). Contaminant concentrations may fluctuate over time (Metcalf, 1989), so in order to measure maximum concentrations of contaminant, a sampling procedure may be utilised that is triggered by a chemical or physical change in water quality. For example, Liess *et al.* (1996) used a sampler triggered by changes in conductivity to monitor xenobiotic input to streams. This type of monitoring allows samples for analysis to be taken when contaminants are likely to be entering the freshwater environment. Furthermore, using sediment samples allows the measurement of accumulated contaminants that are absorbed onto sediment particles. For example, Spalding and Snow (1989) reported the use of sediment sampling and subsequent chemical analysis to estimate pesticide contamination.

There is a clear need to quantify the amount of contaminants in freshwater environments. Indeed, it has been argued that because of the difficulty of detecting biological effects in the field, it is more realistic to monitor chemical residues and relate them to standards of toxicity (Preston, 1979). It is not possible to monitor for all types of xenobiotic that may be present, especially the breakdown products of a chemical. Furthermore, the interpretation of the effect of chemically measured xenobiotic concentration is complicated because of the need to integrate the toxic action with physico-chemical factors such as dissolved oxygen concentration, temperature and other variables, possibly including the presence of other xenobiotics (James and Kleinow, 1993).

The fate and behaviour of chemicals is an important factor in their availability to organisms and is dependent on the properties of the chemical and physico-chemical variables in the environment. For example, some organic xenobiotics will adsorb to organic particles and consequently are present only in very low concentrations in the water column. Thus, water-column organisms are unlikely to be exposed to such chemicals whereas sediment-dwelling organisms may be exposed to higher concentrations of the chemical. The fate and bioavailability of metal contaminants are affected by many factors that may control their partitioning between sediments, interstitial water and the water column. The dynamics of metal movement, their availability and possibly their toxicity are influenced by chemical and physical factors such as oxygen/redox gradients, pH, temperature, adsorption, sedimentation, complexation and precipitation (Burton, 1991). In addition a variety of common sediment bacterial communities can metabolise and alter metal and metalloid valence states via oxidation-reduction reactions, thereby altering chemical fate and toxicity (Wood, 1987; Drotar *et al.*, 1987).

The physico-chemical properties of contaminants will affect their uptake into organisms. Properties such as the lipophilicity, hydrophobicity, presence of acidic or basic groups and the size of the molecule both separately and together

influence the uptake from water, sediment or from food in the gastro-intestinal tract in animals (James and Kleinow, 1993). Molecules that are highly water soluble may be easily taken up by animals through membrane surfaces, highly lipid soluble compound may accumulate in tissue and be persistent in the environment.

1.1.2 Biological monitoring.

The use of biological monitoring counters many of the problems of chemical monitoring. Assessing the toxic effect of xenobiotics ensures that only bioavailable xenobiotics are considered. Furthermore, the bioconcentration of xenobiotics in the tissues of living organisms can be used to integrate xenobiotic exposure concentrations with time. Organisms can thus be used to estimate the toxic effect of xenobiotics as well as being used as monitors of xenobiotic concentration.

Monitoring of bioavailable contaminants can be assessed by analysis of body burdens of organisms in contaminated environments. For example, some pollutants, notably heavy metals and organochlorine compounds, occur in much higher concentrations within aquatic organisms, sometimes by as much as a factor of 10^3 to 10^6 (Phillips, 1980). However, each species and contaminant will exhibit its own degree of integration with time. Therefore, a knowledge of the accumulation strategy of the organism is essential to the use of organisms as useful monitors of contaminants (Phillips, 1980). Furthermore, the use of organisms in water quality monitoring assesses the amount xenobiotic exerting toxic effect rather than the total amount of xenobiotic in the environment.

Multi-species monitoring.

Many systems for assessing the quality of freshwaters have been derived based on the increase or reduction in numbers of species or taxa present in the community. Assessment of the response of the entire community to stress is impractical and consequently most assessment systems have focused on particular parts of the community in the ecosystem (Metcalf, 1989). The saprobic approach to water

quality monitoring relates the presence of organisms and their dependency on decomposing organic substances as a food source to a pollution grading (Kolkwitz and Marsson, 1908, 1909). This system has been applied using the saprobic index, which is based on the presence of indicator species that are mainly bacteria, algae, protozoans and rotifers but also includes some benthic invertebrates and fish. Species have been assigned saprobic values based on their pollution tolerance. Values range from 0 to 8; the higher the value the more tolerant the organism. Pollution tolerances of individual species are determined by observations on their relative occurrence under specifically-defined conditions of water quality (Sladeczek, 1973). The saprobic approach is considered to be of limited use because of its rigidity and because all indicator organisms occur in unpolluted water (Chutter, 1972). The system is also considered to give little information of the community as a whole as each taxon is considered as a separate entity (Jones *et al.*, 1981).

The assessment of stream water quality is most commonly assessed using benthic macroinvertebrates (Metcalf, 1989). Macroinvertebrate communities are heterogeneous and consist of representatives of several phyla. Thus, it is expected that species will be differentially sensitive to pollutants of various types, producing a graded response to increasing degrees of stress. Furthermore, benthic invertebrates are relatively sedentary and are therefore representative of local conditions (Cook, 1976; Pratt and Coler, 1976; Hellowell, 1977).

Approaches to the biological monitoring of water quality have been based on diversity indices and biotic indices. Diversity indices use the components of community structure to describe the response of a community to water quality. These components are the number of species present (richness), the uniformity in the distribution of individuals among species (evenness) and the total number of organisms present (abundance) (Mackay *et al.*, 1973; Balloch *et al.*, 1976; Hellowell, 1977). In their simpler forms diversity indices express species richness of a community (e.g. Margalef, 1956). This does not take account of the number of individuals per species (i.e. evenness). Evenness and species richness is

accounted for in the more complex Shannon-Weiner index (Wilhm and Dorris, 1968). There are many diversity indices which all place emphasis on a slightly different aspect of community structure resulting in a different index value (Pratt and Coler, 1976). Furthermore, there are additional complications which can alter index values such as the method of sample collection, sample size, natural variations in diversity and the taxonomic level used (Pratt and Coler, 1976; Green, 1979).

Diversity indices do not contain information about the pollution tolerance of individual species and therefore cannot provide an explanation for the community structure (Cook, 1976). In contrast, biotic indices are derived from the knowledge that different species have varying tolerances to pollution. Many assign 'scores' to certain taxonomic groups which reflect their pollution tolerance, the combination of these scores giving the index value. Biotic indices include the Trent Biotic Index (TBI), which is based on presence/absence data (Woodiwiss, 1964) and Chandler Biotic Score (CBS), which takes abundances into account (Chandler, 1970). Biotic indices such as the TBI and CBS have been criticised because they are too complicated and are only applicable to upland rivers (Metcalf, 1989).

A standardised biotic system was developed by the Biological Monitoring Working Party (BMWP) (ISO, 1979). The BMWP system scores families of macroinvertebrates to reflect their pollution tolerance based on a knowledge of distribution and abundance. Values range from 1, pollution tolerant to 10, pollution sensitive (Metcalf, 1989). The BMWP score may not accurately reflect the water quality of the river sampled because the score improves with increasing diversity, so sites containing different families due to habitat types will yield different scores. Moreover, larger sample size will increase the score because additional taxa found can only increase the score. A remedy to these problems is the use of the Average Score per Taxon (ASPT) (Armitage *et al.*, 1983; Metcalf, 1989). This method is obtained by dividing the BMWP score by the number of taxa used to calculate the score. Therefore, as well as being less

dependent on habitat variation and sampling effort the ASPT limits all values to within a range of one to ten and is less sensitive to seasonal change. The ASPT can only increase with sampling effort if the additional taxa found are higher scoring than the existing mean.

Diversity and biotic indices describe community structure and can only provide information on water quality by reference to known uncontaminated communities. A scheme has been developed to predict the impact of river pollution, namely; the River Invertebrate Prediction and Classification Scheme (RIVPACS). This scheme is based on the prediction of the type of macroinvertebrate community at a site using environmental data.

Macroinvertebrate community data and physico-chemical information has been obtained for 438 reference sites in the UK. These communities have been classified by two-way indicator species analysis (TWINSPAN) and environmental factors are used to predict the expected fauna at an unpolluted site (Wright *et al.*, 1984; Furse *et al.*, 1984; Moss *et al.*, 1987; Armitage *et al.*, 1987; Wright *et al.*, 1989). RIVPACS can be used to predict BMWP and ASPT scores for sites based on their physical and chemical characteristics (Wright *et al.*, 1989).

Predictions of ASPT are more accurate than predictions of BMWP and Wright *et al.* (1989) suggested that predictive equations for ASPT could provide target values against which observed values could be compared.

Although RIVPACS has an advantage over diversity indices and biotic indices in that it attempts to predict the species assemblage at the site if it were unpolluted, it is based on correlation rather than a mechanistic understanding of pollutant effects. Moreover, RIVPACS may detect changes late as assessment is based on the presence or absence of species or families, therefore these groups must be eradicated before change between predicted and observed community is detected. Furthermore, although biological surveillance methods such as diversity indices, biotic indices and RIVPACS detect ecological changes indicative of water quality changes, they do not identify the specific cause of change. The best use of

biological methods is by detection of changes that indicate effect and the use of physico-chemical methods to identify the cause.

Single-species monitoring.

Xenobiotic contaminants affect populations and communities. However, they exert toxic effects on individual organisms and these effects can be manifest at different levels of biological organisation. The use of single-species toxicity monitoring is an indirect method of assessing water quality in relation to toxic effect on the ecosystem (Slooff and De Zwart, 1983) and the data gained usually have low ecological significance. Whereas, multi-species assessments, whilst providing good ecological information have a low specificity to toxic effects. It has been indicated that methods of community assessment and single-species assessments of toxicity are complementary and should be applied simultaneously to obtain information on macroinvertebrate distribution in relation to possible causative factors (Williams *et al.*, 1984). It has been argued that there is no 'universal' test organism applicable for every toxicant and situation, and in most cases it is suitable to use the most sensitive and locally important species (Buikema *et al.*, 1982). Indeed, invertebrate species belonging to the same group have been reported to show as much variability in susceptibility as species from different groups (Slooff, 1983). Individual species have been used to relate toxicological data to ecological effect. For example, Williams *et al.* (1984) reported that *Gammarus pulex* and *Baetis rhodani* were excluded from a river in which zinc concentrations exceeded those found to be lethal to these organisms on short-term exposure in the laboratory.

The use of *in situ* studies using a single species may prove useful to the assessment of toxicity in contaminated sites (Burton *et al.*, 1996). Using this type of assessment, the effects of environmental variables are integrated with the toxic response. Indeed, *in situ* toxicity assessment may be used to study these interactions, for example, the effect of photoinduced toxicity of polycyclic aromatic hydrocarbons (PAHs) has been reported (Ireland *et al.*, 1996). Furthermore, the increase of toxicity associated with increased input of

xenobiotics may be subject to uncertainty in timing and is best assessed using *in situ* assessments of toxicity (Maltby *et al.*, 1995; Crane *et al.*, 1995). The assessment of toxicity in exposure-response relationships can be measured by sampling or deploying of animals along a suspected contamination gradient. The use of *in situ* assessment may involve the deployment of 'caged' animals, in which case there may be an effect of containment, or the assessment of natural populations, in this case the selection of control or reference sites with which to compare toxicant induced responses is necessary (Burton *et al.*, 1996).

The use of single-species tests may allow a more mechanistic approach to be followed, such that an elucidation of the mechanisms of toxicity can be made (Maltby and Calow, 1989). This may involve estimation of the toxicity of xenobiotic(s) in bioassay. A bioassay being a biological action in response to, or as the result of, a stimulus. In the context of the assessment of contaminant toxicity, it is an experiment in which a single-species is exposed in the laboratory to concentration(s) of the xenobiotic of interest. Stricter definitions are that the exposure is to samples of water or sediment from the field that contain suspected xenobiotics (Hill *et al.*, 1993).

The uptake and entry of xenobiotic contaminants into organisms will depend on the bioavailability of the substance which in turn depends on the physico-chemical properties of the substance and the environment. The accumulation of contaminants in the tissues of organisms is known as bioaccumulation. Some chemicals are known to accumulate in the tissue of organisms more than others (Phillips, 1980). Bioaccumulation of contaminants will be an important factor in toxicity, since organisms may accumulate a sufficient amount of the xenobiotic within the early stages of exposure to cause their eventual death. When such a threshold has been accumulated death may be inevitable even if the chemical is no longer present in the water (Williams *et al.*, 1984).

Bivalve mollusc species have been used in many studies of the uptake and effects of xenobiotics in the aquatic environment (Willows and Page 1993; Boryslawskyj

et al. 1987; Boryslawskyj *et al.* 1988). Their sedentary nature and filter feeding habit ensures that water-borne pollutants are readily in contact with the body tissues of the organism and thus are often accumulated. However, aquatic crustacean species are often in contact with both the water column and the substratum, and thus are likely to be in contact with both water-borne and sediment-bound xenobiotics. Indeed, accumulation and effects of xenobiotics have been reported in freshwater crustacean species (e.g. Thyband and Le Bras, 1988; Le Bras, 1990).

Environmental bioassays involve the use of biological systems of varying levels of complexity to predict or assess the impact of pollutants on ecosystems (Maltby and Calow, 1989). The use of single-species bioassays to assess the effect of toxic xenobiotics has been discussed above. Particular test results are used to make links between the effect on species and the effects on ecosystem health (Calow, 1989). Test organisms in predictive tests and indicators in assessments are often chosen because they are typical or sensitive (Cairns, 1986). However, particular effects caused by a chemical on an organism may not be any guarantee that these effects will be manifest with a different chemical (Maltby and Calow, 1989). Thus, in order to make more mechanistic links between the toxic action of a chemical and its biological effects an understanding of the effect of a chemical on particular biological systems is needed.

1.2 BIOMARKERS.

In general a biomarker may be considered as a biological response to a chemical or chemicals that gives a measure of exposure and sometimes also of effect (Weeks, 1995). Like all biological measurements, biomarker techniques integrate exposure in time and space, avoiding the difficulties of chemical concentration measurements due to differences in bioavailability (Peakall, 1994). The levels of biological response that can be considered, range from changes at the molecular level to the structure of communities and even to the structure and functioning of ecosystems (Peakall, 1994). Peakall and Walker (1994) stated that all biomarkers are markers of exposure and indeed all biomarkers demonstrate an effect of some

kind. Ernst and Peterson (1994) limited a biomarker definition to biochemical, physiological and morphological changes to measure exposure. Depledge and Fossi (1994) have argued that biomarkers are able to allow estimation of the departure from health of individual organisms and may give some indication of their 'fitness'. Although the discussion of the terminology suggests that there are many definitions for the concept of a biomarker (e.g. Van Gestel and Van Brummelen, 1996), the term is more commonly used in a more restrictive sense, namely physiological and biochemical sublethal changes resulting from individual exposure to xenobiotics. Biomarkers have been demonstrated at the physiological, tissue and cellular and molecular/ biochemical levels of organisation, and range from non-specific stress responses to highly specific markers of exposure (Huggett *et al.*, 1992).

Physiological markers of effect such as scope for growth (SfG) have been demonstrated, using fish (e.g. Warren and Davis, 1967), marine mussels (e.g. Bayne *et al.*, 1985) and freshwater crustaceans (e.g. Maltby *et al.*, 1990). SfG represents the difference between the energy gained from food and the energy lost by respiration and excretion. SfG values can be positive, indicating that energy is available for growth and reproduction, zero when energy input balances energy loss or negative when the animal must use its energy reserves for essential metabolism. SfG has been reported to be a reliable indicator of pollutant-induced stress (Donkin and Widdows, 1986; Maltby, 1994). When SfG is used to measure the impact of a stress, the observed changes in the energy budget are assumed to be closely related to changes in individual growth rate and fecundity (Naylor *et al.*, 1989).

The measurement of SfG has advantages over the direct measurement of growth and fecundity in that results can be obtained rapidly. Furthermore, SfG provides insight into which particular components of the energy budget are affected by the stress. Scope for growth can be reduced by a reduction in feeding rate, a reduction in absorption efficiency and/or an increase in respiration or excretion rates (Naylor *et al.*, 1989). The particular effects may vary with exposure to

different stresses. With regard to xenobiotics, Willows (1994) reported that different mechanisms of toxicity had differential effects on the components of SfG. For example, xenobiotics such as alcohols, esters and ketones exert non-specific narcotic effects that decrease feeding rate but have no apparent effect on respiratory energy loss. In contrast, some organic compounds such as pentachlorophenol that are known to inhibit ATP synthesis and cause metabolic inhibition, affect both respiration and feeding rate (Willows, 1994). A disadvantage of SfG is it is not specific to toxicant exposure and has been shown to indicate other environmental stresses (Bayne and Widdows, 1978). Thus, the interpretation of field experiments requires a large database on organisms from normal, unpolluted conditions and/or reference sites for comparison (Lagadic *et al.*, 1994).

Estimates of energy reserves have been used as markers of energy use, for example, Bhagyalakshari *et al.* (1984), Thomas *et al.* (1981), Baturu *et al.* (1995) have reported the estimation of glycogen concentrations and Holland (1978), Voogt (1983) reported the estimation of lipid concentrations. Glycogen represents the readily mobilisable storage form of glucose for most animals. Both glycogen level and glycogenolysis enzymes can be used as biomarkers of environmental stress and both increases and decreases in glycogenolysis can occur due to toxicant induced stress (Baturu *et al.*, 1995). However, the increased energy demand associated with stress usually results in depletion of glycogen reserves (Lagadic *et al.*, 1994).

Adenylate energy charge (AEC) can be considered as a measure of the metabolic energy available to an organism from the adenylate pool and is an indicator of the metabolic state of cells (Atkinson, 1977). AEC is a direct calculation based on the measured concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP). This biomarker is based on the use of energy by organisms under stress, such that a decrease in the concentration of ATP would be expected in animals under stress. AEC has been

reported as a useful indicator of physiological status in aquatic animals (Ivanovici, 1980; Haya *et al.*, 1983).

The disadvantages of energy yielding substrates and indices of energy use are that these, like SfG, are not necessarily indicative of toxicant induced stress.

Furthermore, in the case of AEC, they may be influenced by age, sex, season and geographical area (Giesy *et al.*, 1981; Dickson and Giesy, 1981; 1982).

Glycogen concentrations may be subject to differential mobilisation in tissue types (Baturu *et al.*, 1995) and may also be subject to the influence of intrinsic and extrinsic variability (Mayer *et al.*, 1992).

Histopathological biomarkers are lesions that signal effects resulting from prior or on-going exposure to one or more toxic agents. Individual lesion types are rarely pathognomic of exposure to a single toxicant (Hinton *et al.*, 1992). However, if an appropriate target organ is selected, and a higher frequency of lesions is correlated with analytical chemistry, these lesions can be used as biomarkers of effect. For example, cellular necrosis and cellular carcinoma of the liver in fish exposed to contaminants have been reported by Hendricks *et al.* (1984).

Although, most tissue damage in aquatic organisms is reported in fish, tissue damage due to xenobiotic exposure in invertebrates has been reported. For example, Blockwell *et al.* (1996a) report damage of the hepatopancreas of the freshwater crustacean *Gammarus pulex* exposed to the pesticide lindane.

Biomarkers at the sub-cellular level such as lysosomal disruption have been described (e.g. Moore *et al.* 1987, Weeks and Svendsen, 1996). Lysosomes are noted for their responses to many types of cellular injury and have also been shown to compartmentalise and accumulate a wide range of injurious chemicals (Moore *et al.* 1987). Moore *et al.*, (1987) reported that lysosomes react in a number of ways to injury, showing increases or decreases in lysosomal contents such as hydrolytic enzymes, and changes in the rate of membrane fusion events and lysosomal permeability. Weeks and Svendsen (1996) report the use of the method described by Lowe *et al.* (1992) for a histochemical staining technique

using neutral red stain in an earthworm species (*Lumbricus rubellus*). Loss of dye accumulated in isolated cells being indicative of toxic effect. Lysosomal membrane stability is affected by both chemical and non-chemical factors. Thus, this assay may have utility as an integrative biomarker of multiple stressors. However, the difficulty in interpreting alterations in lysosomal membrane stability due to xenobiotic exposure will be in discriminating them from changes due to non-chemical factors (Mayer *et al.*, 1992).

At the level of the molecule, alterations in DNA have been described in the assessment of exposure to genotoxic compounds. For example, DNA adducts are a type of structural change involving covalent attachments of a chemical or its metabolites to DNA. Several analytical procedures are currently used to monitor the formation of DNA adducts directly, the most prominent of these is the ³²P-postlabelling technique (e.g. Watson, 1987; Livingstone *et al.*, 1994). This technique has been reported for use in marine organisms (Livingstone, 1993; Livingstone *et al.*, 1994) showing the increased formation of bulky hydrophobic adducts with increasing levels of contaminant exposure.

Toxic chemicals may also cause secondary modifications to the DNA molecule. This may include strand breaks, changes in base composition or increases in DNA repair. Strand breaks have been detected in aquatic organisms exposed to hydrocarbons (Shugart, 1988). DNA alterations provide evidence of specific exposure that has passed many of the mechanisms that protect the cell from chemical damage (Shugart *et al.*, 1992). Thus, characterisation of specific DNA alterations may ultimately lead to the identification of a group of genotoxic chemicals of environmental concern. It is assumed that most DNA adducts and alterations are damaging, however, this assumption might not be entirely correct since some adducts might be readily repaired with out further consequences. Furthermore, some changes in DNA structure may not produce adverse effects (Shugart *et al.*, 1992).

1.2.1 Biochemical biomarkers.

Changes at the biochemical level may offer distinct advantages over changes at higher levels of biological organisation for two major reasons. Firstly, biochemical or molecular alterations are usually the first detectable, quantifiable responses to environmental change, including changes in the chemical environment. Secondly, alterations can serve as biomarkers of exposure and effect; by definition a chemically induced change in biochemical systems represents an effect of the chemical exposure (although not necessarily a harmful one).

A number of biomarkers at the biochemical level have been identified that are both specific and general in terms of their induction by chemical exposure or stress. Certain proteins can be produced in response to specific chemical exposure, such as metallothioneins and also to non-specific stress such as heat shock proteins. The stress protein response includes two major groups of gene products namely the heat shock proteins (hsp) and the glucose-regulated protein (grp) group (Welch, 1990). Synthesis of hsp is dramatically increased by exposure to heat and a variety of other physical and chemical stressors, while synthesis of grp is increased in cells by such factors as deprivation of glucose or oxygen. These two protein groups are closely related, having similar biochemical and immunological characteristics (Stegeman *et al.*, 1992).

Metallothioneins (MT) are small cysteine-rich proteins that can combine with a variety of metal ions and have been reported to occur in many invertebrate groups (Engel and Brouwer, 1984; 1987; Hamer, 1986). The synthesis of MT can be induced by exposure to metal ions such as cadmium, mercury, zinc and copper. Metallothioneins have been considered potential biomarkers for metal exposure in the environment (Stegeman *et al.*, 1992). The disadvantages of the use of metallothioneins as markers of metal exposure is that they may be induced by agents other than metals. Furthermore, some metals notably copper and zinc bound to metallothionein does not necessarily reflect exposure to these metals.

For example, it has been demonstrated that factors such as temperature and nutritional status may have profound effects on the copper/zinc ratios bound to metallothionein in marine invertebrates (Engel and Brouwer, 1987).

Biochemical biomarkers linked with specific mode of action have been identified, namely acetylcholinesterase (AChE) and non-specific cholinesterase (ChE). The activities of AChE and ChE are potential tools for assessing the effect of organophosphate and carbamate pesticides on vertebrates (Greig-Smith, 1991) and invertebrates (Edwards and Fisher, 1991). Other contaminants such as arsenic, copper and mercury, also exhibit a strong inhibiting action on ChE activity (Galgani *et al.*, 1992; Mayer *et al.*, 1992) and a weak effect on AChE (Bocquene *et al.*, 1995). Exposure to organophosphate pesticide has caused increases in inhibition of ChE in aquatic invertebrates. For example, the freshwater crayfish, *Procambarus clarkii*, showed a 10 % ChE inhibition on exposure to trichorfon (Repetto *et al.*, 1988). It is suspected that the existence of many isoforms of ChE and AChE coupled with possible resistance to organophosphate exposure may lead to variability in AChE and ChE inhibition (Edwards and Fisher, 1991). Thus, this may affect the use of these enzymes as biomarkers of pesticide exposure.

Detoxification enzymes.

Throughout their evolution animals have been exposed to plant and microbial secondary metabolites which they cannot completely metabolise and utilise for their own life processes (George, 1993). Many of these compounds are biologically active and are used as drugs (e.g. morphine and digitalis) or pesticides (e.g. pyrethrins). Enzyme systems enabling detoxification and consequent removal of xenobiotic chemicals have thus evolved. These enzyme systems tend to have relatively low activities and broad substrate specificities.

The metabolism of xenobiotic compounds can conveniently be divided into two phases; phase I and phase II. Phase I is the alteration of the original xenobiotic

molecule so as to expose or add on a functional group, the product can then be conjugated in phase II (Figure 1.1).

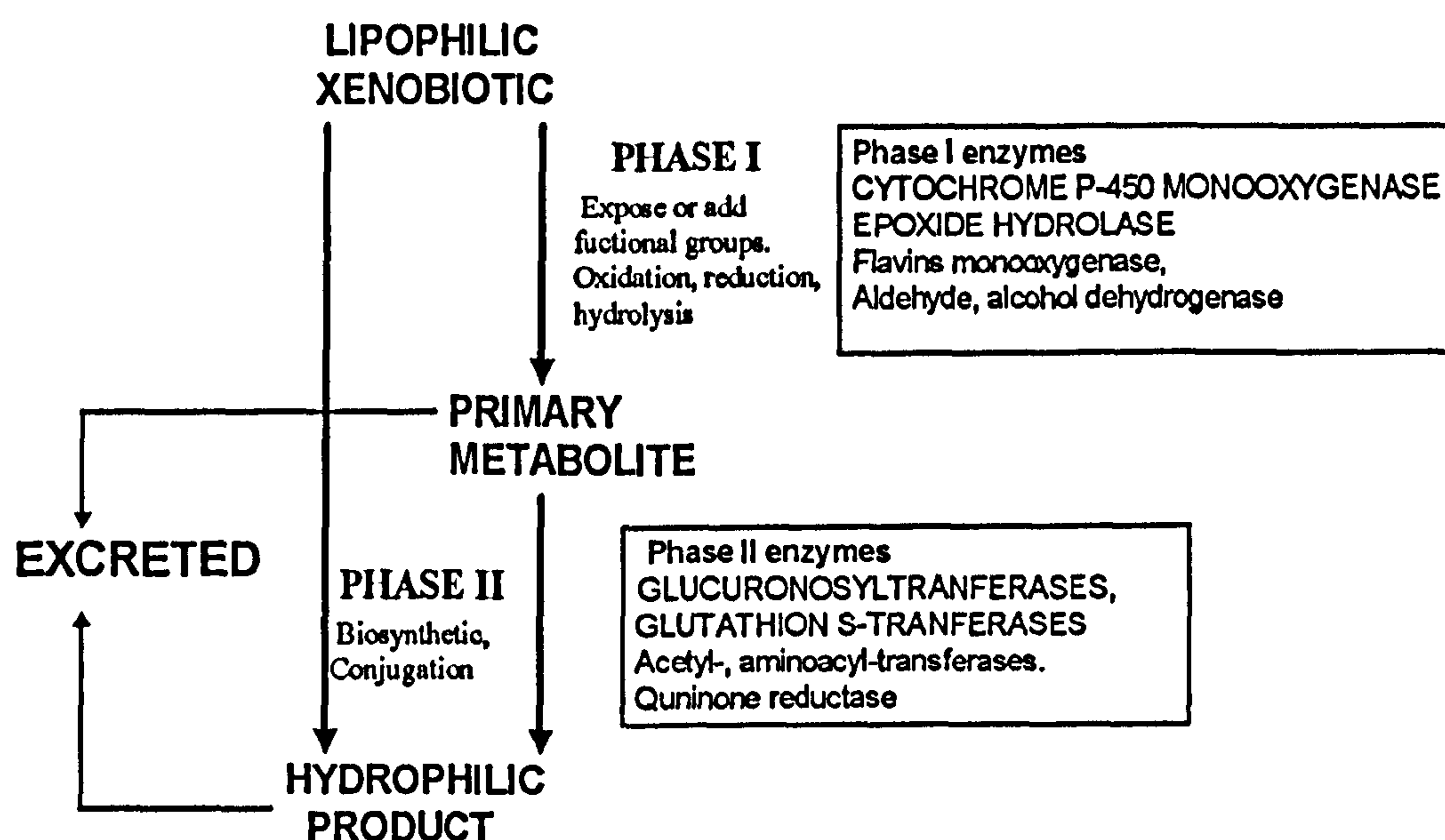


Figure 1.1. Phase I and phase II pathways of xenobiotic metabolism. Lipophilic xenobiotics may undergo phase I biotransformation and be directly excreted, or be further metabolised by phase II detoxification, or be directly conjugated in phase II detoxification, prior to excretion. After George (1993).

The major phase I reactions are oxidation, reduction and hydrolysis. The majority of oxidation reactions are catalysed by mono-oxygenase enzymes which are located in the smooth endoplasmic reticulum of the cell. They are thus known as microsomal enzymes, mono-oxygenases or mixed function oxidases (MFO). Other oxygenation enzymes are located in the mitochondria (e.g. amine oxidases) and the cytosol (e.g. xanthine oxidase and alcoholdehydrogenase). The microsomal reactions may be subdivided into: aromatic hydroxylation, aliphatic hydroxylation, alicyclic hydroxylation, heterocyclic hydroxylation, N-, S- and O-dealkylation, N-oxidation, N-hydroxylation, S-oxidation, desulphuration, deamination and dehalogenation (Ortiz de Montellano, 1986). The majority of the microsomal reactions are catalysed by one enzyme system; the cytochrome P450 mono-oxygenase system. The P450 system is a collection of isoenzymes all of which possess an iron protoporphyrin IX as the prosthetic group. Cytochrome

P450 are known to exist in a multiplicity of forms with overlapping substrate specificities. This multiplicity accounts for the diversity of the reactions catalysed and the substrates accommodated (Timbrell, 1991).

P450 systems in fish and mammals, have been shown to be induced by their substrates, and by compounds structurally related to their substrates (Stegeman, 1981; Kleinow *et al.*, 1987). Induction is the process by which a chemical increases the amount of enzyme, generally involving the synthesis of new messenger RNA and, subsequently, new enzyme protein. Either P450 enzyme activity, protein, or mRNA might be measured to detect induction (Stegeman *et al.*, 1992). The structure and function of microsomal cytochrome P450 proteins that metabolise foreign compounds have been studied most extensively in mammalian systems, primarily in the liver. Fish and invertebrate species possess microsomal P450 systems which are in many ways similar to those present in mammals (Bend and James, 1978; Stegeman, 1981; Lech *et al.*, 1982; Lee, 1982). The properties of MFO systems in marine invertebrates have been reported (Lee, 1982; Livingstone, 1989; James, 1989a; Livingstone, 1991). The induction P450s has been reported consistently in molluscs and crustaceans, the levels of cytochrome P450 in some species being comparable to those reported in teleost fish (Livingstone, 1989; James, 1989a).

Glutathione s-transferase.

Many xenobiotic compounds can be metabolised by phase II pathways (Figure 1.1), the major route being dependent upon the chemical nature of the compound and the characteristics of the enzymes. The major pathway for electrophilic compounds is conjugation with glutathione, while for nucleophilic compounds, conjugation with glucuronic acid is the major route. Glutathione is one of the most important molecules in the cellular defence against toxic compounds (Hinson and Kadlubar, 1988). This protective function is due in part to its involvement in conjugation reactions (Timbrell, 1991). Glutathione is a tripeptide composed of glutamic acid, cysteine and glycine (glu-cys-gly). The presence of the cysteine provides a sulphhydryl group, which is nucleophilic and so glutathione

will react probably as the thiolate ion (GS⁻), with electrophiles (Douglas, 1988). These electrophiles may be chemically reactive, metabolic products of a phase I reaction, or they may be more stable xenobiotic compounds. Glutathione thus protects cells by removing reactive metabolites by conjugation. The conjugating moiety (glutathione) is not activated in some high energy form, whereas the substrate often is in an activated form. Glutathione conjugation may be enzyme-catalysed reactions or simply a chemical reaction. The glutathione conjugation produced by the reaction may then be either excreted or the conjugate further metabolised (Mannervik, 1985). Enzyme catalysed glutathione conjugation involves the enzyme glutathione s-transferase (GST) in the initial conjugation of the electrophilic compound with glutathione (Figure 1.2). This involves several steps; the removal of the glutamyl and glycyl groups and acetylation of the cysteine amino group to yield a mercapturic acid (Figure 1.2) or N-acetylcysteine conjugate.

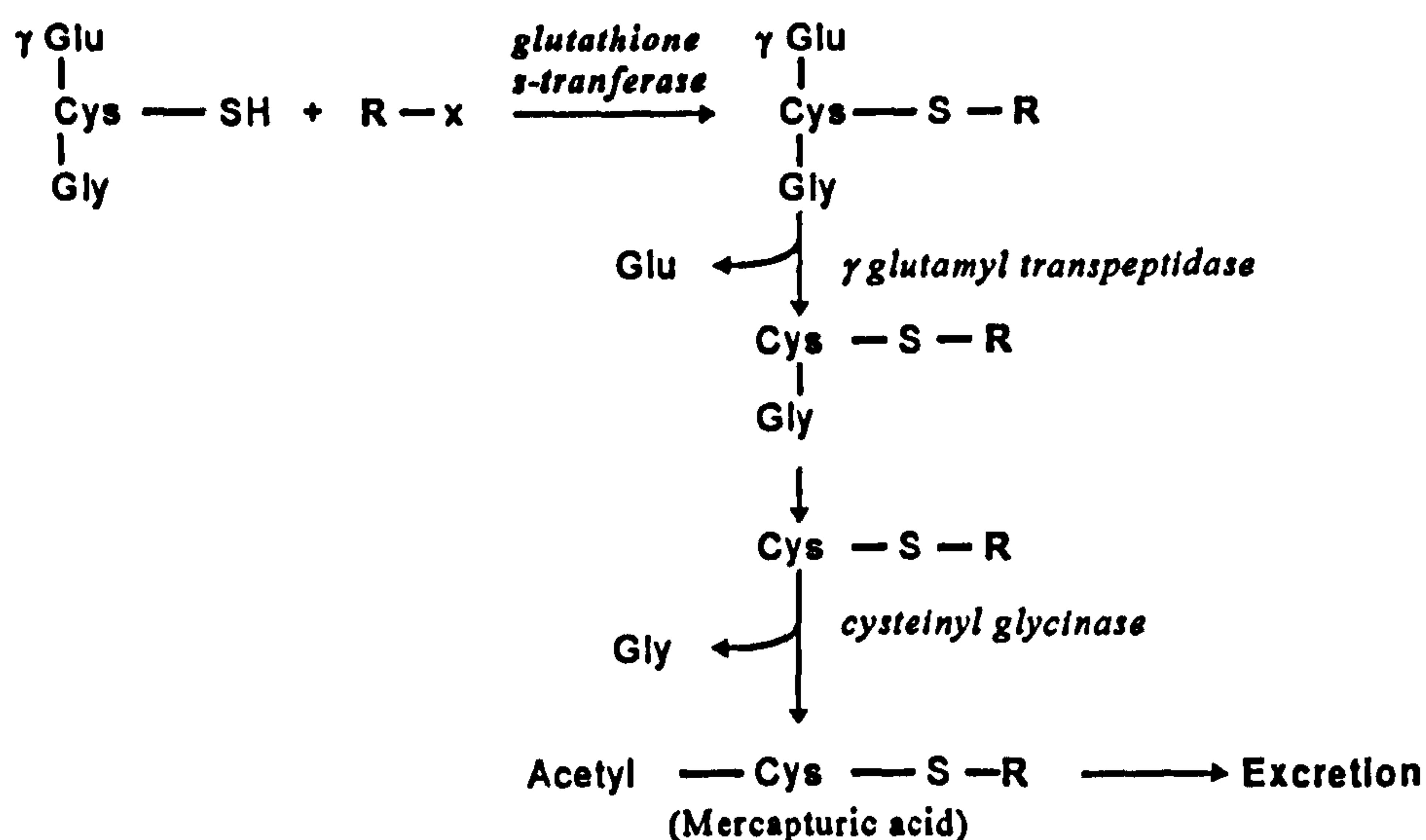


Figure 1.2 Mercapturic acid pathway for excretion of GSH conjugates. Modified from Mannervik (1985).

The GSTs (E.C. 2.5.1.18) are a multigene superfamily of dimeric, multifunctional, primarily soluble enzymes. They occur ubiquitously, having been identified in prokaryotes, yeasts, higher plants, molluscs crustaceans, insects, fish amphibians and mammals (Stenersen *et al.*, 1987). The GSTs are believed to play major roles in isomerisation of steroids and selenium-independent GSH

peroxidase activity towards organic peroxides including steroid and DNA hydroperoxides as well as in the irreversible binding of reactive electrophilic xenobiotics such as azo-dyes carcinogens and polycyclic aromatic hydrocarbons (PAHs) (George, 1993). Moreover, of particular interest in the use of GST as an enzymic biomarker is the involvement of GST in the catalysis of glutathione (GSH) conjugation with electrophilic centres in a variety of compounds as the first step in the formation of mercapturic acids (Figure 1.2).

Cystolic GST is made up of a number of distinct isoenzymes that can be divided up into alpha (α), mu (μ), pi (π) and theta (θ) groups (Ketterer *et al.*, 1988). With the exception of microsomal GST (Morgenstern *et al.*, 1990), the GSTs are soluble, dimeric proteins (comprised of two protein sub-units) (Ketterer *et al.*, 1988). Sub-units from the same class form heterodimers, share 75 -95 % amino acid sequence identity, and have cDNAs that cross-hybridise; sub-units from the different classes do not form heterodimers (Ketterer *et al.*, 1988; Mannervik, 1985; George 1993). With the exception of θ -class enzymes all other GSTs conjugate the artificial substrate 1-chloro-2,4-dinitrobenzene (CDNB). A further class of GST is found located on the endoplasmic reticulum of the cell, microsomal GST is structurally distinct from cystolic GST (Morgenstern and Depierre, 1988). While the microsomal and cystolic GSTs conjugate a similar spectrum of substrates such as CDNB, the microsomal GST is most notable for its peroxidase activity, which is a strong indication for a functional role in inhibition of lipid peroxidation (Morgenstern and Depierre, 1988; Morgenstern *et al.*, 1990).

It is clear that GST, because of its ubiquity in nature, its broad substrate specificity and induction as well as its almost universal conjugation with CDNB, can be used a biomarker of a number of organic xenobiotics. However, induction of increased GST activity may be indicative of exposure but not necessarily of detrimental effect.

Detoxification enzymes as biomarkers.

Enhanced levels of detoxification enzymes provide direct evidence of exposure to xenobiotics. In the biomarker area, invertebrate multi-function oxidases (MFO) and glutathione s-transferase (GST) have received much attention (Lagadic *et al.*, 1994). GST is characterised by substrate specificity to a large range of organic xenobiotics (Clark, 1989a). It is involved in the metabolism of different classes of organic xenobiotics such as polycyclic aromatic hydrocarbons (PAH) and pesticides (Boryslawskij *et al.*, 1988; James, 1989b). These characteristics render GSTs as potentially suitable as specific stress indices for organic xenobiotic pollution. Induction of GST in various marine invertebrates from polluted areas has been reported (e.g. Rodriguez-Ariza *et al.*, 1992; Sheehan *et al.*, 1991) Fewer studies have been carried out on freshwater invertebrates although increased GST activity in molluscs on exposure to single classes of contaminant have been reported (Blat *et al.*, 1988; Boryslawskji *et al.*, 1988; Beverly, 1994; Baturu and Lagadic, 1996).

1.2.2 Biomarkers of exposure and effect.

Homeostasis can be considered to be the maintenance of bodily function within a range of 'normal' parameters for the organism (Depledge *et al.*, 1992). For many physiological processes invertebrates do not maintain true homeostasis, but tend to conform to environmental changes such that the net effect of the flux on particular physiological systems is stability, or a tendency toward it (Magnum and Towle, 1977). Depledge (1989) adapted a hypothesis from the field of pollutant monitoring in occupational health (Hatch, 1962) to describe the departure from homeostasis and the eventual onset of disease in organisms due to xenobiotic exposure in the aquatic environment. The original hypothesis of Hatch (1962) was concerned with early disturbances that may be the precursors of human disease, this concept is illustrated in Figure 1.3 in which a distinction is made between impairment and disability.

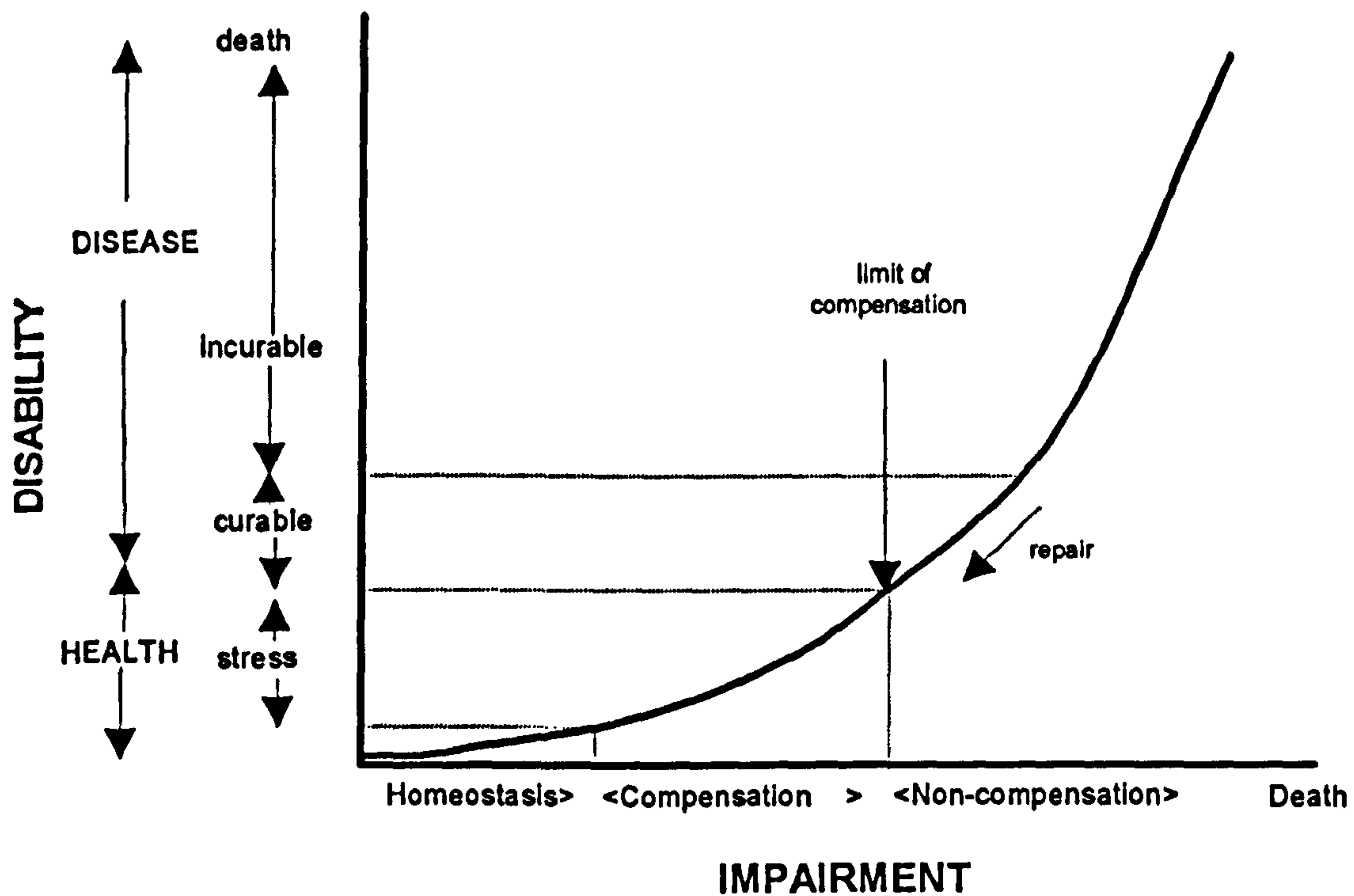


Figure 1.3 Impairment and disability as indicators of xenobiotic toxicity. After Hatch (1962) as modified by Depledge (1989).

The scales represent the underlying disturbance of the system (impairment) and the consequence of such disturbance in terms of identifiable disease (disability). Starting with 'normal' health, there is a progression with increasing exposure (e.g. to a chemical) along the scale of impairment and of disability, ultimately to death. Early departures from health (impairment) are accompanied by little disability and normal homeostatic processes insure adequate adjustment to offset stress. Beyond this zone of early change, compensatory processes maintain the overall function of the system without serious disability. However, further increase in impairment beyond the limits of compensation are accompanied by a rapid increase in disability and consequent movement into the zone of disease (Figure 1.3). Hatch (1962) makes the point that as increasingly sensitive measures of response on the impairment scale are selected to demonstrate disturbance, these become increasingly non-specific. Furthermore, such non-specific disturbances only have meaning after it is demonstrated that the exposed population have a higher incidence of disturbance than the general population. Moreover, it was put forward by Hatch (1962), that the particular terminal disease state may not, itself, bear a direct relation to the environmental stress.

Depledge (1989) used an adaptation of Hatch's hypothesis (Hatch, 1962), to describe departures from physiological and behavioural functioning in invertebrates. Indeed, Depledge (1989) used an example of a macroinvertebrate to illustrate the use of Hatch's hypothesis for the detection of the early effects of pollutants. "A crab exposed to metal pollution will depart from a healthy condition and there will be compensation by the activation of excretory and detoxification mechanisms until the limit of compensation is exceeded. At this stage physiological and behavioural processes fail (osmoregulation may be disturbed and feeding may cease) and the animal is disabled. Unless rapidly returned to a clean condition death ensues" (Depledge 1989).

This hypothesis can also easily be applied to the use of biochemical biomarkers. Indeed, Depledge and Fossi (1994) illustrated that it could be used to describe departures from ecosystem integrity using biochemical and physiological biomarkers of exposure and effect. It is assumed that a healthy individual exposed to increasing pollutant load will suffer a progressive deterioration in health which is eventually fatal (Figure 1.3). The shape of the curve in Figure 1.3 shows that early departures from health are not apparent as overt disease, but are associated with the initiation of biochemical and physiological responses. When compensatory responses are activated, the survival potential of the organism may already have begun to decline because the ability of the organism to mount compensatory responses to new environmental challenges may have been compromised. Figure 1.3 illustrates that if an organism has acquired a pollutant load that cannot be tolerated, detoxified or excreted, then pathological processes will result in the development of overt disease and finally death. Beyond the limit of compensation it is unlikely that any response to newly arising environmental challenges could be mounted successfully. However, provided conditions improve sufficiently and quickly enough, an organism that has not moved too far into the non-compensatory zone may still be able to recover if repair mechanisms can restore compensatory responses (Figure 1.3).

In the context of the use of biochemical biomarkers of exposure to xenobiotics in the present study, initial departures from homeostasis would be detected by increased activity of GST. Furthermore, induction of increased GST activity is one of the first mechanisms to counter exposure to xenobiotic as it is a detoxification enzyme. Thus, increased GST activity is expected to be induced at low levels of impairment. As Figure 1.3 shows the impairment scale is more sensitive to pollutant effect than the disability scale, thus, monitoring impairment of a biochemical response such as GST activity will, in theory, provide 'early warning' of the onset of disabilities. By using physiological markers of exposure and stress such as scope for growth and use of energy reserves such as glycogen, an assessment of the compensatory mechanisms (or at least the amount of energy uptake by those mechanisms) that counter the effect of continued exposure is given. Moreover, the sensitivity of GST as a biomarker of exposure and thus early warning of a departure from normal functioning (homeostasis) can be assessed.

1.3. LINKING LEVELS OF ORGANISATION.

From an ecological point of view, environmental changes at community and ecosystem levels are mostly assessed by the use of indicators of community or ecosystem change rather than biomarkers (Lagadic *et al.*, 1994). Furthermore, risks to an ecosystem and its components are expected to increase as the amount of pollutant entering the system increases (Depledge and Fossi, 1994). There will be a degree of self-compensation in each ecosystem which will tend to preserve structure and function to some extent (Webster *et al.*, 1975; Harwell *et al.*, 1978). This is analogous to the compensatory responses exhibited by individual organisms exposed to pollutants (Depledge, 1989). Conceptually, a suite of biomarkers and higher level response indicators can provide evidence to test hypotheses about the linkage between exposure to xenobiotics and ecologically relevant effects. The rationale for this approach is indicated in Figure 1.4, which illustrates the relationship between responses at different levels of biological organisation and the relevance and times scales of responses.

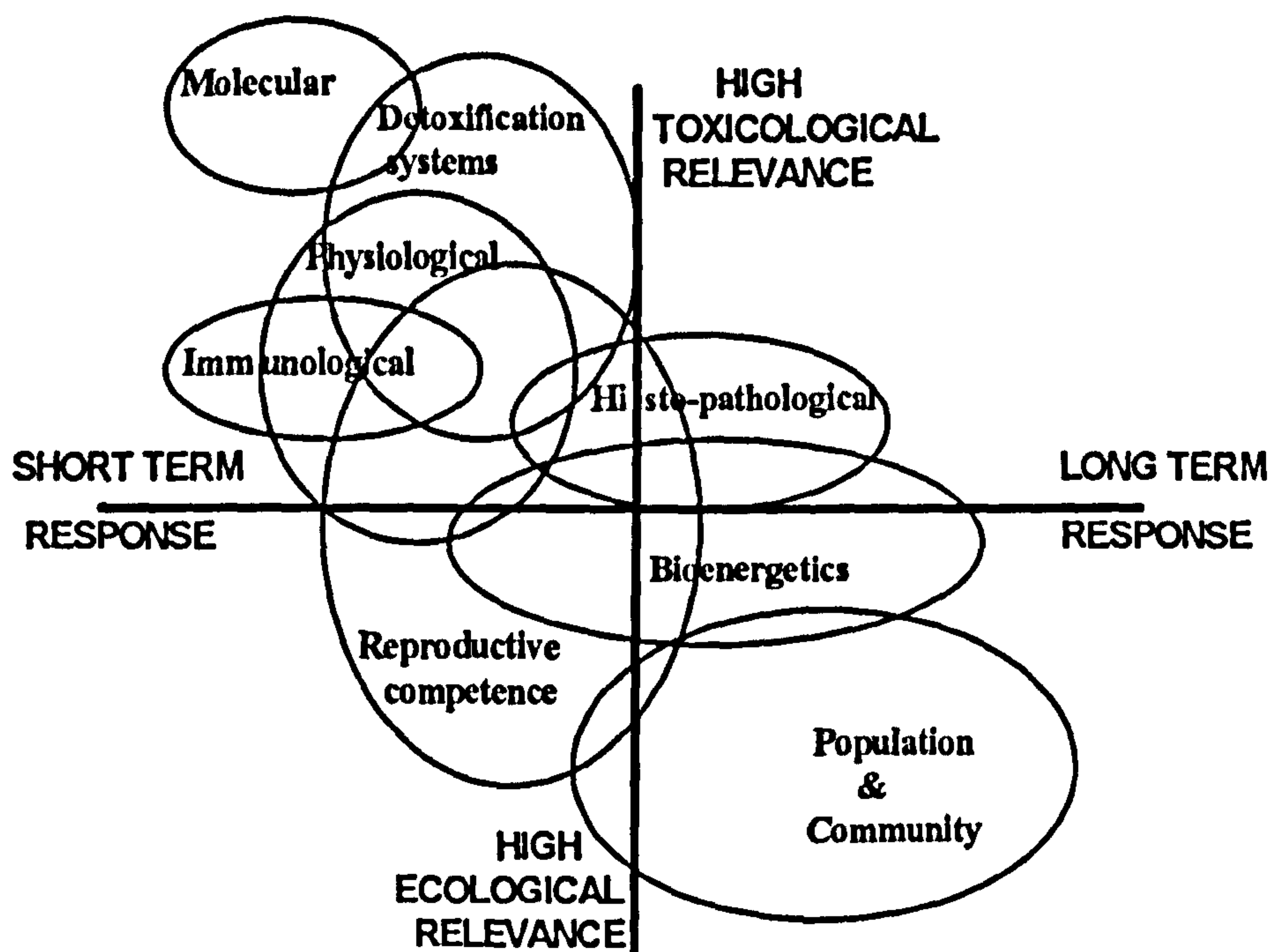


Figure 1.4 The relationship between responses at levels of biological organisation and the relevance and time scales of responses. Adapted from Adams *et al.* (1989).

Responses measured at the lower levels of biological organisation such as DNA damage or enzyme activity often provide sensitive and specific responses to particular toxicants. These biomarkers therefore offer advantages as measures of exposure, and may be diagnostic of the type of contaminant to which the organism is exposed (McCarthy and Shugart, 1990). However, the biological significance to the overall structure and function of a population or ecosystem is unclear. Conversely, responses at higher levels of biological organisation, such as changes in population abundance or species diversity are directly relevant to concerns about ecological effects, but cannot by themselves, prove whether differences among sites are due to pollutants or to natural ecological factors. It is illustrated in Figure 1.5 that changes at the molecular and biochemical level are usually the first detectable and therefore most sensitive responses which are quantifiable, to xenobiotic exposure. Responses at the organism level are less sensitive but have more ecological relevance. Effects at the population level are more relevant to changes at the ecological level. However, responses showing

increasing relevance to the ecological level are the least sensitive of responses (Figure 1.5).

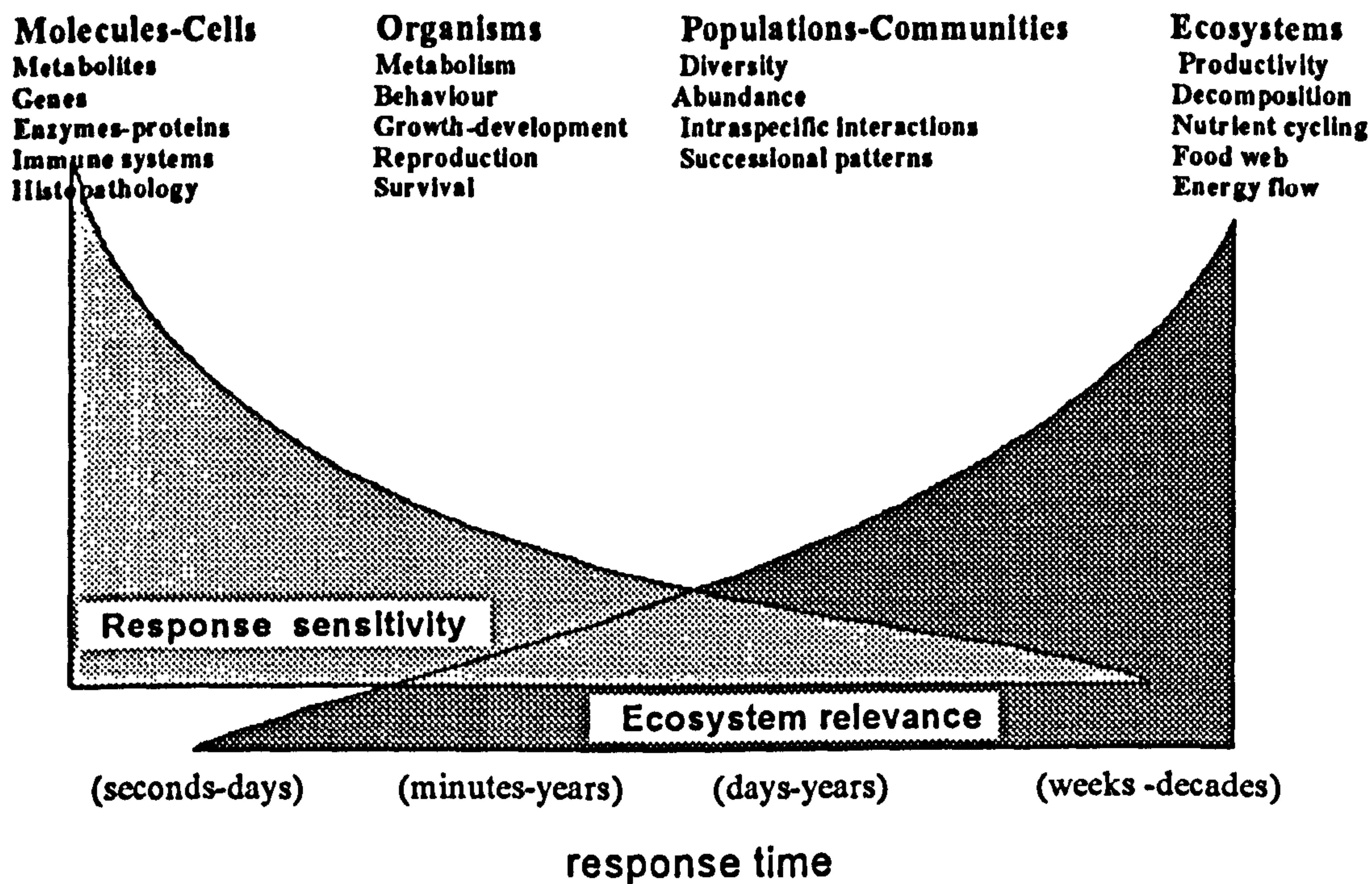


Figure 1.5 General relationship between biological endpoints, level of toxicant sensitivity, ecosystem relevance and response time. After Burton (1991).

A biological response to chemical exposure is expected to follow a concentration dependent or 'dose response' relationship. It is clear that under a set of constant conditions there will be a varying response to varying chemical concentration. However, under non-constant conditions the effects of chemical exposure may be depressed or enhanced by other variables from temperature to the effect of other chemicals. These other variables may greatly confound extrapolation of conclusions gleaned in the laboratory to field situations. Within any exposure scenario, dose-response relationships are expected, but each set of exposure conditions may give rise to a different relationship and in a fluctuating environment that may be constantly changing (Depledge *et al.*, 1992). However, the identification of the early onset of changes in otherwise healthy organisms may represent a departure from homeostasis and with continued exposure the onset of disease. This represents a more mechanistic approach to linking levels of organisation in their response to toxicant exposure. Early detection of

exposure to xenobiotics using GST activity marks the detection of exposure and the induction of a protective mechanism that counters exposure. Exposure will have an energetic cost because of the increase in metabolism involved with compensation systems induced by exposure including synthesis of enzyme protein. This energetic cost can be measured by SfG. The use of stored energy to counter the increased use of energy can be estimated by measuring glycogen concentration. Finally, by measuring changes in the energy available for growth and reproduction in an organism, a direct link is made between physiological function and effect at the population level. Furthermore, the use of a species that is important in the turnover of energy input into an ecosystem may link changes in population to changes in ecosystem functioning.

1.4 AIM OF THIS STUDY.

Organic xenobiotic contamination in freshwater streams may exert a toxic effect on biota at all levels of biological organisation. Biochemical and physiological markers can be used to assess exposure and effects at sublethal concentrations. A biochemical biomarker can be used to assess exposure to specific pollutants and physiological markers can be used to assess the effects of pollutants on energy balance. The main aim of this study was to assess the use of the detoxification enzyme, GST, as a biomarker of organic xenobiotic exposure and effect and to relate toxicant-induced changes in GST to changes in energy status and physiological energetics.

In order to link the changes at the biochemical level (GST) and physiological level (SfG) to changes that may be manifest at the community and ecosystems levels, a test species that has an important role in the functioning of the ecosystem should be selected. The use of macroinvertebrates in the assessment of aquatic pollution has been discussed (section 1.1.2). The relative sedentary nature of benthic freshwater macroinvertebrates gives the advantage of exposure of individuals from the same population in the same site which may then be sampled. The use of macroinvertebrate populations for biomarker measurements has the advantage that populations are often numerous, so that samples can be readily

taken for analysis without significantly impairing population dynamics (Depledge and Fossi, 1994).

In order to use a single species in any assessment of toxicity it is desirable that its selection is based on a number of criteria which are dependent on the objective of the assessment (Hill *et al.*, 1993). In this study the measurement of a biochemical marker of xenobiotic exposure (GST) in laboratory and field animals is related to the measurement of physiological markers of effect (SfG and glycogen concentration). Based on these objectives a species is to be selected that is available in large numbers throughout the year in freshwater streams and which may be maintained under laboratory conditions. As determination of biochemical parameters are likely to be effected by life-stage (Lagadic *et al.*, 1994), ease of identification of life-stage is important. Furthermore, the functional role of species in the ecosystem is of importance as effects on the organism will determine ecosystem effects (Maltby, 1992).

The freshwater amphipod *Gammarus pulex* (L.) has a wide distribution in alkaline streams in northern Europe (Gledhill *et al.*, 1976). It is regarded as one of the most important invertebrate species in chalk streams in terms of biomass (Westlake *et al.*, 1972) and is an important source of food for fish (Smyly, 1957; Maitland, 1966; Welton, 1979). *Gammarus pulex* consumes coarse particulate organic material (CPOM) and in the process of feeding and egestion produces fine particulate organic material (FPOM). The CPOM it utilises may be allochthonous or autochthonous. In many small streams the major energy input is allochthonous leaf material which can be incorporated into animal biomass, via the action of shredders such as *G. pulex* (Willoughby and Sutcliffe, 1976; Welton, 1979; Willoughby and Earnshaw, 1982). Thus, *G. pulex* has a key role in the incorporation of terrestrially fixed organic material into the freshwater food web (Maltby, 1994). Any disruption of this process, either through reduced abundance or reduced feeding activity, could have considerable implications for the structure and functioning of the ecosystem as a whole (Maltby, 1992).

As well as its importance in stream ecosystems, much information is available on the biology, feeding habits and population dynamics of *G. pulex* (e.g. Sutcliffe *et al.*, 1981; Welton *et al.*, 1983; Gee, 1988). Moreover, *G. pulex* has been the subject of many toxicity tests on a number of chemical classes including heavy metals (e.g. McCahon and Pascoe, 1988), organic chemicals (e.g. Taylor *et al.*, 1991) and pesticides (e.g. Stephenson, 1983). The effect of different classes of chemical at sublethal concentrations has also been reported for metals (e.g. Maltby and Naylor 1990) and organics (e.g. Malbouisson *et al.*, 1995; Blockwell *et al.*, 1996b).

1.4.1 Hypotheses and approach.

Exposure to toxic chemicals in the environment may pose a serious threat to life and organisms possess detoxification mechanisms involving inducible metabolic processes to render many poisons harmless. There is a metabolic cost in maintaining mechanisms for detoxification of such chemicals. Detoxification enzymes systems will be one of the first systems to respond to organic xenobiotic exposure, this is because they are induced by these chemicals, and are directly involved in their metabolism. Measurement of the extent of induction can therefore be used as a marker of exposure to xenobiotic chemicals.

The increased activity of detoxification enzymes are expected to precede a departure from normal metabolic functioning, damage and disease and therefore act as an early signal of the onset of a continuum of impairment to the functioning of the organism that would be apparent with increased exposure.

The production of protective proteins such as detoxification enzymes, will necessitate protein synthesis. This will demand a greater amount of energy from a finite energy resource acquisition. Resource acquisition is finite because either the resource is finite and/or an organism's mechanisms for uptake of energy will always limit its capacity for uptake and thus the amount of resource it can use (Calow and Townsend, 1981). Therefore, increased allocation of energy to the production of protective proteins will result in decreased allocation of resource energy to other processes, such as storage, growth and reproduction. The

energetic cost of exposure will include the energetic cost of detoxification enzyme (and other protective proteins) production as well as the cost of repair mechanisms (when protective mechanisms can no longer render the xenobiotic harmless). The increased amount of energy expenditure due to xenobiotic exposure at a particular level may be estimated by the measurement of the component parts of energy uptake and expenditure.

As detoxification enzymes are one of the mechanisms of tolerance to xenobiotic exposure, animals subject to xenobiotic exposure are expected to increase activity of these enzymes by induction and this will incur an associated energetic cost. The amount of induction is expected to be related to the amount of chemical exposure. Exposure over a number of generations may select animals able to sustain higher levels of detoxification enzyme (i.e. adaptation). Conversely, low level exposure may induce increased levels of detoxification enzyme that enable increased induction on further exposure by a process of acclimation.

In this study the effect of exposure to organic xenobiotics on GST activity in an organism that plays a key role in the functioning of freshwater stream ecosystems, namely *Gammarus pulex*, was determined. By investigating the effect of exposure on energy acquisition and expenditure, the effects of exposure could be related to the energetic cost of exposure, the cost of GST production being incorporated into these energetic costs. The effect of xenobiotic exposure can be related to changes at the population level, because estimation of energy expenditure and acquisition allows estimation of the amount of energy available for growth and reproduction. Long-term exposure to xenobiotic will lead to increased GST activity that may allow an increased tolerance of xenobiotic exposure. Increased GST activity in animals exposed in the field over a number of generations may be the result of selection of increased induction of GST or acclimation by pre-exposure.

It was predicted that on short-term exposure to sublethal concentrations of xenobiotics, there would be an increase in GST activity and that this would be

detected under laboratory and natural conditions. A second prediction was that the increased expenditure of energy due to exposure would be measurable using SfG and the increased use of energy would also be reflected in the depletion of glycogen energy reserves as less energy was allocated to storage.

The measurement of GST activity in animals from sites historically exposed to xenobiotic for a number of generations allows the assessment of GST as a marker of xenobiotic contamination. It was predicted that *G. pulex* from contaminated sites would possess greater GST activity than animals from clean, non-contaminated sites. The amount of induction of GST in contaminated site animals would be dependent on the amount of contamination in the site. It was predicted that the removal of the inducing chemical (i.e. the contaminant) would lead to similar GST activity in animals from both sites after long-term holding in clean conditions. Conversely, it was predicted that long-term exposure to low 'environmental' concentrations of xenobiotic would lead to similar increased activity in animals from both sites. The energetic cost of long-term exposure can be measured using SfG and the consequent effect on the allocation of energy to storage (energy reserves) estimated by measuring glycogen concentration. It was predicted that long-term exposure increased the demand on acquired energy because of an increased metabolic energy cost due to the production of protective proteins systems one of which is GST. Increased further exposure after long-term exposure would lead to increased energy expenditure due to protein production and repair as protective enzyme systems are no-longer able to cope with exposure and prevent tissue damage. Furthermore, it was predicted that increased production of GST would enable a greater tolerance of further exposure limiting the onset of tissue damage and therefore repair. Thus, the energetic cost of further exposure would not be as great in these animals. Specific objectives were set to test these predictions.

1. To establish a method for the detection of GST activity in *Gammarus pulex*, to evaluate the effects of environmental variables on GST activity and to establish a baseline of GST activity (Chapter Two).

A rapid and precise method to measure GST in individual *G. pulex* was developed. The effects of body size, environmental temperature and feeding on GST activity were assessed.

2. To describe the GST response on exposure to organic xenobiotics (Chapter Three).

The generality of the GST response to organic xenobiotics was assessed in the laboratory using the organochlorine pesticide lindane and the surfactant alcohol ethoxylate. The detection of the response under natural conditions was assessed using an outdoor mesocosm.

3. To assess the use of GST as a biomarker of pesticide exposure in the field (Chapter Four).

The GST activity of animals collected from reference and pesticide contaminated sites was compared both in the field and on further laboratory exposure to pesticide. The difference in GST activity between animals from different sites was investigated by long-term exposure to pesticide in the laboratory in order to elucidate whether differences were the result of acclimation or adaptation.

4. The use of a measure of energy balance and energy reserves in order to calculate the energetic cost of short-term and long-term pesticide exposure on *G. pulex* (Chapter Five).

SfG and glycogen concentration were measured on short-term exposure to pesticide to investigate the energetic cost of exposure. The effect on these physiological parameters (SfG and glycogen concentration) was determined on longer term exposure to elucidate the energetic cost of exposure to 'environmental' concentrations of pesticide.

CHAPTER TWO

Measuring the activity of the detoxification enzyme glutathione s-transferase in *Gammarus pulex*.

2.1 INTRODUCTION.

2.1.1 Glutathione s-transferase activity in crustaceans.

Glutathione s-transferase activity has been found in all vertebrates and invertebrates thus far studied (Stenersen *et al.*, 1987). In addition the activity of GST has been reported in prokaryotes (Sheehan and Casey, 1993) and plants (Marrs, 1996). GST activity has been demonstrated in a number of crustacean species (Table 2.1), and of particular relevance to this study is the work of Aceto *et al.* (1991) and Dierickx (1984) who studied the *Gammarus* species, *Gammarus italicus* and *Gammarus pulex*, respectively.

Table 2.1 GST specific activity in crustacean species. Activities shown with the substrate 1-chloro 2,4-dinitrobenzene (CDNB). Animals assayed are whole body extracts except hepatopancreas (h) and gill tissue (g). Error (where shown) is standard error of the mean (*) or one standard deviation (§).

Species	Specific activity µmole/min/mg protein	Reference
<i>Daphnia magna</i>	0.6 ± 0.1 *	LeBlanc & Cochrane (1985)
<i>D.magna</i>	0.38 ± 0.02 *	LeBlanc <i>et al.</i> (1988)
<i>Ceriodaphnia reticulata</i>	0.6 ± 0.04 *	LeBlanc & Cochrane (1985)
<i>Artemia salina</i>	3.5	Stenersen <i>et al.</i> (1987)
<i>Oniscus asellus</i>	4.0	Stenersen <i>et al.</i> (1987)
<i>Callinectes sapidus</i>	0.46 ± 0.73 (h) § 0.44 ± 0.17 (g) §	Tate & Herf (1978) Stenersen <i>et al.</i> (1987)
<i>Eupagurus bernhardus</i>	3.05	
<i>Carcinus maenas</i>	0.25 ± 0.03§	Lee (1988)
<i>Procambarus clarkii</i>	0.63 ± 0.22§	Almar <i>et al.</i> (1987)
<i>Gammarus italicus</i>	0.85	Aceto <i>et al.</i> (1991)
<i>Gammarus pulex</i>	0.21	Dierickx (1984)

It can be seen from Table 2.1 that most of the specific activities of GST are within an order of magnitude. Notable exceptions are *Artemia salina*, *Oniscus asellus* (Stenersen *et al.*, 1987) and *Eupagurus bernhardus* (Tate and Herf, 1978) which show higher activities. It should be noted that activities are reported as specific activity (i.e. per milligram protein) therefore activities may vary as a result of the purity of the enzyme extract. The specific activity of GST for *G. pulex* as reported by Dierickx (1984) was 0.21 $\mu\text{g}/\text{min}/\text{mg}$ protein and thus the lowest value of those reported in Table 2.1.

2.1.2. Isoenzymes of glutathione s-transferase.

GST may exist as isoenzymes belonging to four subfamilies, namely alpha, mu, pi and theta (Ketterer *et al.*, 1988). These different isoenzymes can be differentiated by their activity with a number of model substrates (Habig and Jakoby, 1981a). The subfamilies or classes of GST show differential activity with these model substrates (Table 2.2).

Table 2.2. Presence of activity of subfamilies and isoenzymes of GST with specific model substrates. Subfamilies are named according to the scheme of Ketterer *et al.* (1988) and isoenzyme class (Mannervik *et al.*, 1985). Modified from Egaas *et al.* (1993).

Substrate	Subfamily	Isoenzyme class	Reference
1-chloro-2,4-dinitrobenzene (CDNB)	all	all except 5-5 & 9-9	Habig <i>et al.</i> (1974)
1,2-dichloro-4-nitrobenzene (DCNB)	mu	3-3, 3-4	Ketterer (1988)
ethacrynic acid (ETHA)	pi	7-7	
1,2-epoxy-3-(p-nitrophenoxy) propane (ENPP)	theta	5-5	
Coumene hydroperoxide	alpha,theta	2-2, 5-5	

Almost all isoenzymes show activity with 1-chloro-2,4-dinitrobenzene (CDNB) (Habig *et al.*, 1974; Jakoby and Keen, 1977; Ketterer *et al.*, 1988; Mannervik, 1985; Clark, 1990) and has been consequently referred to as the 'universal' (Ketterer *et al.*, 1988) or 'general' (Mannervik, 1985) substrate. CDNB is widely used to assay total GST activity but,

because of its low specificity, is less suitable for detecting specific isoenzymes (Clark, 1990).

GST activity is dependent upon the substrate used in the assay (Table 2.3). Tate and Herf (1978) measured GST activity in the blue crab (*Callinectes sapidus*) using CDNB, DCNB, p-nitrobenzyl chloride and epoxide, ENPP (1,2-epoxy-3-[p-nitrophenoxy] propane). Activity was demonstrated with each of these substrates but was greatest for CDNB. Activity using CDNB was an order of magnitude greater than for PNBC and two orders of magnitude greater than for ENPP and DCNB. Stenersen *et al.* (1987) investigated activity toward the substrates CDNB, DCNB and ETHA in 72 species representing nine phyla, three species of which were Crustacea. GST activity was demonstrated with CDNB showing it to be two orders of magnitude higher than with ETHA or DCNB in *Artemia salina*. These differences are even greater in *Oniscus asellus* and *Eupagurus bernhardus* which only exhibited activity with CDNB (Table 2.3). LeBlanc and Cochrane (1985) found that whereas activity with CDNB was comparable between two species of cladoceran, with ETHA, *Daphnia magna* exhibited nearly twice the activity of *Ceriodaphnia reticulata* (Table 2.3). They concluded that *Daphnia magna* and *Ceriodaphnia reticulata* produce two enzymes, one primarily involved in CDNB conjugation and the other in ETHA conjugation and that the relative abundance of these two isoenzymes varied between the two species. There is also evidence for the presence of two isoenzymes of GST in *Gammarus italicus* namely GST II and GST III (Aceto *et al.*, 1991). These isoenzymes were isolated by GSH affinity chromatography and chromatofocusing. Furthermore, using whole animal cytosolic fraction, Aceto *et al.* (1991) demonstrated that these isoenzymes were both active with CDNB as a substrate (Table 2.1).

Table 2.3. GST activity with different specific substrates in some species of Crustacea. Substrates shown are 1-Chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (ETHA), 1,2-Dichloro-4-dinitrobenzene (DCNB), 1,2-Epoxy-3-(p-nitrophenoxy)propane (ENPP) and p-nitrobenzyl chloride (PNBC). Activities are from whole body extract except where indicated from specific tissues, hepatopancreas (h) or gill (g). Error (where shown) is indicated * standard error of the mean and § one standard deviation. Where no activity for a substrate was detected this is indicated by N/D.

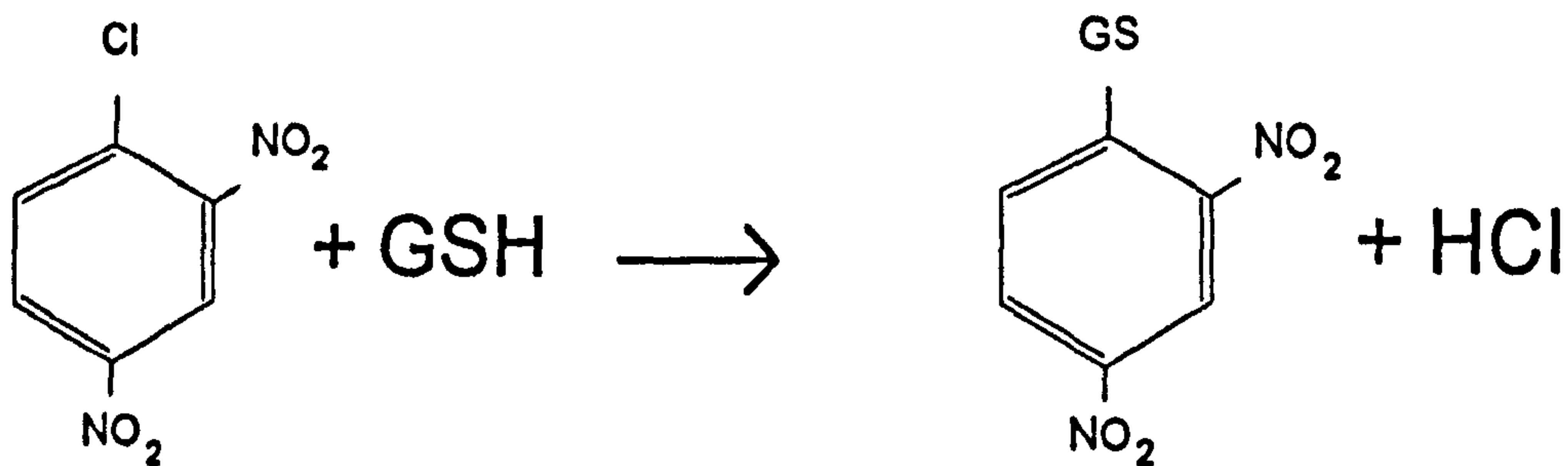
Species	Conjugate substrate	Specific activity $\mu\text{mole}/\text{min}/\text{mg}$ protein	Reference
<i>Daphnia magna</i>	CDNB	0.584 ± 0.099 *	LeBlanc & Cochrane (1985)
	ETHA	0.0095 ± 0.0014 *	
	DCNB	N/D	
	ENPP	N/D	
<i>Ceriodaphnia reticulata</i>	CDNB	0.603 ± 0.035 *	LeBlanc & Cochrane (1985)
	ETHA	0.0067 ± 0.0026 *	
	DCNB	N/D	
	ENPP	N/D	
<i>Artemia salina</i>	CDNB	3.456	Stenersen <i>et al.</i> (1987)
	ETHA	0.0192	
	DCNB	0.0162	
<i>Oniscus asellus</i>	CDNB	4.032	Stenersen <i>et al.</i> (1987)
	ETHA	0.006	
	DCNB	0.00486	
<i>Eupagurus bernhardus</i>	CDNB	3.048	Stenersen <i>et al.</i> (1987)
	ETHA	ND	
	DCNB	ND	
<i>Callinectes sapidus</i>	CDNB	0.461 ± 0.072 § (h)	Tate & Herf (1978)
		0.44 ± 0.172 § (g)	
	DCNB	0.0043 ± 0.0013 § (h)	
		0.0011 ± 0.0002 § (g)	
	PNBC	0.036 ± 0.015 § (h)	
		0.018 ± 0.004 § (g)	
	ENPP	0.0018 (h)	
		0.003 ± 0.0003 § (g)	

2.1.3. *In vitro* determination of GST activity.

Most workers estimating the activity of GST utilise the spectrophotometric method as described by Habig *et al.* (1974). Other methods of assaying GST activity have also been demonstrated and many are described by Habig and Jakoby (1981a).

Spectrophotometric assay.

The principle of spectrophotometric assays for GST activity is that they detect thioether formation and depend upon a direct colour change in the absorbance of the substrate when it is conjugated with glutathione (GSH). It must be noted that there is a finite rate of non-enzymatic reaction and care is taken to reduce this rate by minimising substrate concentrations and by reducing pH whenever necessary (Habig and Jakoby, 1981a). The spectrophotometric assay with the universal substrate CDNB depends on the maximum absorbance wavelength change of the electrophilic 1-chloro-2,4-dinitrobenzene when conjugated to nucleophilic glutathione.



Equation 2.1

The reaction shown in Equation 2.1 shows the conjugation of CDNB with GSH to form product S- (2,4 dinitrophenyl) glutathione (DNP-GSH). This product has a maximum absorbance at 340 nm wavelength. The increase in product concentration can be monitored with a spectrophotometer over the linear range of the product absorbance and thus enzyme activity can be monitored. The main advantage of the spectrophotometric assay for GST activity is its simplicity and the ease with which the assay can be conducted. It is perhaps because of this that it is the selected method for many workers assessing the activity of GST .

Titrimetric method.

The titrimetric conjugation of alkyl halides with GSH can be measured by acid production, which accompanies many of the transferase-catalysed reactions in which thioethers are formed. The rate of NaOH addition to maintain a constant pH in a stirred reaction vessel is measured using a recording titrimer. However, titrimetry is only used when more convenient assays are not available (Habig and Jakoby 1981a).

Colorimetric method.

Colorimetric techniques have been used to measure nitrite production during enzyme-catalysed conjugation of GSH with compounds with labile nitro groups. Nitrite is released when GSH reacts with nitroalkenes or with organic nitrate esters. The nitrite is assayed as the limiting factor in a diazotization reaction with sulphanimide that produces a readily quantifiable pink dye. The reaction with nitroalkenes involves attack of an electrophilic carbon atom leading to the formation of a thioester. In the case of nitrate esters the attack is on an electrophilic nitrogen.

A cyanide assay has been described (Habig and Jakoby, 1981a) in which GST catalyses the attack of the glutathione thiolate ion on the electrophilic sulphur atom of several organic thiocyanates, resulting in the formation of an asymmetric glutathionyl disulphide and cyanide. Cyanide is readily quantified by a colourmetric method.

Antibody recognition methods.

Monoclonal and polyclonal antibodies to GST isoenzymes have been used to detect GST isoenzymes in tissue samples (Baldwin and LeBlanc, 1996; Alin *et al.*, 1985). To produce antibodies, the antigen of interest (GST protein) is introduced into a mammal, initiating an immune response, in which many antibodies are produced. These polyclonal antibodies are produced in response either to different antigenic determinants of the original antigen or to other immunogenic substances in the introduced material. Monoclonal antibodies (MAb) may be produced by cloning from a single antibody-producing

lymphocyte. MAbs are more specific than polyclonal antibodies, allowing the recognition of a specific antigen by the same antibody in a very large number of assays. MAbs are useful for the detection of specific GST isoenzymes which may be quantified using enzyme-linked immuno-sorbent assay (ELISA).

Polyclonal antibodies have been prepared against various GST isoenzymes but generally do not exhibit high specificity (Alin *et al.*, 1985). Wang *et al.* (1986) described the detection of multiple sub-units of GST by monoclonal antibodies in mammals and Baldwin and LeBlanc (1996) described the production of monoclonal antibodies for distinct classes of GST in the cladoceran *Daphnia magna*. Antisera to human uterus GST III (class mu) and GST V (class pi) developed by Di Ilio *et al.* (1988) and to human skin GST 8.5 (class alpha) developed by Del Boccio *et al.* (1987) were used by Aceto *et al.* (1991) to identify GST isoenzyme classes in *Gammarus italicus*.

2.1.4 'Baseline' GST activity and sources of variability.

The successful use of GST activity as a marker of exposure to xenobiotics depends upon a knowledge of the activity of unexposed animals and the variability in this "baseline" activity. GST activity in samples may be subject to intrinsic and extrinsic factors. GST may be induced as a consequence of handling and/or laboratory holding conditions. For example, Beverley (1994) found that GST activity increased in the bivalve mollusc *Sphaerium corneum* collected from the field and kept in clean conditions; elevated activity being evident up to 192 hours from collection. Conversely, Collier and Varanasi (1991) observed a progressive decrease in GST activity in fish (*Pleuronectes retulus*) following laboratory acclimation. The effect of diet on the GST activity of the cabbage moth (*Manestra brassicae*) was considered by Egaas *et al.* (1993) who found that the activity was significantly higher (270 %) in larvae held on a cabbage rather than semi-synthetic diet. There may also be an effect of fasting on GST activity; Almar *et al.* (1987) showed that fasted crayfish (*Procambarus clarkii*) have significantly lower GST activity. This may indicate, as was suggested by Egaas *et al.* (1994), the role of the GST in the metabolism of plant alleochemicals. Yu (1982) reported tenfold differences in GST activity in fall

army worms (*Spodoptera frugiperda*) fed on different natural diets. The effect of laboratory holding temperature on GST was reported by Beverley (1994) in *Sphaerium corneum*. Animals collected in winter and held at 8 °C showed an increase in GST activity to a maximum at 72 hours holding, decreasing to levels that were the same as at collection after 192 hours. Animals collected in the summer and held at 15 °C showed greater variability in response although the changes in GST showed no logical pattern over time.

Intrinsic factors affecting variation of GST activity such as age have also been documented. Differences in activity between stages of cabbage fly (*Delia floralis*), meal worms (*Tribolium confusum*) and pine weevils (*Hyllobius abietis*) were demonstrated by Stenersen *et al.* (1987) and in the mosquito (*Aedes aegypti*) by Hazelton and Lang (1983). The effect of body size in *Sphaerium corneum* was demonstrated by Beverley (1994). Both weight and protein based GST activity decreased with increasing weight and protein concentration. An allometric relationship between body size and GST activity was demonstrated such that GST activity was subsequently expressed as a function of a standard body size.

Stability of GST during storage.

GST is relatively stable group of enzymes when stored frozen in tissue (Darby, 1973), crude tissue supernatants (Boyland and Chasseaud, 1968) or purified preparations (Hayakawa *et al.*, 1974; Pabst *et al.*, 1974). In contrast, the activity of enzyme incubated with CDNB substrate declined with time (Nimmo and Spalding, 1985; Nimmo, 1985; Nimmo, 1986). The effect of temperature on GST activity of tissue extracts from trout liver were investigated by Förlin and Andersson (1985). They found that the activity of GST was unaffected by storage at -80°C in 0.15 M KCl phosphate (pH 7.4) buffer for up to 49 weeks. Freezing in liquid nitrogen or on dry ice did not affect GST activity although it did affect the activity of other detoxification enzymes (e.g. ethoxyresorufin-O-deethylase). Beverley (1994) considered the effect of storage temperature on GST activity in the freshwater mussel (*Sphaerium corneum*) and found that there

was a decrease in activity after overnight storage of 14 % at 4 °C and 5 % at -20 °C. Storage for 7 days at -20 °C resulted in a 10 % decrease in activity.

2.1.5. Kinetics of glutathione s-transferase.

The GST activity of different preparations can be compared by studying the enzyme kinetics. At high substrate concentrations the rate of enzyme-catalysed reactions is almost constant (zero order kinetics), but at low concentrations of substrate the rate of reaction shows a direct relationship to concentration (first order kinetics).

A Lineweaver-Burk plot (Figure 2.1) is produced in which the reciprocal of reaction velocity (v) measured as activity is plotted against the reciprocal of the substrate concentration ($[S]$) to give a straight line.

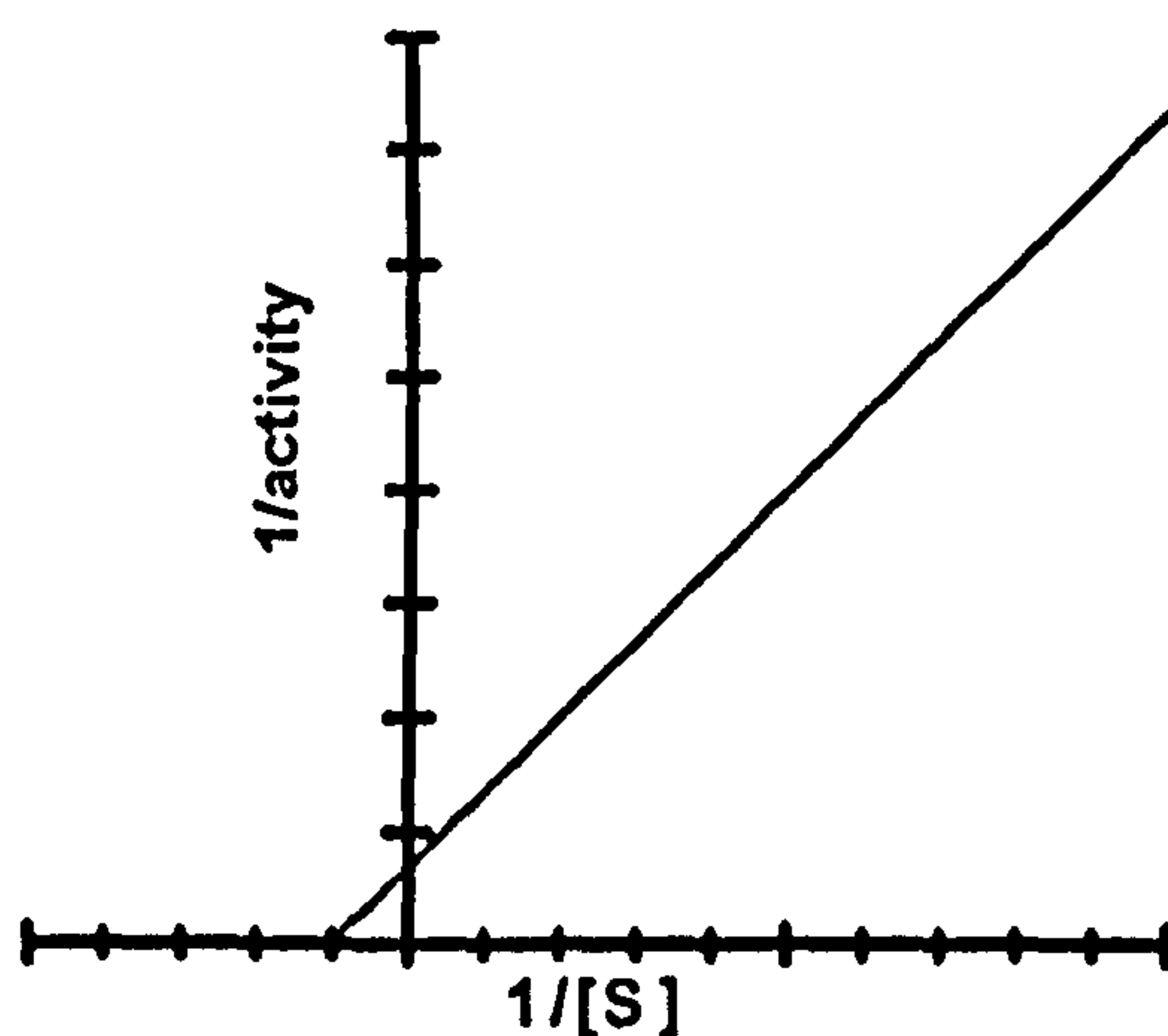


Figure 2.1 Lineweaver-Burk plot (double reciprocal plot) showing the reciprocal of the substrate concentration on the abscissa and the reciprocal of activity on the ordinate axis. When extrapolated, the intercept on the y axis gives the reciprocal of activity for the maximum velocity (V_{\max}) the intercept on the x axis gives the negative reciprocal of half V_{\max} the Michealis constant (K_m).

The regression line of the reciprocal of activity versus the reciprocal of substrate concentration is described by Equation 2.2

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \times \frac{1}{[S]} + \frac{1}{V_{\max}} \quad \text{Equation 2.2.}$$

The intercepts on the plot are described by Equations 2.3 and 2.4

$$\frac{1}{[S]} = -\frac{1}{K_m} \quad \text{Equation 2.3}$$

$$\frac{1}{v} = \frac{1}{V_{\max}} \quad \text{Equation 2.4}$$

The V_{\max} and K_m values have been determined for GST extracted from aquatic invertebrates and fish (Table 2.4). For example, K_m values for the freshwater mussel *Sphaerium corneum* were reported to be 5.1 mM CDNB an unpurified and 5.6 mM CDNB for a partially purified GST extraction (Beverley, 1994).

Table 2.4. Kinetic values derived for various isozymes and purifications of GST. Isoenzymes or fraction are named according to authors schemes.

Species	Tissue	Isozyme/ fraction	$K_{m\text{CDNB}}$	$K_{m\text{GSTII}}$	Reference
Rat (<i>Rattus norvegicus</i>)	Liver	A	0.06	0.2	Habig <i>et al.</i> (1974)
		B	0.8	0.2	
C		0.1	0.1		
	Liver	Y	0.039	0.067	
Thorny back Shark (<i>Platyrhinoides triserata</i>)	Liver	Y ₁	0.38	0.22	Sugiyama <i>et al.</i> (1981)
		Y ₂	0.3	2.08	
		purified Y ₂	0.28	4.3	
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Kidney		4.5	0.4	Nimmo & Spalding (1985)
	Liver	cationic	0.4	0.2	Ramage & Nimmo (1984)
		anionic	1.9	0.7	
Gill		1.0	1.9	Nimmo (1985)	
Freshwater pea mussel (<i>Sphaerium corneum</i>)	Whole body		5.1	0.37	Beverley (1994)
	Gill		8.89	0.34	
	Digestive gland		6.42	0.39	
		GST II		0.77	
		GST III	5.6	2.9	
Blue crab (<i>Callinectes sapidus</i>)	Excretory gland		0.44		Tate & Herf (1978)
	Hepatopancreas		0.32		

It can be seen from Table 2.4 that there is considerable variation in the values determined for K_{mCDNB} and K_{mGSH} in the species shown. The values are dependent on the isoenzyme used in the determination and the tissue assayed. For example, for the rainbow trout *Oncorhynchus mykiss*, K_{mCDNB} is greatest for kidney tissue and least for the cationic fraction of liver tissue. In contrast K_{mGSH} is greatest for gill tissue and least for the cationic fraction of liver tissue.

2.1.6. Aims and objectives.

The aim of the work detailed in this chapter was to assess the activity of GST in *Gammarus pulex* and to establish which factors affected activity. First, a method for measuring GST activity in a large number of extracts of *G. pulex* homogenate was developed. Several methods based on the spectrophotometric method were assessed. Determination of optimum conditions and kinetic values allowed comparison with a purified GST value.

Second, both the effects of extrinsic (i.e. holding temperature and feeding) and intrinsic (i.e. body size) factors on GST activity were assessed. The final objective was to determine a baseline level of GST activity in *G. pulex*. This would enable the effects of chemical exposure to be assessed in subsequent experiments (Chapters Three and Four).

2.2. MATERIALS AND METHODS.

2.2.1. Reagents.

All reagents were supplied by Sigma Ltd (UK) and were stated to be of 99% purity or above. The assay was carried out in a phosphate buffer made up from equal volumes of 0.1 M dipotassium hydrogen phosphate (K_2HPO_4) and 0.1 M potassium dihydrogen phosphate (KH_2PO_4), both solutions were made up in distilled water. The phosphate buffer was at pH 6.5. This was checked with a Russell pH probe and meter appropriately calibrated with pre-prepared buffers at pH 4, 7 and 9.2. The pH was adjusted (if necessary) to 6.5 with 10 % hydrochloric acid (HCl) or 10 % potassium hydroxide (KOH). A stock solution of EDTA (ethylene diamine tetraacetic acid, disodium salt) was made up to 100 mM and stored at 4°C. The buffer was made up with EDTA to give a final concentration of 1 mM.

The buffer was used as diluent in the assay and as a homogenisation buffer. The buffer used in homogenisation differed only in that phenylmethylsulphonyl fluoride (PMSF) was added. PMSF was made up in 95% ethanol to a concentration of 100 mM and stored for up to one week at 4 °C in darkness. A volume of 10 µl was added to the homogenisation mixture to give a PMSF concentration of 1mM.

2.2.2. Collection and holding of animals.

Gammarus pulex used for these assays were collected from Craggs stream near the village of Clowne in Derbyshire, National Grid Reference SK 497745 (for map see Appendix 1.1). The stream is spring fed and uncontaminated. Craggs stream is 0.7 m wide and had a depth of 0.25 m at the point of collection, it has a macrophyte cover, the substrate is fine gravel and silt. The total hardness of the water is 204 to 808 mg/l as calcium carbonate ($CaCO_3$) with conductivity ranging from 48 to 78 µmho. Animals were collected using a fine mesh (0.25 mm) sieve and transported back to the laboratory in stream water. In the laboratory animals were sorted by sex and held in Artificial Pond Water (APW) (Appendix 1.2) that had been left to equilibrate to the laboratory temperature and aerated for 24 hours

prior to use. Animals were held in perspex tanks containing 10 litres of aerated APW and maintained at a constant temperature of 15 ° C (± 1 ° C) and lighting regime of 12 hours light to 12 hours dark for at least 1 week prior to use in experiments, unless otherwise stated. Animals were fed *ad libitum* with *Cladosporium* sp. conditioned alder (*Alnus glutinosa* (L.)) leaves (see Appendix 1.3 for preparation). Animals were held individually in holding tubes (Figure 2.2) during experiments to prevent cannibalism and allow the identification of individuals.

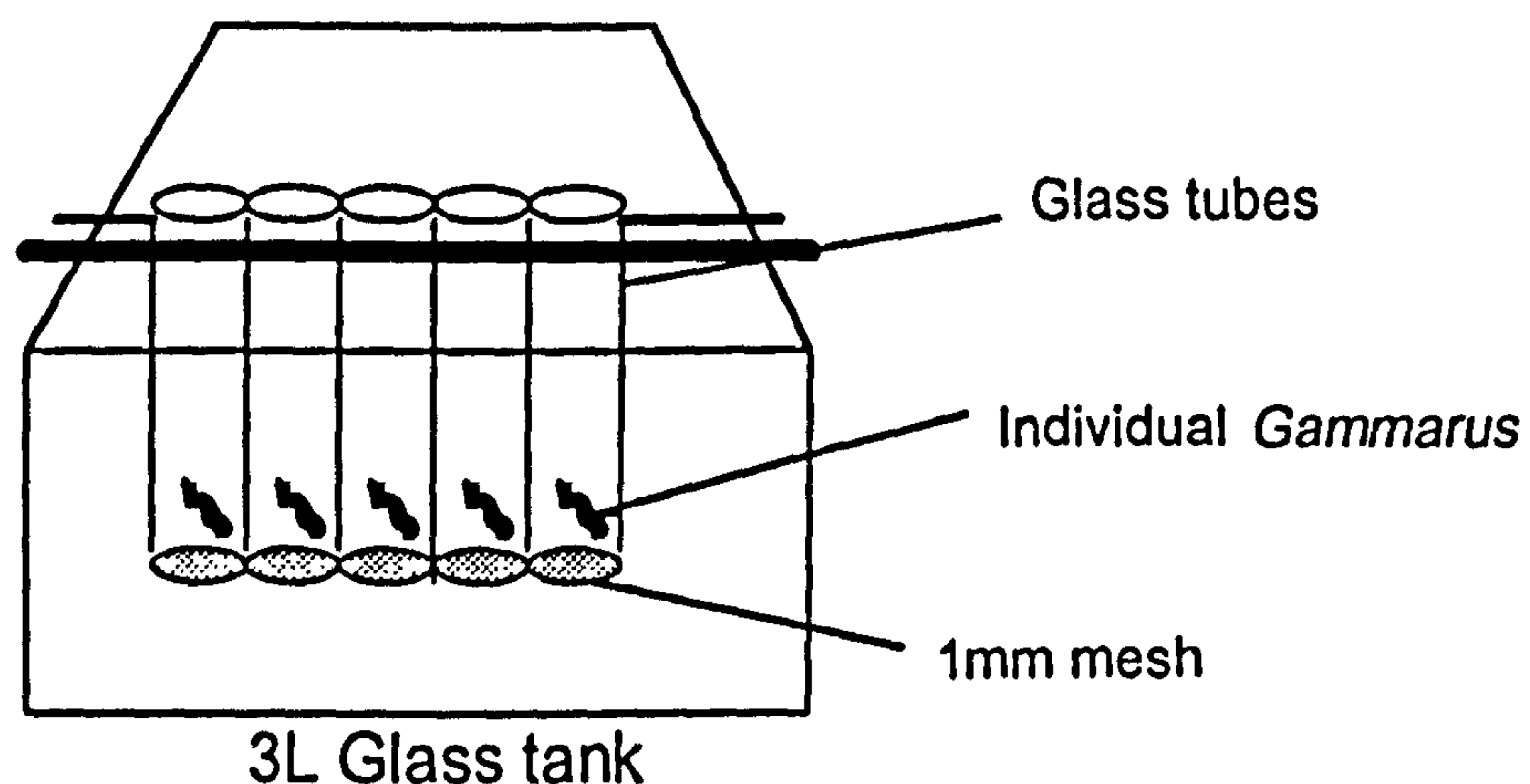


Figure 2.2 Individual holding tubes (80 x 15 mm) constructed of glass bonded to other tubes in a set of five with fungicide-free silicon based aquarium sealant. Up to 7 of sets of holding tubes could be suspended in a three litre tank.

2.2.3. Preparation of animal material for GST assay.

Large males were lightly blotted with a clean paper tissue to remove excess water and weighed (alive) on a Mettler ME 30 balance (accuracy: ± 1 μ g). They were then killed by immersion in liquid nitrogen (-196 °C) for twenty seconds.

Samples were stored for assay in 1.5-ml polythene 'Eppendorf' tubes that were placed in expanded polystyrene 'Eprak' boxes and held at -70 °C in a Kelvinator Ultra cold (series 100) deep freeze.

Initially, for each assay animals were homogenised in a 5-ml hand-held glass tissue grinder. Samples were homogenised in 5 ml of ice-cold phosphate buffer at pH 6.5 (containing 1 mM EDTA and 1 mM protease inhibitor PMSF) for 2

minutes. The number of animals were initially varied between one and five. The ratio of animals to millilitres of homogenisation buffer was always 1:1. The homogenate was decanted into 5-ml MSE Coolspin centrifuge tubes and held on ice. The tubes were sealed and the samples centrifuged at 30,000 x g for 30 min at 4 °C. The supernatant was decanted and passed through a plug of clean glass wool to remove any lipid globules before being used in the assay. The preparation of samples for the microplate assay differed from the above method in that individual animals were always used. Assay samples were centrifuged in 1.5-ml 'Eppendorf' tubes at 20 000 x g for 20 minutes at 4 °C in a Hereaus Biofuge.

2.2.4. Spectrophotometer assay.

GST was assayed in a dual cell double beam spectrophotometer (Pye Unicam SP 8 -100) with a thermostatic cell holder set at 25 °C. An assay mixture was prepared by adding 0.25 ml of 20 mM reduced glutathione solution and 1.45 ml phosphate buffer (pH 6.5) to each of a matched pair of 3-ml quartz-glass cuvettes. To one cuvette was added 0.75 ml *G. pulex* homogenate and to the other 0.75 ml of buffer. To start the reaction 0.05 ml of 50 mM CDNB was added to each cuvette. The total volume of the assay was 2.5 ml resulting in substrate concentrations of 2 mM GSH and 1mM CDNB. The assay reagents were mixed by rapid inversion of the cuvette. The assay was monitored continuously over 3 minutes at 340 nm at a temperature of 25 °C. The spectrophotometer was set at 1 nm band width with a full scale deflection of 1 absorbance unit. Absorbance over time was recorded on a chart recorder set at 20 seconds per cm. Assays for kinetic parameters and temperature and pH optima were executed using a 1 ml assay mixture made up with 100 µl of 100 mM GSH solution, 640 µl phosphate buffer (pH 6.5), 250 µl enzyme solution or *G. pulex* extract and 10 µl of 100 mM CDNB as assay starter. Substrate concentrations were varied for kinetic measurements as were pH and temperature for optima assays (section 2.2.9).

2.2.5. Multi-cell spectrophotometer.

The use of a Shimadzu 1200 UV/visual variable wavelength spectrophotometer enabled the simultaneous monitoring of five sample cells and a reference cell. The sample preparation for this method was identical to that in section 2.2.4. The Shimadzu UV/Vis 1200 spectrophotometer did not possess a thermostatic cell holder therefore assays were conducted in a 15 °C constant temperature room. The temperature of the assay mix was monitored using a thermocouple temperature probe. An assay mixture was prepared with 1.64 ml phosphate buffer and 0.3 ml 50 mM reduced GSH solution to which was added 1 ml homogenate supernatant. The assay starter was 0.06 ml 50 mM CDNB, giving an assay volume of 3 ml. The assay solution was mixed by rapid inversion of the cell and the mixture was assayed for 3 minutes at 340 nm against a blank without enzyme (i.e. homogenate). The range of the spectrophotometer was set at 2 absorbance units. Ten measurements were taken over the assay period and the absorbance per minute calculated.

2.2.6. Multi-well plate reader method.

The use of a plate reader enabled up to 96 samples to be monitored simultaneously over time. Falcon Micro-test III sterile micro plates were used, as they did not demonstrate significant protein binding or absorbance at the wavelength used (340 nm). The plate was blanked to ensure variation of absorbance was not due to variation in absorbance over the plate. Each plate was used once and discarded. The maximum volume of the assay cells was 300 µl. This enabled the assay of triplicate samples of an individual animal. A 140 µl assay mixture was prepared containing 10 µl of 20 mM reduced glutathione solution, 100 µl buffer and 30 µl distilled water. The assay mixture was pipetted into the wells using a 'Finnipipette' multi-channel pipette. Using a calibrated P100 Gilson 'pipettman' autopipette, 50 µl of enzyme or homogenate was added to each sample well and 50 µl buffer was added to first three wells and the last three well on the plate. A volume of 10 µl of CDNB was added rapidly to each of the wells using an Eppendorf multi-pipette. The assay was monitored using an Anthos Lab-tech HP11 plate reader with a kinetic programme set up to calculate

the maximum slope of absorbance per minute over a 3 minute period. A measurement filter of 340 nm was selected with a reference filter of 405 nm. An internal temperature monitor ensured that the assay was performed at 25 °C. The assay plate was pre-shaken for 20 seconds prior to the first reading and then for 2 seconds prior to each subsequent reading. A reading was taken every 20 seconds, with a total of 10 readings being taken over a 180 second period. The readout was given as the maximum slope in milli-optical density units (mOD₃₄₀) per minute for each well over the assay period.

2.2.7. Protein measurement.

The concentration of protein was measured in each sample in order to express enzyme activity as specific activity i.e. per mg protein. Two methods were used: the Coomassie method and the Bio-Rad method. The Coomassie method was used to estimate protein concentrations of homogenates using the single cell and multi-cell spectrophotometer GST assay methods. The Bio-Rad method was used to estimate protein concentrations in the micro-plate assay. The Coomassie brilliant blue method is used extensively for general quantification of protein. The principle of the assay is that when the dye complexes with protein it shows a shift in absorption maximum from 464 to 595 nm. The maximum absorbance is developed rapidly (i.e. within 5 minutes) and is stable for at least 1 hour. All samples were prepared in pH 6.5 phosphate buffer, and a calibration curve of bovine standard albumin (BSA) was prepared over the range of concentrations likely to be found in the samples i.e. 0.075 to 1 mg/ml. Standards were prepared from BSA of 75, 100, 250, 400, 500, 1000 and 1500 µg/ml. Samples and standards were prepared for assay by pipetting 0.1 ml into a 16 x 100-mm glass test tube. A volume of 5 ml of Coomassie reagent was added and mixed using a vortex mixer. The absorbance at 595 nm was read using a Pye-Unicam dual beam spectrophotometer against a phosphate buffer blank. Unknown protein concentrations were calculated from the standard calibration curve.

Bio-Rad method.

The Bio-Rad method is recommended by the manufacturers for small volumes of sample. Based on the same principle as the Coomassie assay, it is used as a replacement for the Lowry method (Lowry *et al.*, 1951) of protein determination. A micro assay procedure as recommended by Bio-Rad was used for the measurement of protein solutions of a concentration of 1 to 25 µg/ml protein. The 96 well microplates used were Falcon Microtest III. Prior to assay the plate was blanked to ensure variation of absorbance was not due to variation of absorbance of the plate.

Using a 2 mg/ml BSA protein standard, an intermediate of 30 µg/ml was made up by dilution with phosphate buffer. A series of standards were then made up to 800 µl with buffer such that the addition of 200 µl Bio-Rad protein reagent gave a series of 1, 3, 5, 8, 10, 12, 15, 18, 20, 22 and 24 µg /ml standards. Standards were mixed using a 'Whirlymix' vortex mixer (Fisons). Using a Gilson autopipette, 200 µl of each standard was placed in each of three wells on a 96 well micro-plate.

Samples were prepared by 100 fold dilution of animals homogenated with phosphate buffer by addition of 10 µl of homogenate with a Gilson autopipette to 990 µl of phosphate buffer in a 1.5-ml 'Eppendorf' tube. Samples were mixed using a 'Whirlymix' vortex mixer (Fisons). A volume of 800 µl from each diluted sample was placed in a 1.5-ml 'Eppendorf' tube to which was added 200 µl of Bio-Rad protein reagent, samples were mixed again using a 'Whirlymix' vortex mixer. Using a Gilson autopipette 200 µl of each sample was placed in each of three wells on the 96 well micro-plate (Falcon Microtest III). The standards were also run on each plate.

The Anthos HTII reader was programmed to shake the samples for 10 seconds at high intensity prior to reading. The absorbance was measured at 595 nm with no reference filter. A reagent only standard (blank) was automatically subtracted from each sample. A mean of the three replicates was calculated for each sample.

A plot of OD₅₉₅ (A₅₉₅) verses concentration of standards allowed unknowns to be read from the standard curve. The amount per millilitre of homogenate is calculated from the equation of the regression line and by multiplying by 1.25 as only 800 µl of sample was used in the assay and by 100 as the homogenate was diluted 100 times (Equation 2.5).

$$\frac{A_{595}}{\text{slope}} \times 1.25 \times 100 = \mu\text{g protein/ml} \quad \text{Equation. 2.5}$$

2.2.8. Calculation of enzyme activity.

Enzyme activity was calculated as the amount of product produced per unit time. This is calculated from the molar absorption coefficient and the change in absorption per unit time (ΔA_{340}). The product of GST conjugation of CDNB with GSH is S- (2,4 dinitrophenyl) glutathione (DNP-GSH) and has a molar absorption coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (Habig *et al.*, 1974).

GST activity, given as µmoles of product formed per minute per millilitre was calculated using Equation 2.6, where A_v is the assay volume in millilitres and H_v is the homogenate volume in millilitres.

$$\frac{\Delta A_{340}}{9.6} \times \frac{A_v}{H_v} = \mu\text{moles/min/ml} \quad \text{Equation. 2.6}$$

Calculating activity in micro-plate reader samples.

In micro-plate reader assays path length and, as such, the extinction coefficient (ϵ) is dependent on well volume. It was therefore necessary to determine ϵ for the assay volume used (i.e. 200 µl). To find the “extinction coefficient” for the microplate reader assay at a well volume of 200 µl a range of concentrations of CDNB and GSH were prepared and left incubated with pure equine enzyme overnight. It was assumed that all the CDNB and GSH was conjugated to the product DNP-GSH. The absorbance of these solutions were tested at 340 nm

using the microplate reader and a calibration curve of concentration versus absorbance established (Appendix 1.4). The coefficient determined was 8.9 mM and close to the value of 9.6 mM⁻¹cm⁻¹ (Habig *et al.*, 1974). The microplate assay utilises an assay volume (A_v) of 200 μ l and a homogenate volume (H_v) of 50 μ l. Activity was calculated using Equation 2.7.

$$\frac{\Delta A_{340}}{8.9} \times \frac{0.2}{0.05} = \mu\text{moles/ml/min} \quad \text{Equation. 2.7}$$

Activity can be calculated per milligram weight of animal or by specific activity that is per mg protein (determined as stated in section 2.2.7).

2.2.9 Experiments.

Maximum absorbance and linearity.

The maximum absorbance of the conjugation product of GSH and CDNB, DNP-GSH, was checked by observing the absorbance of the conjugated product over a range of wavelengths. An assay mixture was made up containing 2 mM GSH and 1 mM CDNB (section 2.2.4) using 250 μ l of 10 μ g/ml pure equine GST preparation. The mixture was left for four hours at 25 °C before being measured for absorbance in a Pye Unicam SP-8 100 dual beam spectrophotometer at 340 nm against a blank that did not contain enzyme. Absorbance was measured every five minutes after this period to check that the reaction had stopped. If the absorbance was the same on three consecutive readings then the reaction was assumed to have run to completion. If necessary the mixture was diluted such that the maximum absorbance was within one absorbance unit. The mixture was then scanned against a blank. The spectrophotometer was set to scanning mode and a wavelength speed of one nm per second was selected. The starting wavelength was set to 200 nm and finishing wavelength to 500 nm. The absorbance over the wavelengths was recorded on a calibrated chart recorder running at 20 seconds per cm and set a full scale deflection of one absorbance unit.

Preliminary experiments were conducted to assess GST activity in *Gammarus pulex* and to establish the relationship between activity and homogenate protein concentration. *G. pulex* (five males per sample) were prepared for GST assay as described in section 2.2.3. Homogenate volumes of 0.25 ml, 0.5 ml, 0.75 ml, 1 ml and 2.5 ml each in a total assay volume of 3 ml were prepared. Volumetric differences were made up with phosphate buffer. A second identical set of homogenates were prepared but heat treated to 100 °C to denature the proteins. The assays were conducted as stated in section 2.2.4. Activity as absorbance units per unit time was plotted against total protein content of the assay mix.

Linearity of the relationship between activity and protein was assessed using a pure equine GST preparation measured using the dual beam and microplate methods for GST activity (section 2.2.6). Pure equine GST was obtained from Sigma Chemicals Ltd. It was stated to have an activity of 70 units per milligram protein and 83 units per milligram weight, as assayed with 1.0 mM CDNB and 2.5 mM GSH. Protein was estimated by Sigma by the Biuret method and the enzyme preparation contained 84 % protein. A serial dilution of 0.05 to 0.005 milligram enzyme protein per millilitre was made up in phosphate buffer (pH 6.5). Samples were assayed in triplicate using the microplate reader method as described in section 2.2.6. Total protein was determined using the Bio-rad protein determination on the microplate reader (section 2.2.7). Activity per unit time per ml of enzyme dilution was plotted against total protein content of the enzyme dilution.

Spectrophotometric methods of GST activity estimation were compared using the Pye Unicam SP8 100 dual beam spectrophotometer, Shimadzu UV 1200 multiwell spectrophotometer and the Anthos labtec HP11 microplate reader. Pure equine GST was prepared (as above) and *G. pulex* extract was prepared using the methods described in section 2.2.6. Assays were conducted in phosphate buffer at pH 6.5 with CDNB at 1 mM and GSH at 2 mM at 25 °C with the exception of the multi-well spectrophotometer that was held at 15 °C. Each

enzyme preparation and *G. pulex* extract was read ten times in each type of spectrophotometer against a blank containing no enzyme.

Kinetics.

G. pulex were prepared for assay as described in section 2.2.3. The K_{mGSH} was determined by varying the amount of GSH in the assay and K_{mCDNB} was determined by varying the amount of CDNB in the assay. Glutathione concentrations were 40, 100 and 200 mM and concentrations of CDNB were 10, 50, 100, 200, 250 and 500 mM. In each case, the assay volume was 1 ml and it contained 250 μ l homogenate or diluted enzyme, 10 μ l CDNB stock and 100, 125 or 250 μ l GSH stock. Assays were made up to volume with phosphate buffer (pH 6.5). Triplicate samples of *G. pulex* extract and pure enzyme were run against a blank containing no enzyme or homogenate, the volume being made up with buffer. The Pye-Unicam double beam spectrophotometer automatically deducted the non-enzymatic absorbance from the reading in the reference cell holder and results were recorded on a calibrated chart recorder. Protein concentration was determined as in section 2.2.7. Each assay was run for 2 minutes and change in absorbance per unit time ($\Delta A_{340} \text{ min}^{-1}$) was used to calculate the empirical half saturation concentrations (section 2.2.8). Initial velocities were determined at each substrate concentrations. The Michealis constants were calculated using Equation 2.3.

Kinetic measurements of pure equine GST were performed using the Anthos Labtech HTII microplate reader to compare with values given by the dual beam spectrophotometer. The well volume was kept at a constant 200 μ l using a volume of 50 μ l pure enzyme. Assay mix was made up such that it contained GSH concentration in the range 0.1 to 5 mM. CDNB was added at a constant volume of 10 μ l to give assay concentrations in the range 0.1 to 5 mM. K_{mGSH} was determined by holding CDNB concentration at 1 mM whilst varying GSH and K_{mCDNB} was determined by holding GSH at 2 mM whilst varying CDNB. Both substrates were varied in the range 0.1 to 5 mM in a third treatment to determine the $K_{mGSH/CDNB}$. The assays were run as stated in section 2.2.6 and total

protein concentration was determined as described in section 2.2.7. The Michaelis constants were determined using Equation 2.3.

Temperature and pH optima.

Temperature and pH were altered for set substrate concentrations and both *G. pulex* homogenate and pure enzyme were used for determination of optimum assay conditions. Temperature was varied using a thermostatically control cell holder in the Pye-Unicam spectrophotometer and checked with a thermocouple thermometer. A temperature range of 20 to 50 °C was used at pH 6.5. The pH of the assay was varied using phosphate buffer with pH adjusted to a range from 5.5 to 8 with 10% HCl or 10% KOH. The pH was checked with a Russell pH probe and meter and the assay was performed at 25 °C. A volume of 250 µl of *G. pulex* homogenate or pure enzyme solution was used and concentrations of CDNB and GSH were 1 mM and 2 mM, respectively. Each set of assay conditions was run in triplicate. Activity was calculated as in section 2.2.8 and total protein concentration was calculated as described in section 2.2.7.

Anti-body recognition of GST from *G.pulex* homogenate using Enzyme-linked immunosorbent assay (ELISA).

Strips of twelve microplate wells (Labsystems) were coated with 100 µl of pure equine GST or *G. pulex* assay homogenate (as prepared for enzyme assay, section 2.2.3) as the antigenic material. Well strips were then labelled with indelible marker pen and incubated at 4 °C overnight in sealed polythene bags. The antigenic material was discarded, the wells were then washed four times with 0.1 M phosphate buffered saline with Tween-20 detergent (0.5 %) discarding the residual in between washes. Wells were washed once with 0.1 M phosphate buffered saline (PBS) and rinsed with distilled water. Strips were then dried in a laminar flow cabinet for 10 minutes at room temperature. Well-strips can be stored for 48 hours at this stage in a sealed polythene bag at 4 °C. Wells were then blocked using PBS with 200 µl 2 % bovine standard albumin (BSA) for 30 min. Wells were washed and rinsed as before.

A 50 µl volume of hybridoma supernatant (primary antibody that recognises *Schistosoma japonicum* GST, raised in mouse) obtained from Sigma Ltd. was added to the wells for 60 min at room temperature (20 °C). The hybridoma supernatant was discarded and the wells were washed and rinsed as before. A 1:1000 in PBS secondary antibody solution (Goat anti-mouse antibodies IgM and IgG-horse radish peroxidase conjugate Sigma A-0412) was prepared and 100 µl added to wells. The wells were incubated for 60 minutes at room temperature in a sealed polythene bag after which they were washed and rinsed.

Substrate buffer was made up from 5 ml of 0.2 M sodium acetate, 195 µl of 0.2 M citric acid, 5 µl of 30 % hydrogen peroxide and 5 ml distilled water. Substrate stock was made up from 0.1 g 5,5'-tetramethylbenzidine hydrochloride (TMB) in 10 ml dimethylsulphoxide (DMSO). Working substrate was then made up from 10 ml of substrate buffer and 100 µl of substrate stock. To test the working substrate, a small amount was added to a drop of secondary antibody; colour change indicated the substrate was made up correctly and the enzyme reaction was being catalysed. A volume of 100 µl of working substrate was added to the wells which were then incubated at room temperature for 30 minutes. The reaction was stopped by adding 10 µl of 3 M H₂SO₄. Well strips were fitted onto labelled plate frames and read immediately at 450 nm in a BioTek EL311s automated microplate reading spectrophotometer.

2.2.10 Effect of extrinsic and intrinsic factors on GST activity.

Storage temperature.

The effect of storing *G. pulex* samples on GST activity was investigated by comparing the activity of fresh samples with samples that had been frozen in liquid nitrogen (-196 °C) or frozen in liquid nitrogen and stored at either -10 or -70 °C for 1 month. The activity of stored samples was compared to GST activity in both fresh animal homogenate and low temperature frozen animal homogenate.

Seasonal variability.

Seasonal variability in GST was assessed by collecting animals from Craggs stream throughout 1995. Thirty adult male *Gammarus pulex* (15 to 10 mm in length) were collected from Craggs stream as detailed in section 2.2.2. Animals were weighed and rapidly frozen in liquid nitrogen and stored at -70 °C for subsequent GST assay. GST activity and total protein were analysed by the microplate method (section 2.2.6 and 2.2.7).

Laboratory acclimation.

Acclimation to laboratory conditions may influence GST activity (section 2.1.4) and was assessed by comparing acclimated and non-acclimated animals. A group of thirty large male animals were collected from Craggs stream (section 2.2.2) and either immediately frozen in liquid nitrogen or maintained under laboratory conditions for 1 week or 6 weeks before being frozen and stored. Specific activity was analysed using microplate methods for GST (section 2.2.6) and total protein (section 2.2.7).

Holding conditions

The effect of holding conditions on GST activity was investigated by comparing the activity of animals held in holding tanks and tubes as detailed in section 2.2.2 and illustrated in Figure 2.2, with animals held in tanks only. Three replicate glass tanks were filled with 3 l APW into each of which were placed 15 large (10 to 15 mm) male *G. pulex*. A further three replicate glass tanks were filled with 3 l APW into each of which were placed 15 large male *G. pulex* in individual holding tubes. Animals were held under laboratory conditions of 15 °C, 12/12 hours light/dark for one week. Animals were not fed during this time. At the termination of the experiment all animals were frozen and stored. Specific activity was analysed using microplate methods for GST (section 2.2.6) and total protein (section 2.2.7).

Feeding.

The effect of feeding on GST activity was assessed by comparing male animals fed for 1 week on conditioned alder leaves with unfed animals. Three replicates of twenty animals for each treatment were frozen and stored as described in section 2.2.3. GST activity and total protein were analysed by the microplate method (section 2.2.6 and 2.2.7).

Environmental temperature.

The effect of environmental temperature on GST activity was assessed by maintaining three replicates of thirty unfed male animals at 5, 15 or 20 °C for 14 days prior to analysis. Animals were frozen and stored for subsequent GST and protein assay by microplate reader methods as detailed in sections 2.2.6 and 2.2.7.

Body size.

The effect of body size on GST activity was investigated by collecting *G. pulex* from Craggs stream and acclimating them to laboratory conditions for seven days. They were then sorted into size classes by passing them through a series of six mesh sieves of 3.36, 2.5, 2.0, 1.68, 1.41, and 0.84 mm (Werner, 1994). Individuals retained by the 3.36 mm sieve were referred to as Class one whereas those retained by the 2.5 mm sieve were referred to as size Class two and so on for size classes three to six. The sieves were placed into a 15-l tank of APW at 15 °C with a continuous compressed air supply via an air stone. A sixty watt light source was placed over the tank for six hours causing *G. pulex* to move away from the light source and hence through the sieves (Figure 2.3). Six size classes were collected but only four were used in this experiment, these were classes 2, 3, 5 and 6.

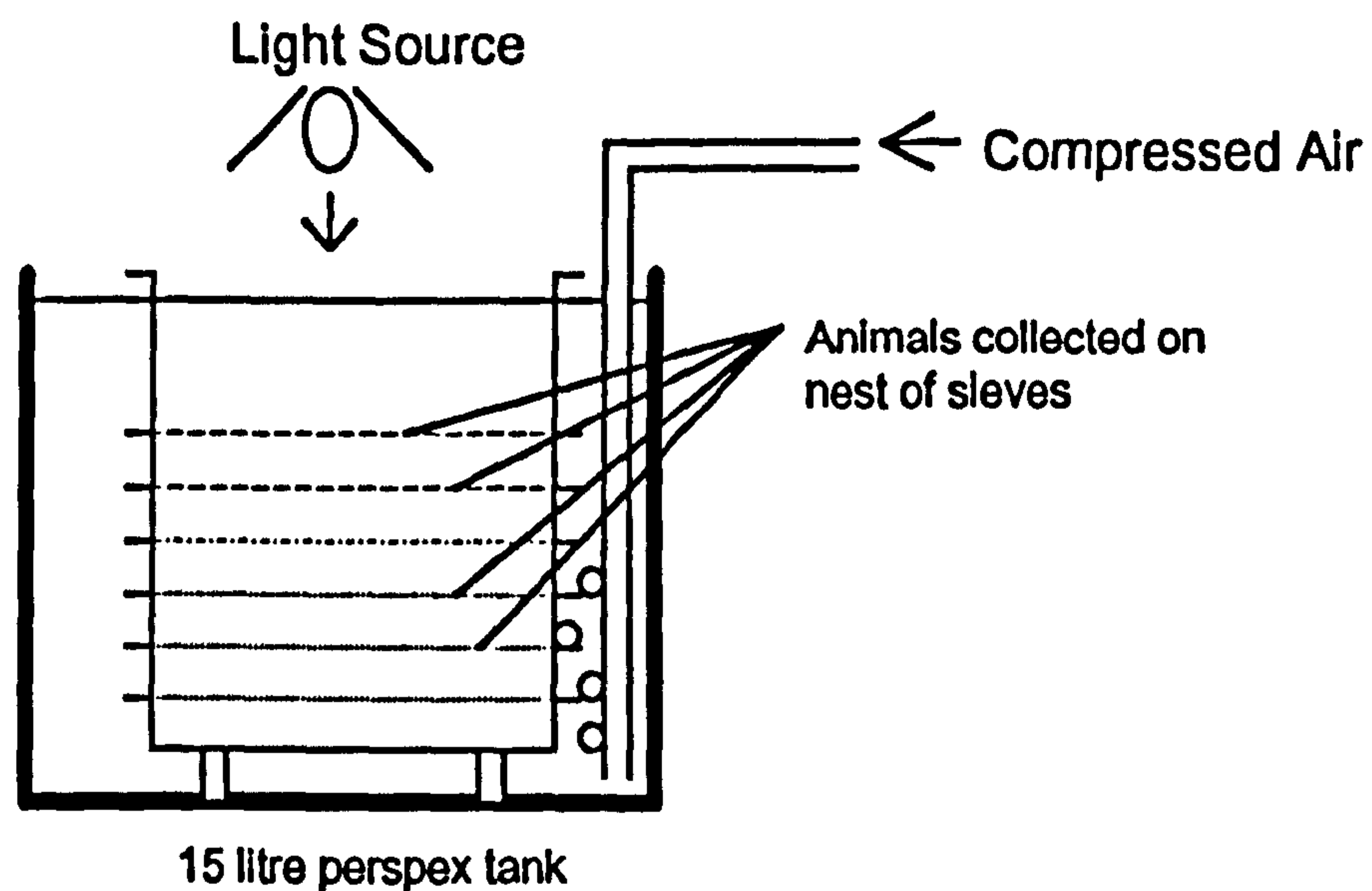


Figure 2.3. Mesh separation of *Gammarus pulex* according to size class. Animals were introduced to the top of the mesh containers. *Gammarus* moved away from the light source and were separated according to size. After Werner (1994).

A sample 20 animals from each size class were weighed (mg, fresh weight) and measured (mm) from the tip of the head to the end of the last body segment. Animals were anaesthetised in carbonated APW for ten seconds prior to measurement. They were then frozen and stored (section 2.2.3) directly for subsequent GST and protein analysis using the microplate methods described in sections 2.2.6 and 2.2.7.

2.2.11. Statistical analysis.

Statistical analyses were performed using the statistical software package Minitab, version 9.2 for Windows. All data were checked for a normality using normal probability plots and Anderson-Darling normality tests. Oneway analysis of variance (ANOVA) were used to assess differences in spectrophotometric methods, storage temperature, activity over the year, laboratory acclimation, size specific activity and weight specific activity. The significance of pair-wise differences in ANOVA was assessed by the multiple comparison test Tukey's test; or significant difference from control groups were analysed using a Dunnett's test; q values stated are derived by the method of Zar (1996). A balanced nested analysis of variance was used to analyse the effect of holding

temperature, holding conditions and feeding on GST activity. Linear least squares regression analysis was performed on activity versus protein and activity versus fresh weight plots for both spectrophotometer and microplate reader experiments. Significance was at $p = 0.05$ unless otherwise stated.

2.3. RESULTS.

2.3.1. Maximum absorbance wavelength and extinction coefficient.

The maximum absorbance of the CDNB-GSH conjugation product was found to be at 340 nm (Figure 2.4).

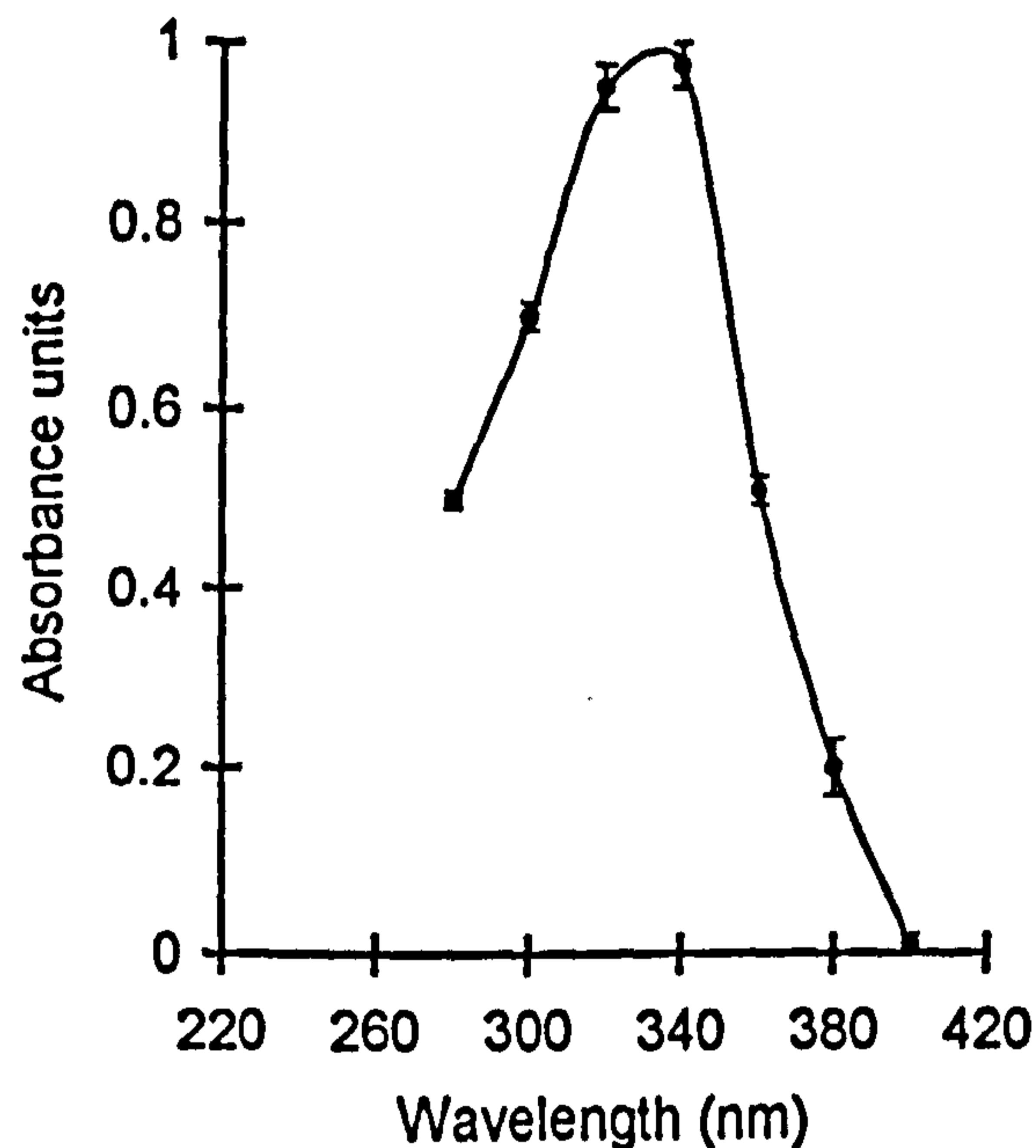


Figure 2.4. Maximum absorbance wavelength. Range of wavelength scan is plotted against absorbance for the conjugation product DNP-GSH. Data points are the mean of three readings, error is one standard deviation.

A value of 9.6 mM cm^{-1} was used as the extinction coefficient (ϵ) for dual-beam spectrophotometer assays (section 2.2.4). This value was checked for use in the microplate assay. The absorbance of a range of concentrations of conjugated product of GSH and CDNB S- (2,4 dinitrophenyl) glutathione (DNP-GSH) were plotted against concentration of product (Appendix 1.4). A regression of these points gave a slope of 8.9 OD mM^{-1} . This value is close to the value given by Habig *et al.* (1974) of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$, but the value of 8.9 OD mM^{-1} was used as the extinction coefficient in all calculations of activity using the microplate reader.

2.3.2. Detection and linearity of activity in pure and *G. pulex* GST.

An assay was performed with *G. pulex* extract that had been heated to 100 °C to denature the proteins. No activity above that for the non-enzymatic activity was shown. The linearity of the GST assay with the amount of protein in the assay was checked with purified equine GST and *G. pulex* extract using the dual beam method of GST assay. GST activity increase was linear to protein content of *G. pulex* extract (Figure 2.5).

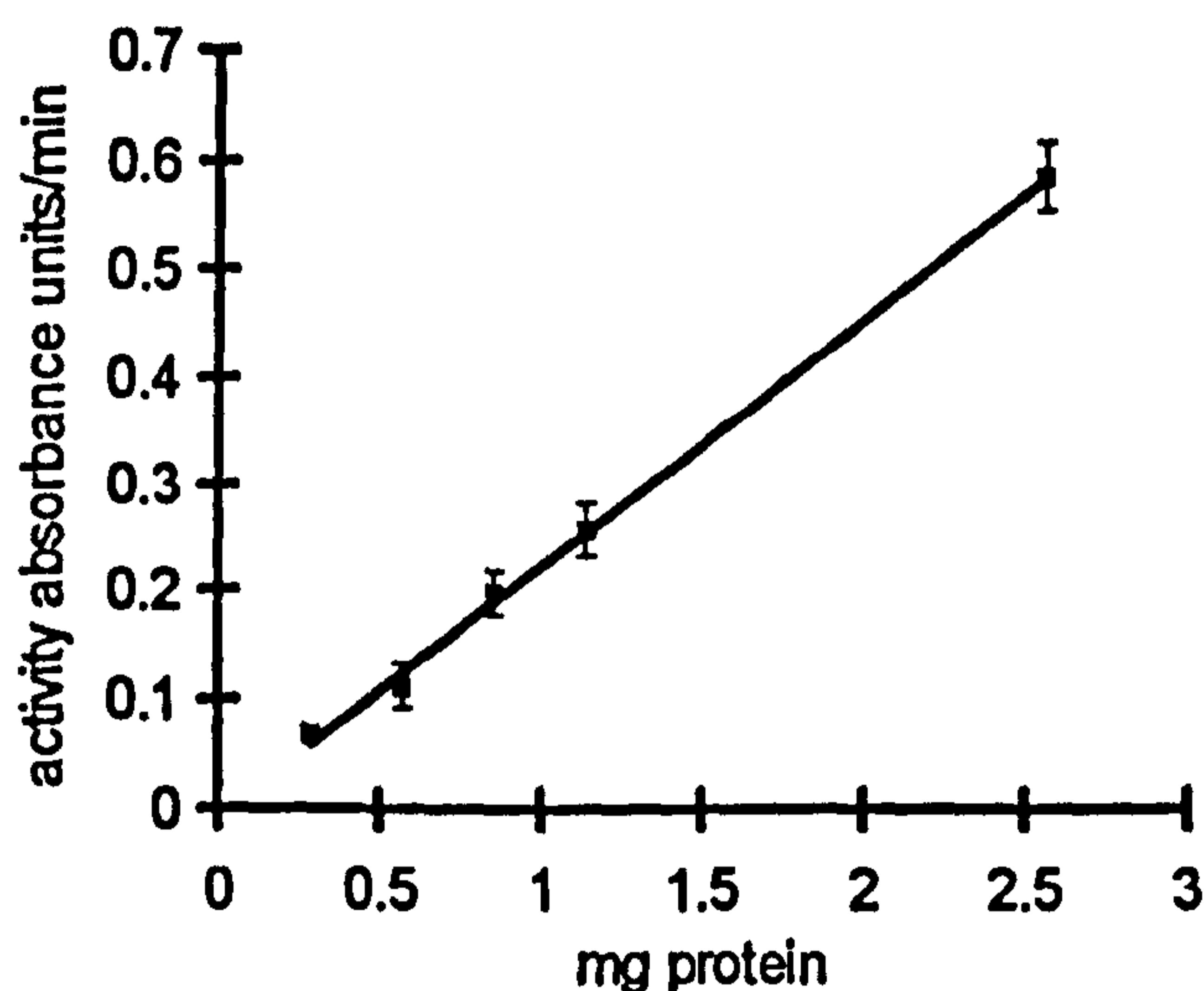


Figure 2.5. Linearity of activity with increasing protein concentration for *G. pulex* extract. Error shown is one standard deviation. A linear regression line is shown plotted described by the equation $y = 0.23x - 0.005$ with a correlation coefficient of 0.99.

Pure equine GST was assayed as described in sections 2.2.4 and 2.2.6 using both the dual beam spectrophotometer and the microplate reader methods. For both methods there was a positive linear relationship between the rate of product formation and the amount of protein in the assay (Figure 2.6).

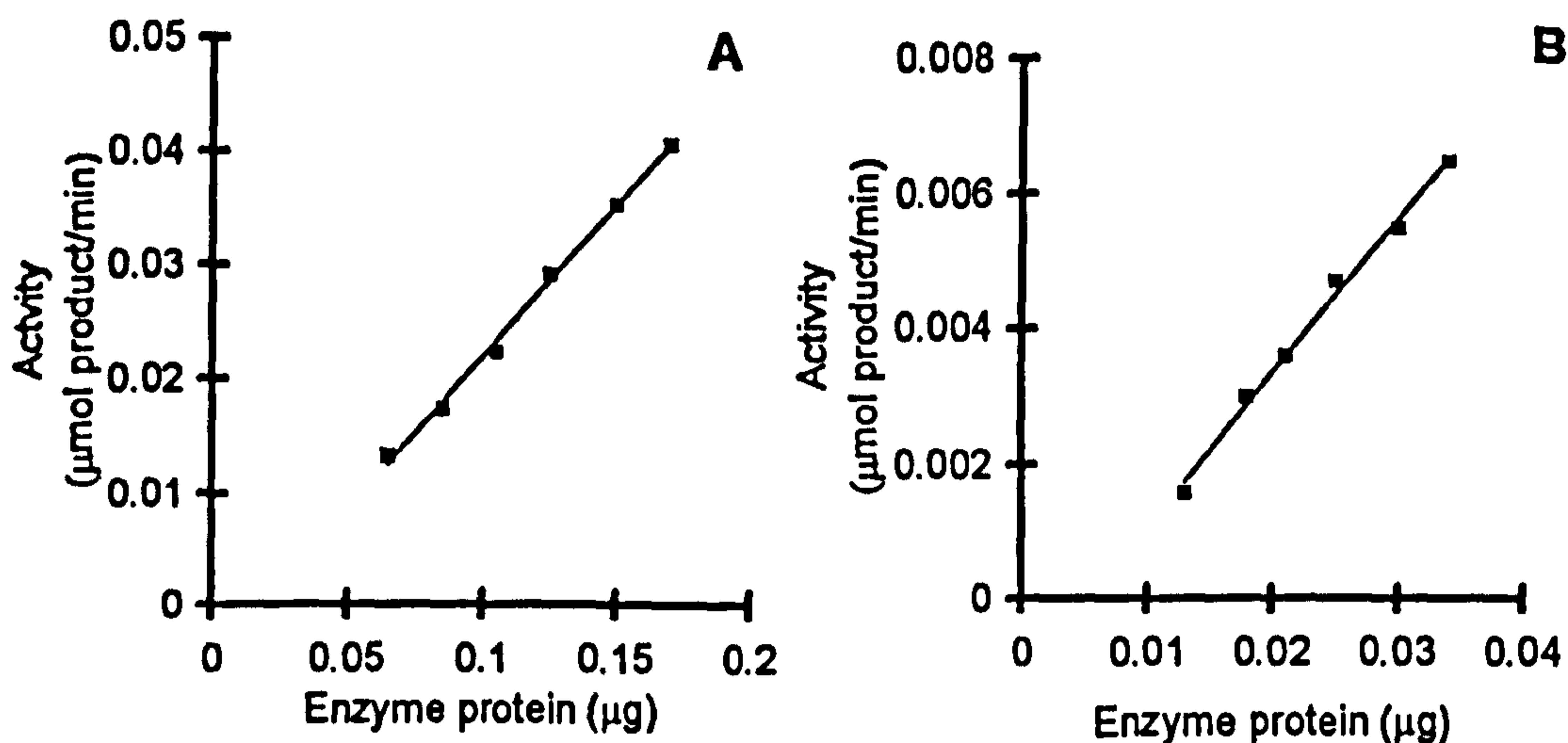


Figure 2.6. Activity of pure equine GST in dual beam spectrophotometer (A) and microplate reader (B). Activity is shown as the amount of product formed per minute and the amount of enzyme is shown as the amount in the assay. Correlation coefficients were $r = 0.99$ and regression equations were $y = 0.265x - 0.0046$ and $y = 0.228x - 0.0012$ for A and B respectively.

The equations for the regression lines show that the microplate method (Figure 2.6 B) gives a lower activity per μg enzyme protein than the dual beam spectrophotometer method (Figure 2.6 A). However, regression coefficients were not significantly different ($t_8 = 1.31$), although elevations were ($t_8 = 4.06$).

2.3.3. Spectrophotometric methods for the assay of GST.

Dual beam, multi-cell and microplate methods were used for the assay of GST in *G. pulex* (see section 2.6). Activities measured by these three methods are compared in Table 2.5. The microplate reader gave lower values than the dual beam spectrophotometer but activities determined using different methods were not significantly different for either pure GST or for *G. pulex* extract ($F_{2,27} < 2.89$).

Table 2.5. Specific activity of pure equine GST and *G. pulex* extract assayed with different spectrophotometer methods. The data shown are mean specific activity as $\mu\text{mol}/\text{min}/\text{mg}$ protein \pm one standard deviation. The number of replicates was 10.

Enzyme source	Dual beam Pye Unicam SP-8	Multi cell * Shimadzu UV1200	Micro-plate Anthos labtec HPII
Pure Equine	84.4 \pm 8.6	no data	79.1 \pm 4.7
<i>G. pulex</i>	0.084 \pm 0.012	0.079 \pm 0.008	0.081 \pm 0.007

* Assay at 15°C (others at 25°C).

2.3.4. Kinetics of pure and *G. pulex* homogenate GST.

Kinetic parameters for GST from *G. pulex* homogenate and purified GST were assessed. The Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined for CDNB and GSH (Table 2.6). $K_{m\text{CDNB}}$ values were found to be significantly different. Regression coefficients for $K_{m\text{CDNB}}$ for pure GST using the spectrophotometer and the microplate method were significantly different ($t_7 = 4.58$). Furthermore, regression coefficients for $K_{m\text{CDNB}}$ for *G. pulex* homogenate and pure GST were significantly different ($t_8 = 11.6$). However, regression coefficients and slopes for $K_{m\text{GSH}}$ and V_{max} for pure GST as measured by dual-beam spectrophotometer and microplate methods were not significantly different ($t_7 = 0.03$ and $t_7 = 1.42$ respectively). The purity of the preparations prevented comparison between V_{max} values derived using pure or *G. pulex* homogenate.

Table 2.6. Kinetic parameters for pure equine GST using both dual beam and microplate methods for GST and protein assay. Values shown are the mean of triplicate readings shown \pm the standard error of the mean. V_{max} and K_m values are determined by Lineweaver-Burk plots. Regression lines were fitted to data with values $r > 0.96$.

Method	GST type	$K_{m\text{CDNB}}$ (mM)	V_{maxCDNB} ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	$K_{m\text{GSH}}$ (mM)	V_{maxGSH} ($\mu\text{mol}/\text{min}/\text{mg}$ protein)
Dual beam	<i>G. pulex</i>	0.66 \pm 0.1	0.17 \pm 0.01	-	-
Dual beam	Pure	1.04 \pm 0.3	116.3 \pm 10.3	0.19 \pm 0.01	147.1 \pm 2.2
Micro-plate	Pure	1.77 \pm 0.3	178.6 \pm 34.2	0.32 \pm 0.01	123.4 \pm 3.1

The K_m and V_{max} by variation of both substrates for pure equine GST gave a K_m $_{CDNB, GSH}$ of 2.1 ± 0.3 (standard error) and a V_{max} $_{CDNB, GSH}$ of 64.5 ± 19.5 (standard error) determined using the micro plate reader method.

2.3.5. Optimum assay conditions for *G. pulex* and pure (Equine) GST.

Optimum assay conditions were determined for pure enzyme and *G. pulex* homogenate. Change in activity over a temperature range is shown in Figure 2.7.

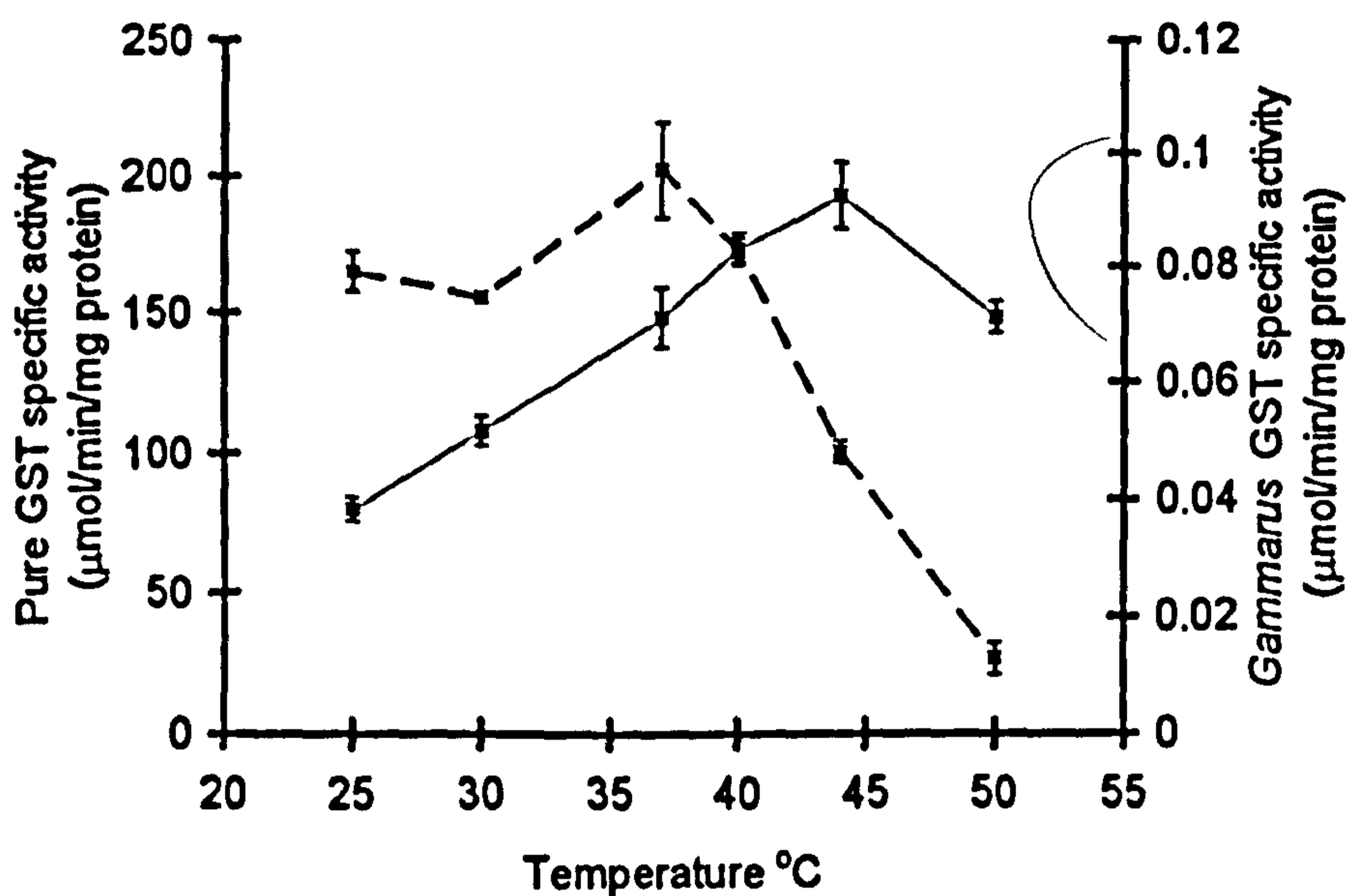


Figure 2.7. Change in specific activity with temperature. *G. pulex* GST activity is denoted by the broken line, pure GST activity by the unbroken line. Data are the mean of three readings \pm one SEM.

G. pulex GST had a maximal activity at 37 °C, although this was not significantly higher than the activity at 25 °C ($t_2 = 3.33$, $p > 0.05$), and there was little change in activity between 25 and 30 °C (Figure 2.7). Beyond 37 °C there was a sharp decline in activity with temperature increase. Pure GST shows a very much higher specific activity than *G. pulex* homogenate due to the relative purity of the preparation. The maximum activity for pure GST was at 44 °C, activity increasing from 25 °C to 44 °C and then declining between 44 and 50 °C (Figure 2.7).

The pH optima of pure and *Gammarus* GST preparations are illustrated Figure 2.8. Again, pure preparation activity is very much higher than that of *G. pulex* GST activity due to the relative purity of the preparations. The profile of the changes in GST activity with increasing pH was, however, similar for both preparations. Both preparations had a maximum activity at pH 7.5 (Figure 2.7)

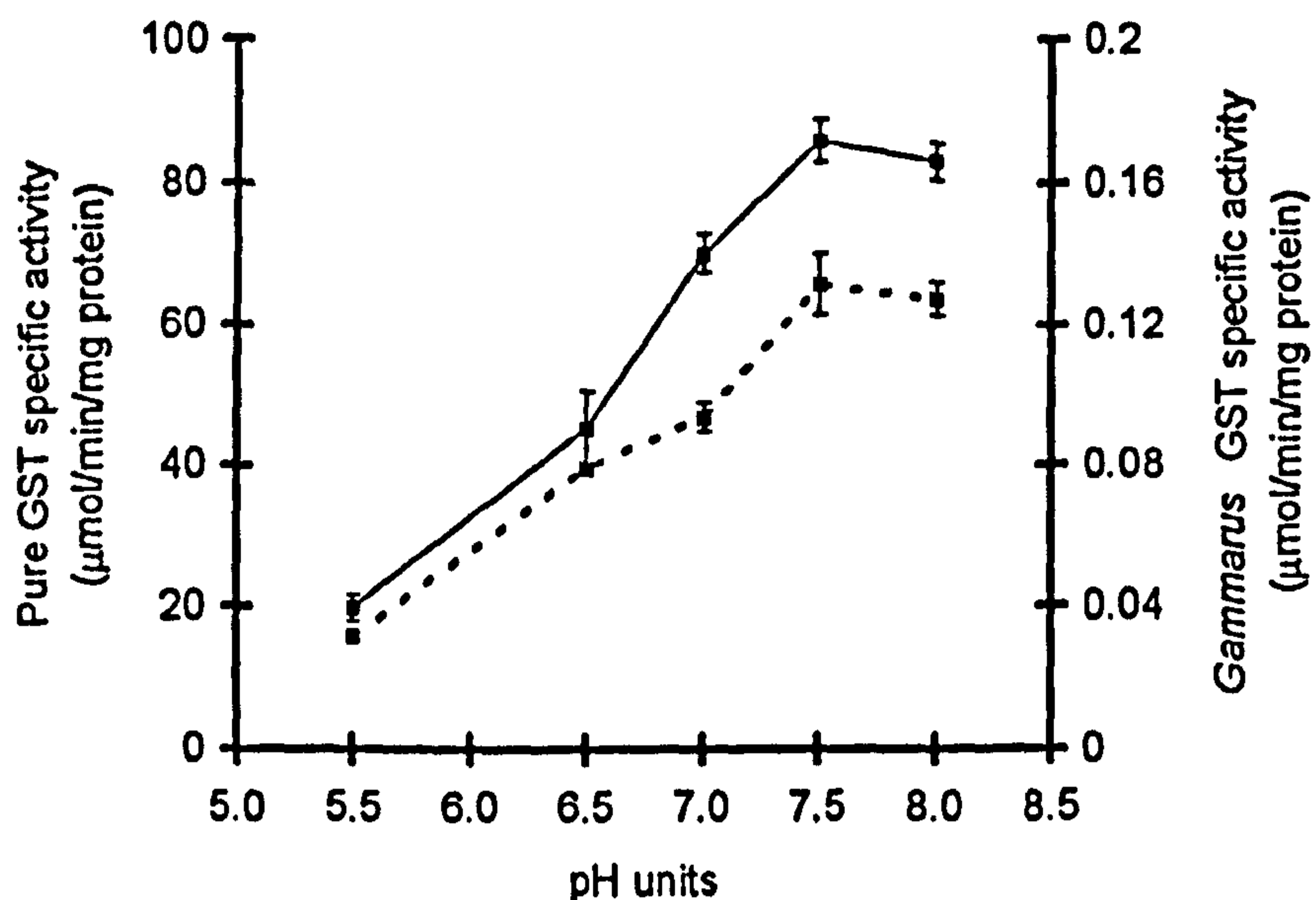


Figure 2.8. GST specific activity with changing pH. *G. pulex* GST activity is shown as a broken line, and purified equine GST activity as an unbroken line. Error shown is standard error of the mean determined from the mean of three readings.

2.3.6. Enzyme linked immuno-sorbent assay detection of GST in *G. pulex*. Antibodies raised to *Schistosoma* GST recognised GST in ELISA using *G. pulex* extract, but not using equine GST preparation. Antibody recognition run against controls without antigen are shown in Figure 2.9. *Gammarus pulex* extract had a significantly higher signal as measured by absorbance than all other preparations ($F_{3,8} = 60$, Tukey's, $q > 4.53$). Pure GST preparation was not significantly different from control (Tukey's, $q < 4.53$).

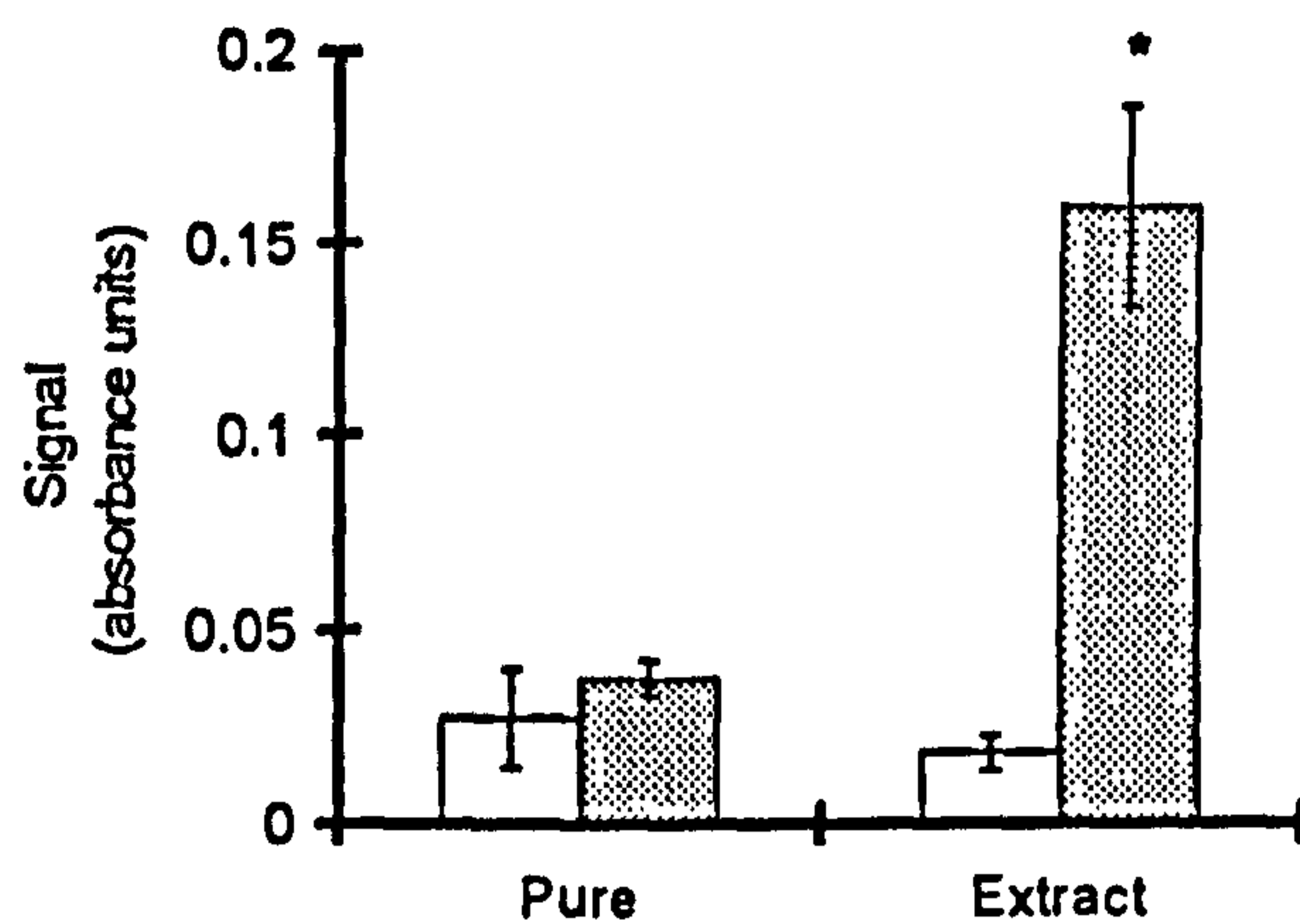


Figure 2.9. Antibody recognition of GST preparations. Control preparations without antigen (open bars) and GST preparations (Solid bars) are shown for ELISA run with pure GST and *G. pulex* extract. Data shown is the mean of three samples. Error bars are one standard deviation. Significant difference between treatments is indicated with an asterisk.

2.3.7. Effects of intrinsic and extrinsic factors on GST activity.

Storage.

There was a significant effect of storage temperature on GST activity in *G. pulex* ($F_{3,68} = 47.2$) Animals that were not frozen in liquid nitrogen prior to assay or stored at -10°C , showed a significant loss of activity as compared with those animals that were frozen in liquid nitrogen only and frozen and stored at -70°C (Tukey's multiple comparison test $q > 3.72$), (Figure 2.10).

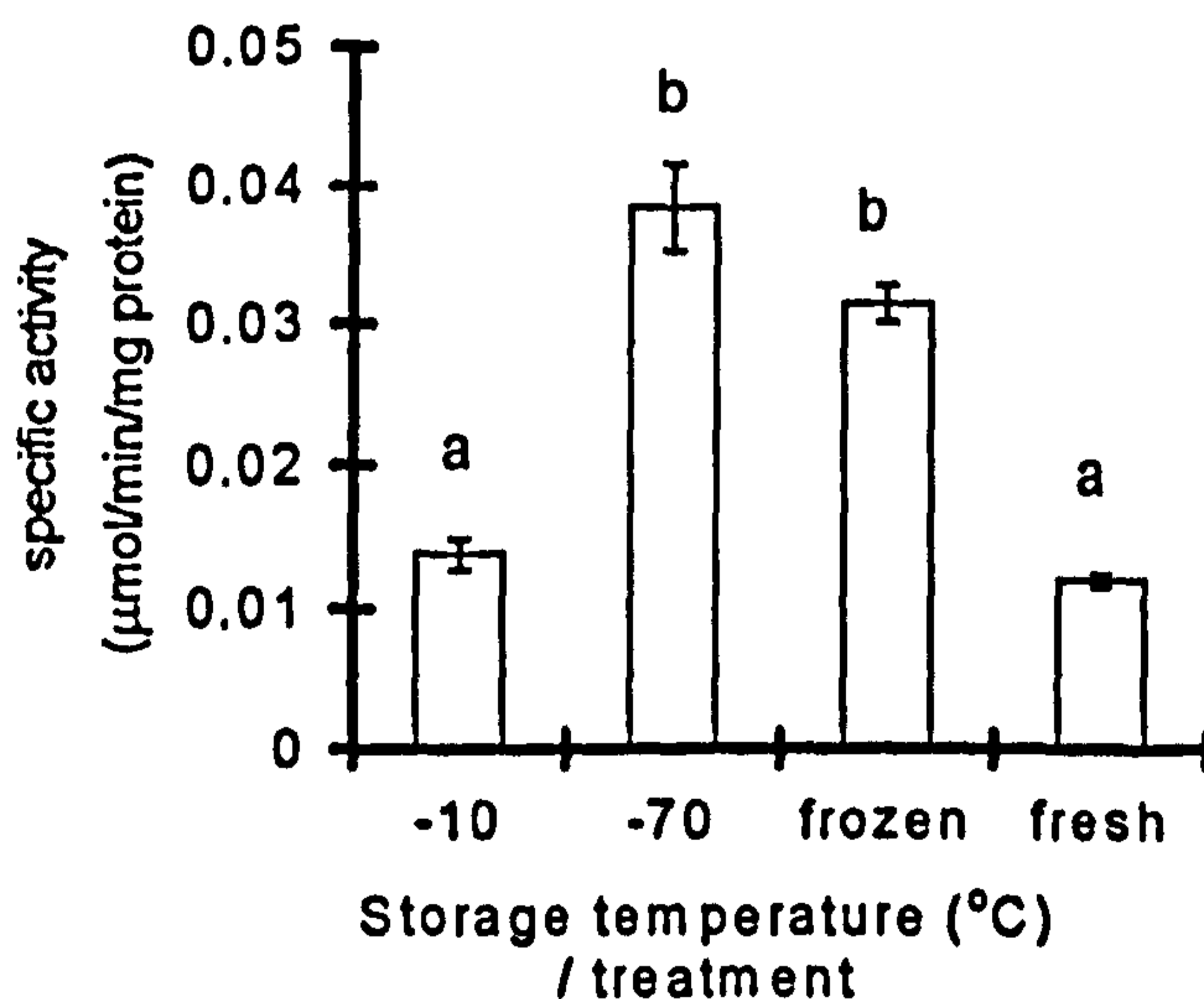


Figure 2.10. Mean specific activity of 18 animals per treatment. The error shown is the standard error of the mean. Data sharing the same letter are not significantly different.

Seasonal variability.

There was significant variation in the specific activity of animals collected from the field site at Clowne Derbyshire during 1995 ($F_{6,133} = 13.71$). There was no significant difference in the GST activity of animals collected in the months from February through to July (Tukey's, $q < 4.23$). However, animals collected in January and October had significantly lower activities than animals collected in other months (Figure 2.11).

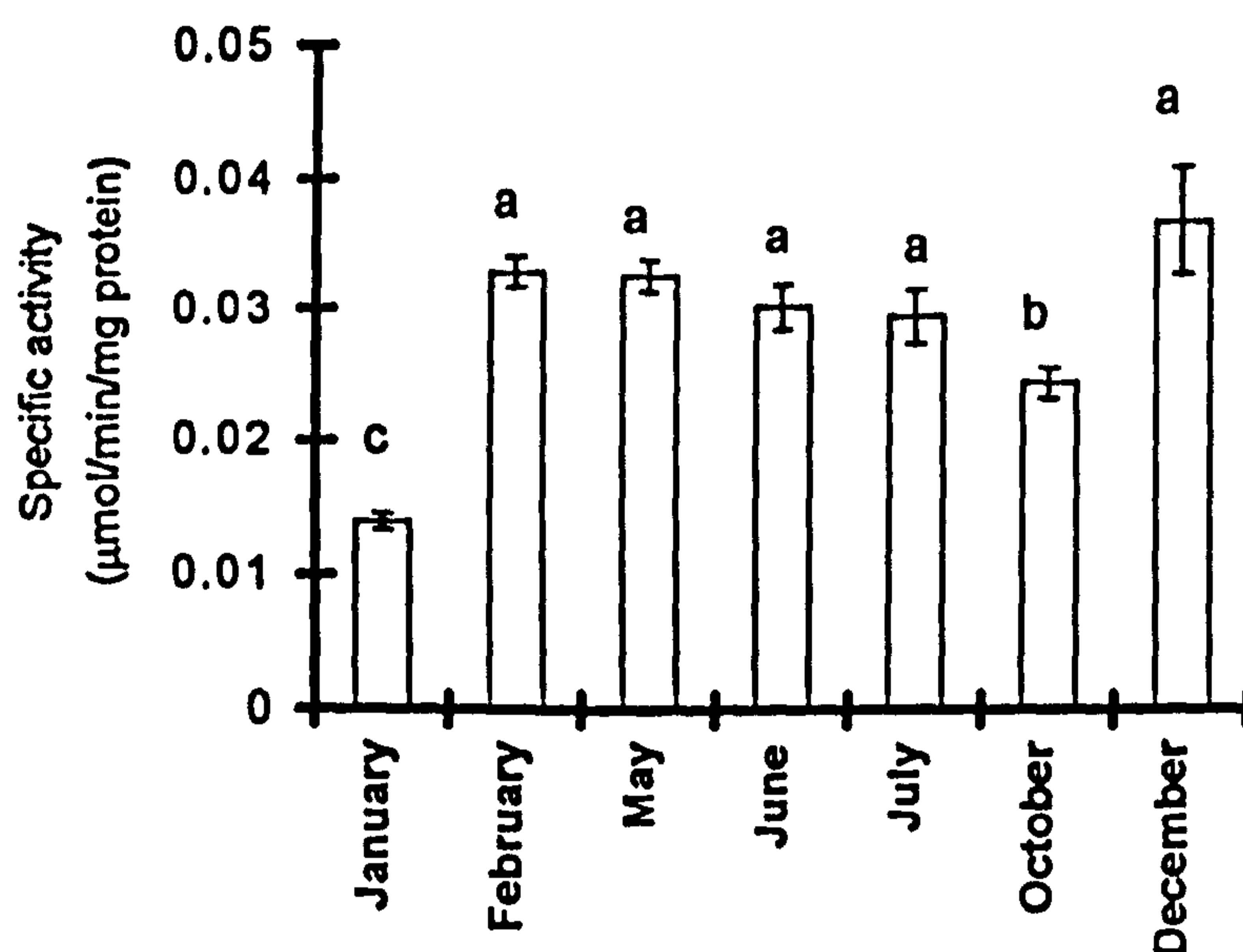


Figure 2.11. Specific activity in *G. pulex* over the year. Animals were collected January to December 1995 and data are the mean of triplicate readings of 20 male animals. Error shown is standard error of the mean. Data sharing the same letter are not significantly different.

Laboratory acclimation.

The effect of acclimation was investigated over a six week period. Maintaining *G. pulex* under laboratory conditions significantly reduced GST activity ($F_{2,57} = 3.79$). Specific GST activity of *G. pulex* maintained under laboratory conditions for 6 weeks was significantly lower than that of animals analysed directly from the field (Dunnett's, $q > 2.77$), (Figure 2.12).

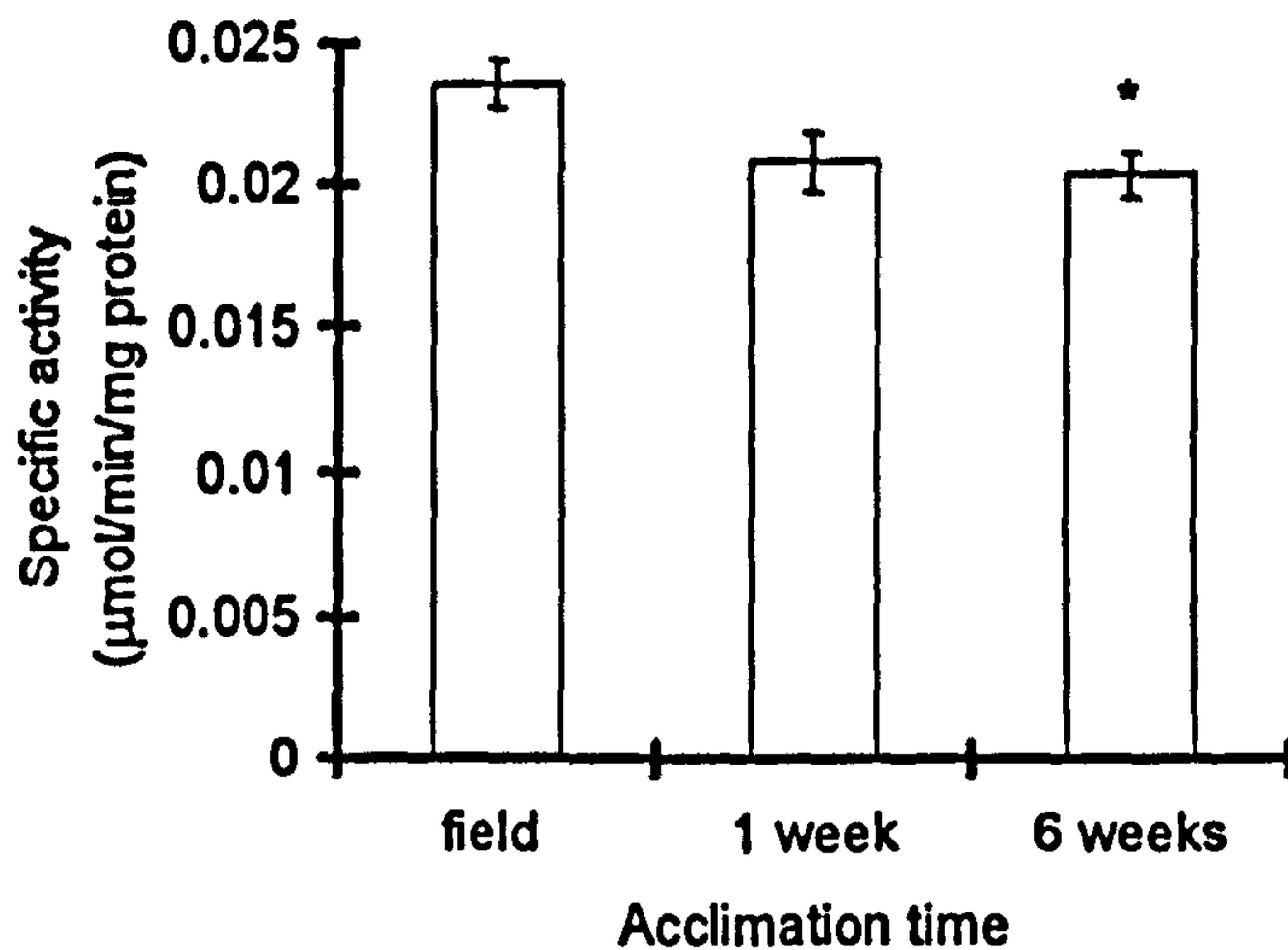


Figure. 2.12. Acclimation time and specific activity for *G. pulex*. Data presented as mean values for 18 animals \pm standard error of the mean. Significant difference from field value is indicated with an asterisk.

Temperature.

The effect of environmental temperature on GST activity is illustrated in Figure 2.13. There was no significant difference between replicates ($F_{6,99} = 0.46$) but there was a significant effect of temperature on the activity of GST determined after one week ($F_{2,99} = 9.84$). Activity was significantly greater at 4 °C than at 15 or 20 °C (Tukey's, $q > 3.36$).

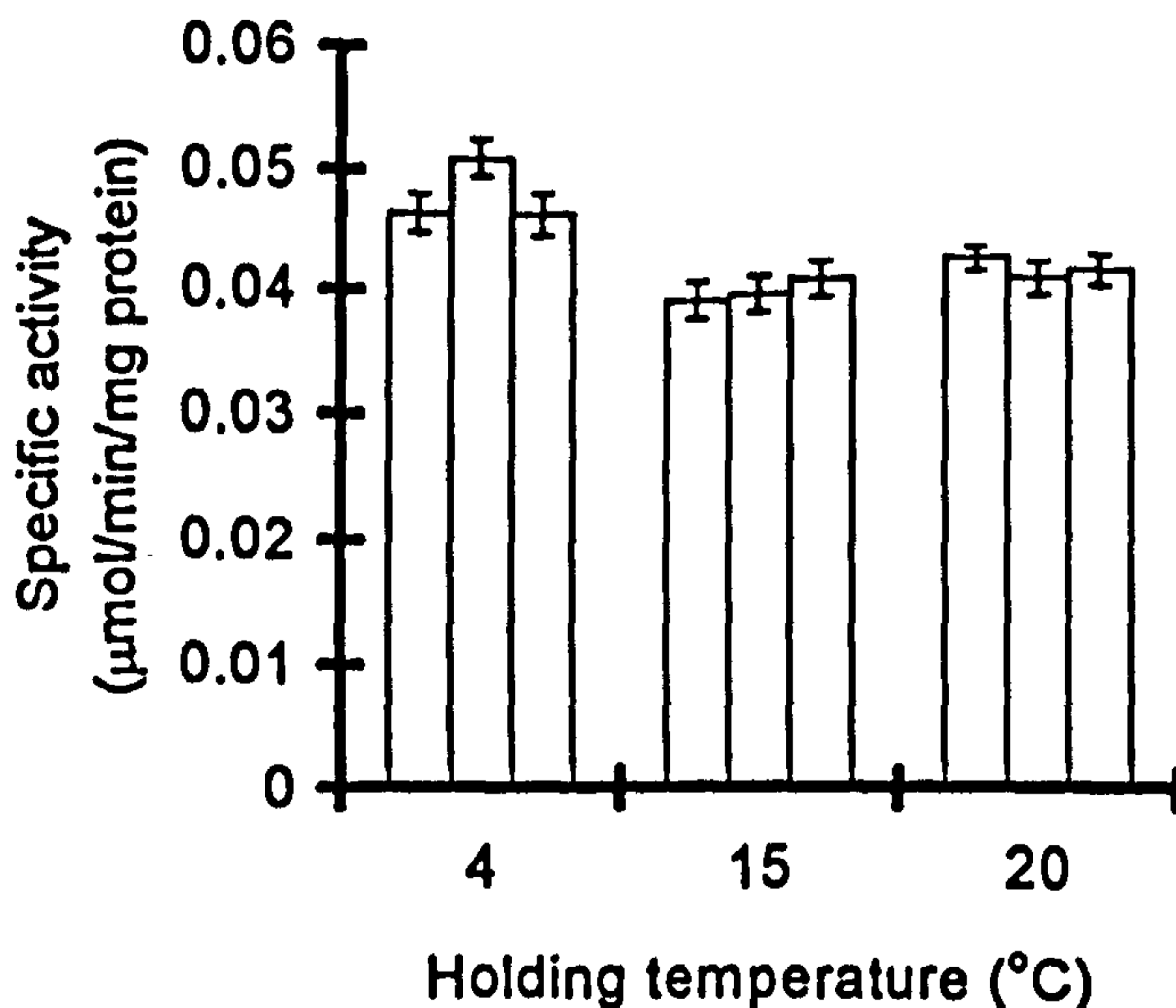


Figure. 2.13. Effect of holding temperature on GST activity in *G. pulex*. Values are the mean of 12 animals and there were 3 replicates for each treatment. Error bars indicate the standard error of the mean.

Effect of holding conditions.

The effect of holding animals in experimental holding tanks and tubes (as illustrated in Figure 2.2) on GST activity is shown in Figure 2.14.

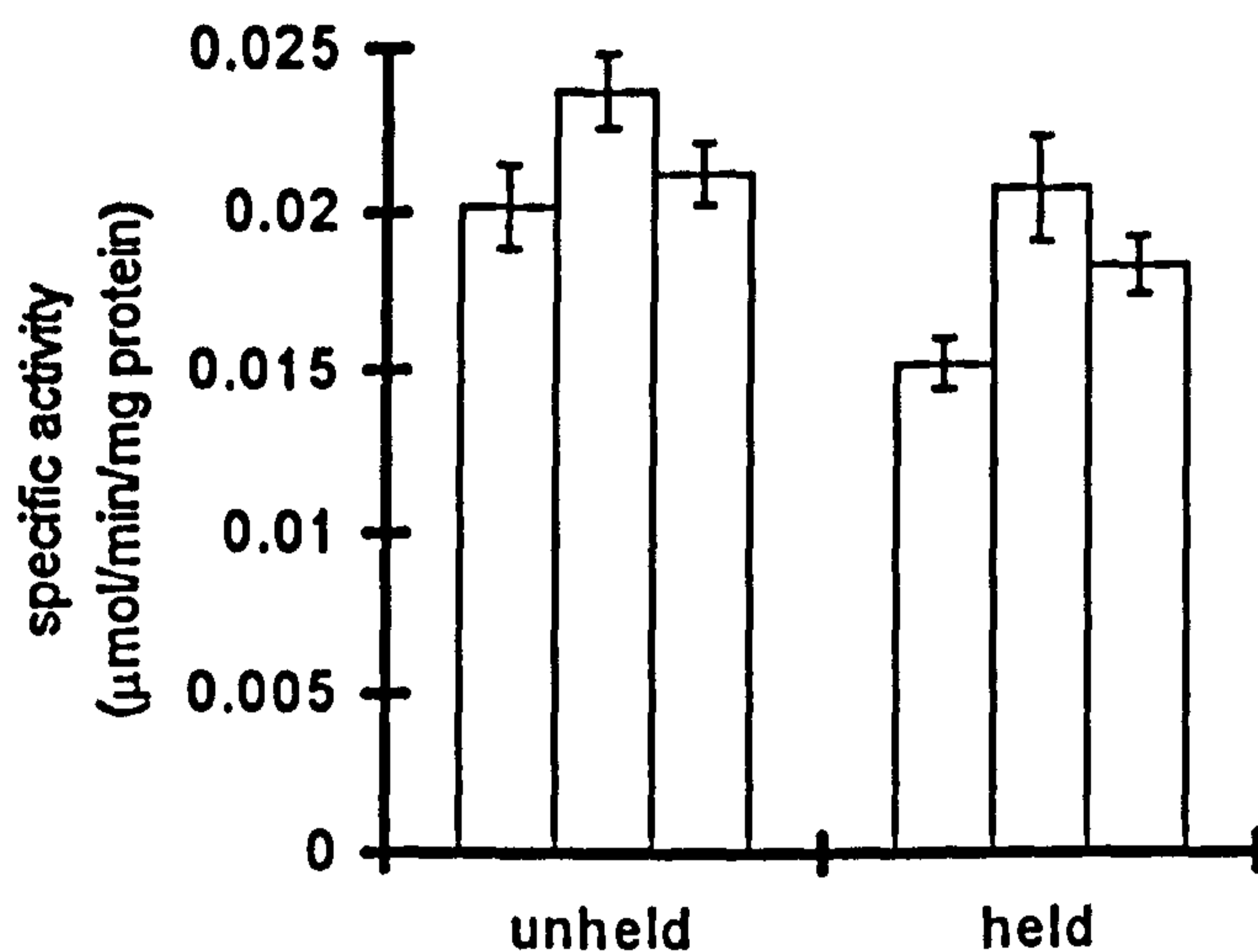


Figure 2.14. GST activity in *G. pulex* in holding tubes (held) and in tanks only (unheld). Three replicates are shown for each treatment. Each replicate is the mean of 15 animals. Error shown is the standard error of the mean.

Nested ANOVA indicated that there was significant variability within ($F_{1,84} = 14.7$) and between ($F_{4,84} = 4.2$) treatments. GST activity of *G. pulex* kept freely in tanks was greater than that of animals held in tubes.

Feeding.

The GST activity of fed and starved *G. pulex* is shown in Figure 2.15. Fed animals had a significantly greater GST activity than unfed animals (nested ANOVA, $F_{1,114} = 23.78$). There was no significant difference between replicates within treatments ($F_{4,114} = 2.19$).

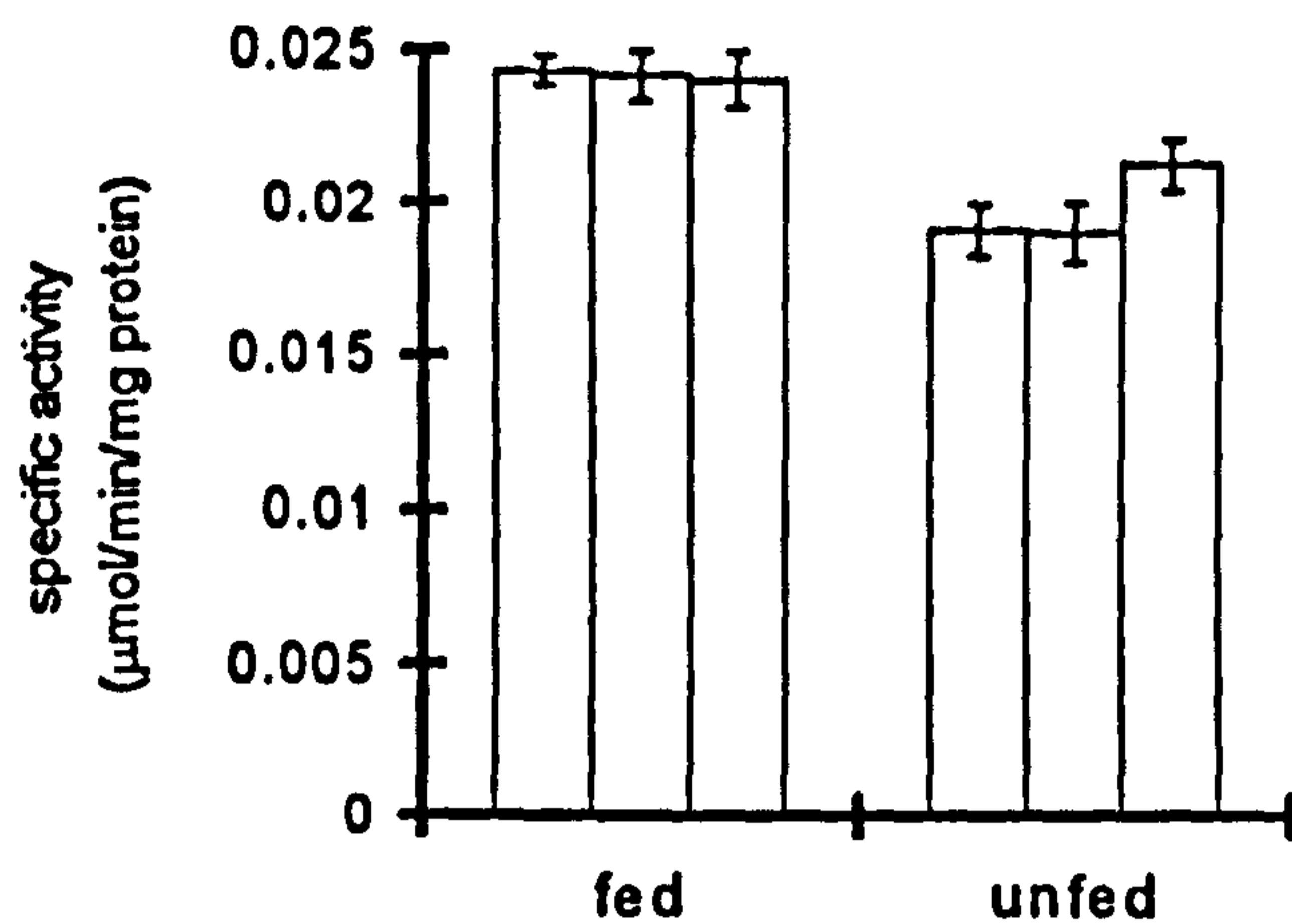


Figure 2.15. GST activity in fed or unfed *G. pulex*. Values shown are the mean of triplicate readings of 10 animals. Three replicates are shown for each treatment and error bars are the standard error of the mean.

GST activity measured using the microplate method and therefore different plates were used to analyse animals from the same treatment. Table 2.7 shows the results of a nested analysis of variance performed on data comparing GST activity in fed and unfed animals.

Table 2.7 Nested ANOVA summary of the total GST activity in fed and unfed individuals. There were: two treatments, six plates per treatment, ten individuals per plate and three replicates per individual.

Source	d.f.	<i>F</i>	% of total variability
treatment	1	69.89 *	16
plate	5	0.59	2
individual	54	19.6 *	37
replicate	120	0.15	4
error	179		41

* $P < 0.001$

Although there was a significant difference between treatments ($F_{1, 179} = 69.89$) There was a no significant difference between plates used in the assay within treatment ($F_{5, 179} = 0.59$). There was a significant difference between individuals ($F_{54, 179} = 19.6$) but not between-replicate samples from each individual. Within a treatment, between-individual variation accounted for 37 percent of the total variation, whereas between-plate variation accounted for only 2 percent.

Body size.

The effect of body size on GST activity was investigated using *G. pulex* from Craggs stream. Animals were divided into size classes on the basis of length (section 2.2.9). However, GST activity is determined on the basis of weight and total protein. The relationship between length and weight is illustrated in Figure 2.16. This was a curvilinear relationship described by the equation $y = -0.004x^2 + 0.33x + 4.47$ ($r = 0.95$).

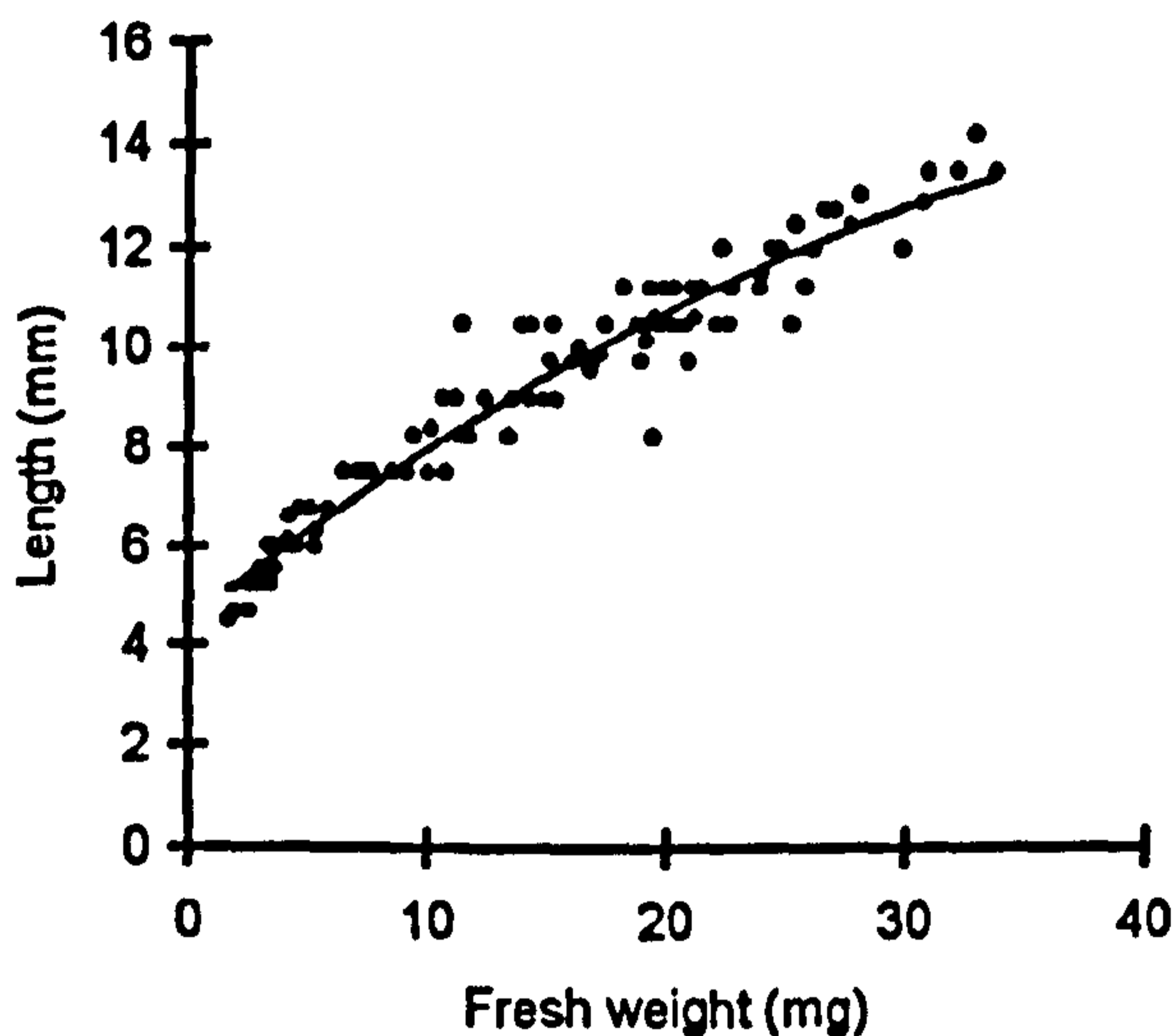


Figure. 2.16. Length-weight relationships for animals from all size groups. A total of 80 animals 20 from each size group were measured.

The relationship between weight and protein content was linear (Figure 2.17) and was described by the equation $y = 0.041x + 0.12$ ($r = 0.83$).

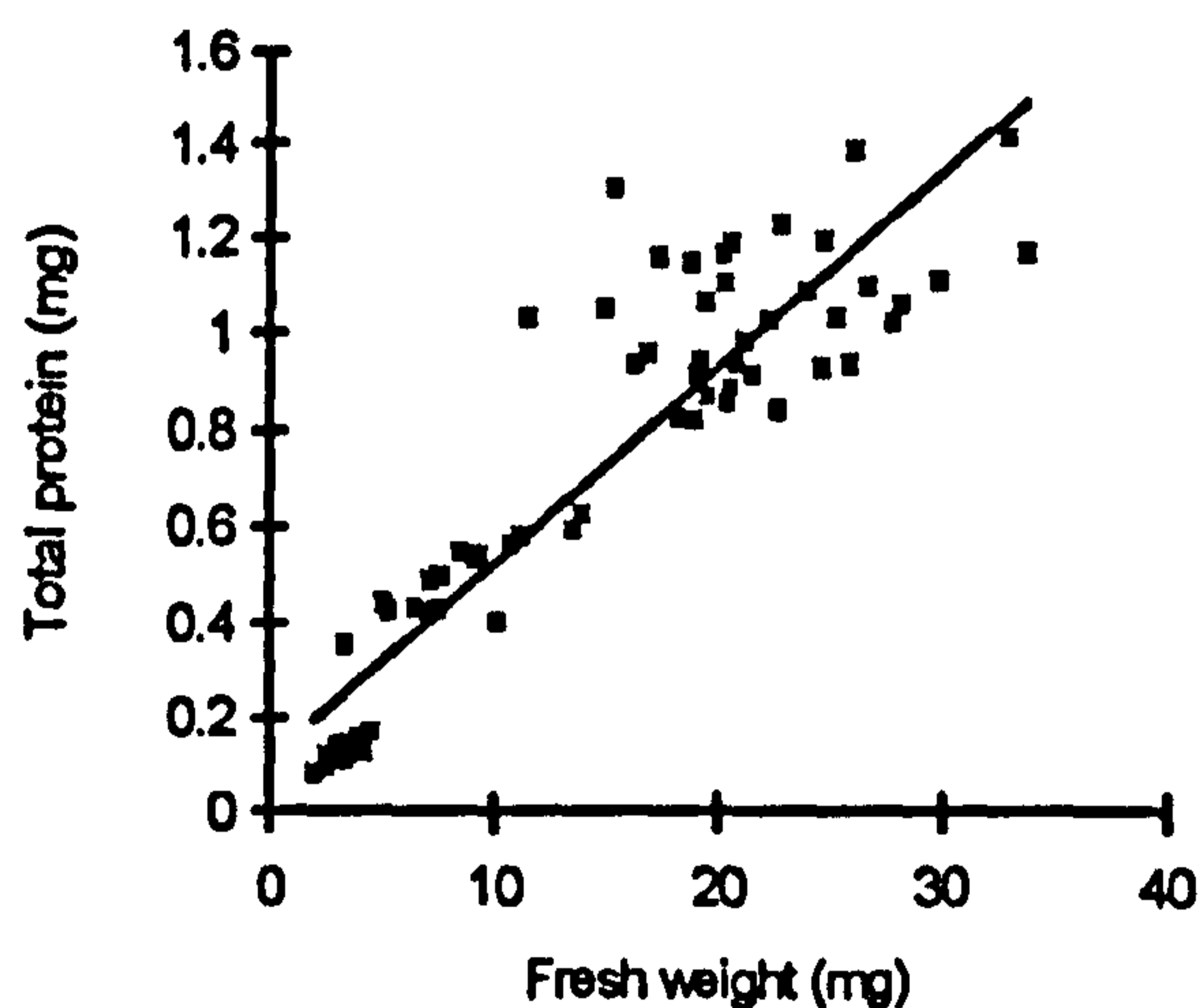


Figure 2.17. Relationship between fresh weight and total protein content of *G. pulex* from four size classes. From each size class a total of 20 animals were analysed.

The linear relationships between activity and weight and activity and protein are illustrated in Figures 2.18 A and B, respectively. These relationships are described by the equations $y = 0.0013x + 0.0018$ ($r = 0.86$) and $y = 0.026x + 0.0014$ ($r = 0.76$), respectively.

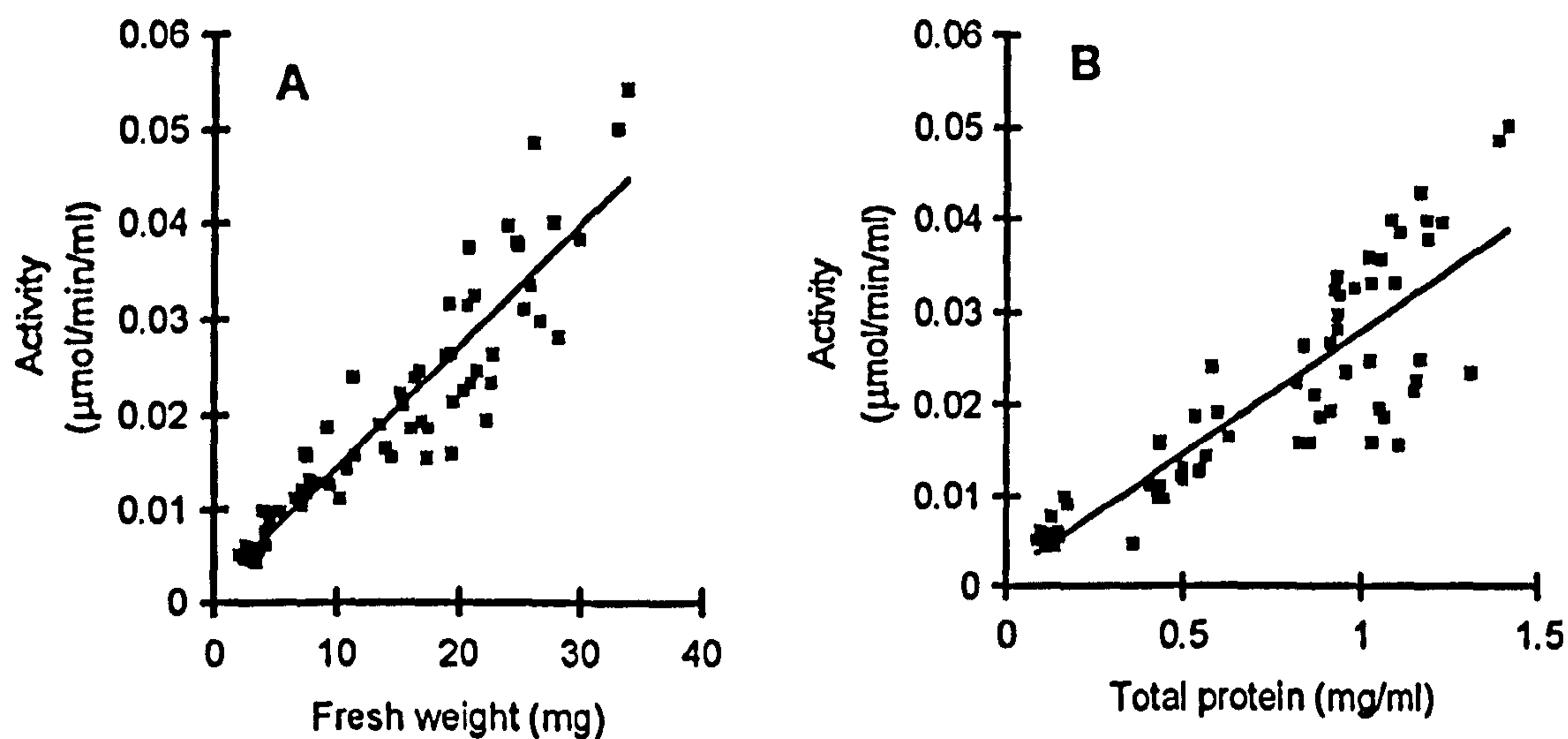


Figure 2.18. GST activity plotted against fresh weight (A) or total protein (B). A total of 80 animals was used (20 from each group). Activity is per ml of *G. pulex* extract used in GST assay. Fresh weight is mg of animal per ml of extract and protein is as mg per ml of extract.

Logarithmic transformation of the data presented in Figure 2.18 B gave a linear regression line described by the equation $y = 0.76x - 1.6$ with a correlation coefficient of 0.84. This suggests that animals with less total protein (i.e. smaller animals) have a proportionately higher GST activity.

Figure 2.19 shows a plot of activity against protein for animals in size class 2 (i.e. 9.6 to 15 mm). This was the size class of adults used in all other experiments. The correlation coefficient was 0.87 and the line was described by the equation $y = 0.0374x - 0.0041$. Logarithmic transformation of the data presented in Figure 2.19 gave a linear regression line described by the equation $y = 1.1x - 1.2$ with a correlation coefficient of 0.85. This suggests that GST activity was proportional to total protein over the range of total protein in this size class.

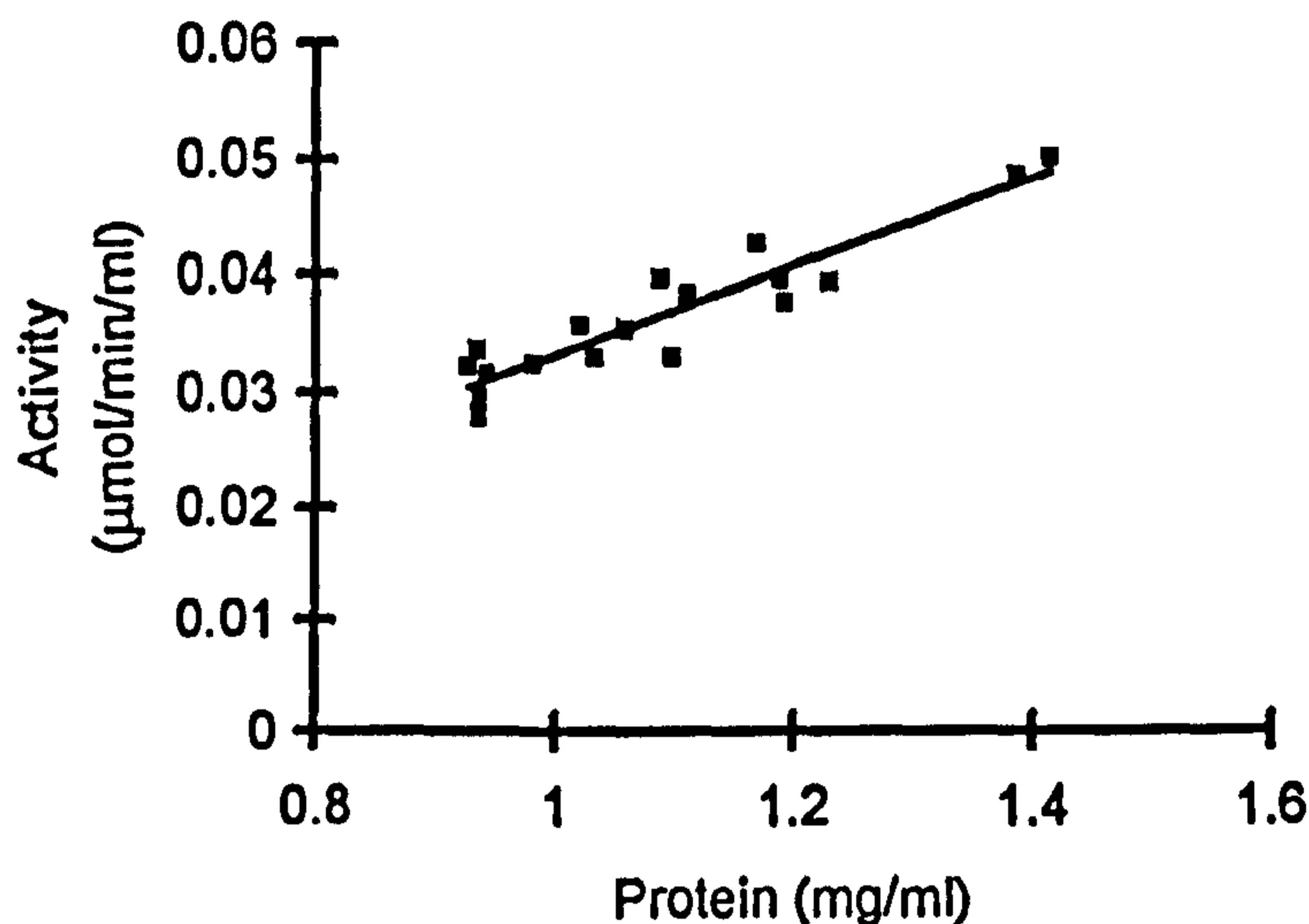


Figure 2.19. Activity and protein in largest (class 2) separated. Values shown are the mean of triplicate readings of 20 animals. Activity is shown per ml of *G. pulex* homogenate in assay. Protein is shown per ml of homogenate. Activity and protein were assayed using microplate reader methods.

The specific GST activity of *G. pulex* in the different size classes is shown in Figure 2.20. Activity differed significantly across size classes ($F_{3,76} = 28.08$) with activity in the smallest animals (class 6, 4.5 - 6.15 mm) being significantly higher (Tukey's, $q > 3.72$) than activity in all other classes. Class 3 animals (8.25 - 12 mm) had the lowest activity.

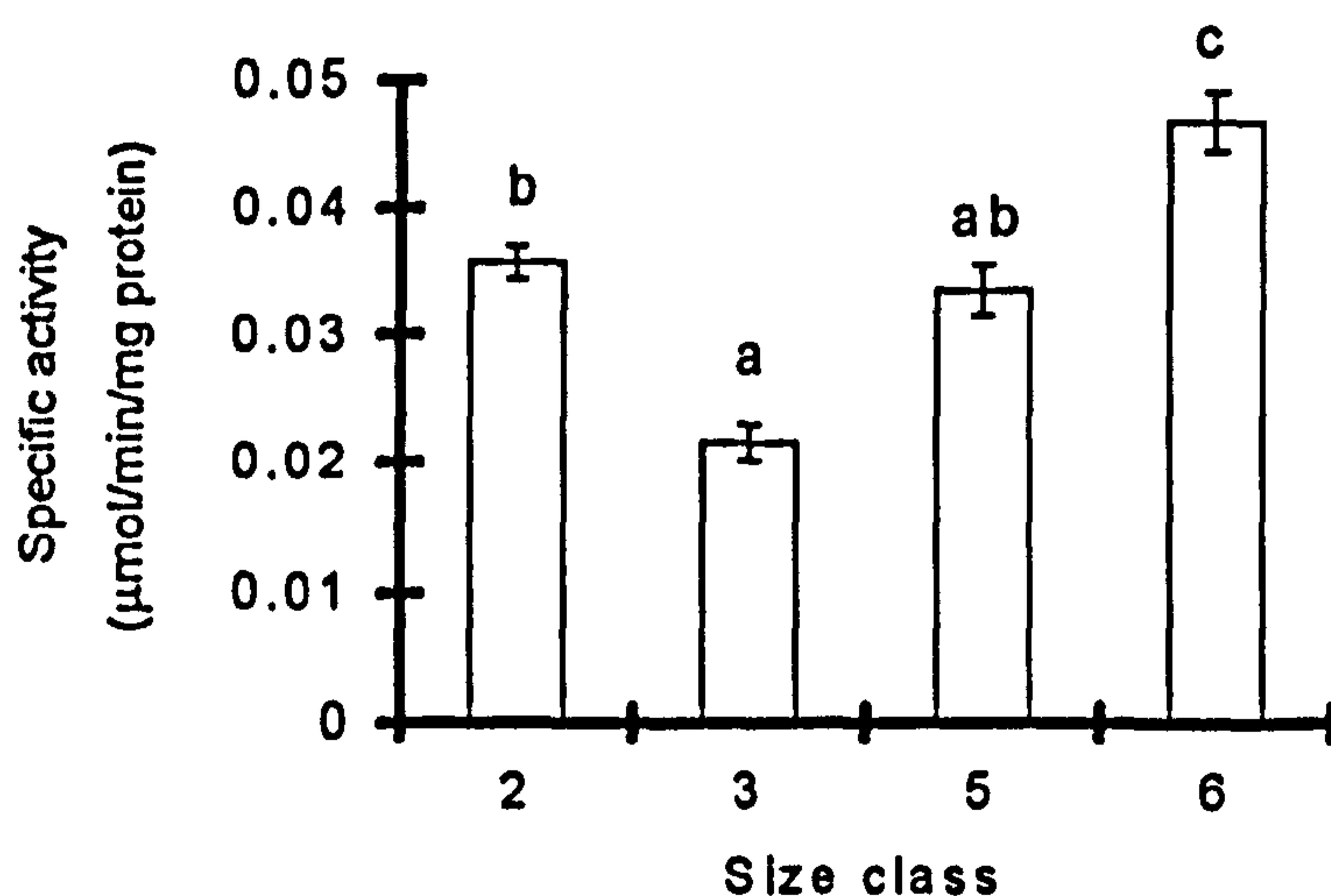


Figure 2.20. Specific activity with size class. Animals separated as described in section 2.2.10 (see Fig. 2.3). Activity is presented as the mean of triplicate readings of 20 animals frozen after 7 days acclimation. Error shown is standard error of the mean. Data sharing the same letter are not significantly different.

A similar pattern was observed when activity was expressed in terms of activity per unit weight (Figure 2.21). On a per unit weight basis, activity in class 3 animals is significantly lower than that of classes 2, 5 and 6 animals ($F_{3,76} = 20.08, q > 3.72$). GST activity in class 2 was significantly lower than that of class 6 ($F_{3,76} = 20.08, q > 3.72$).

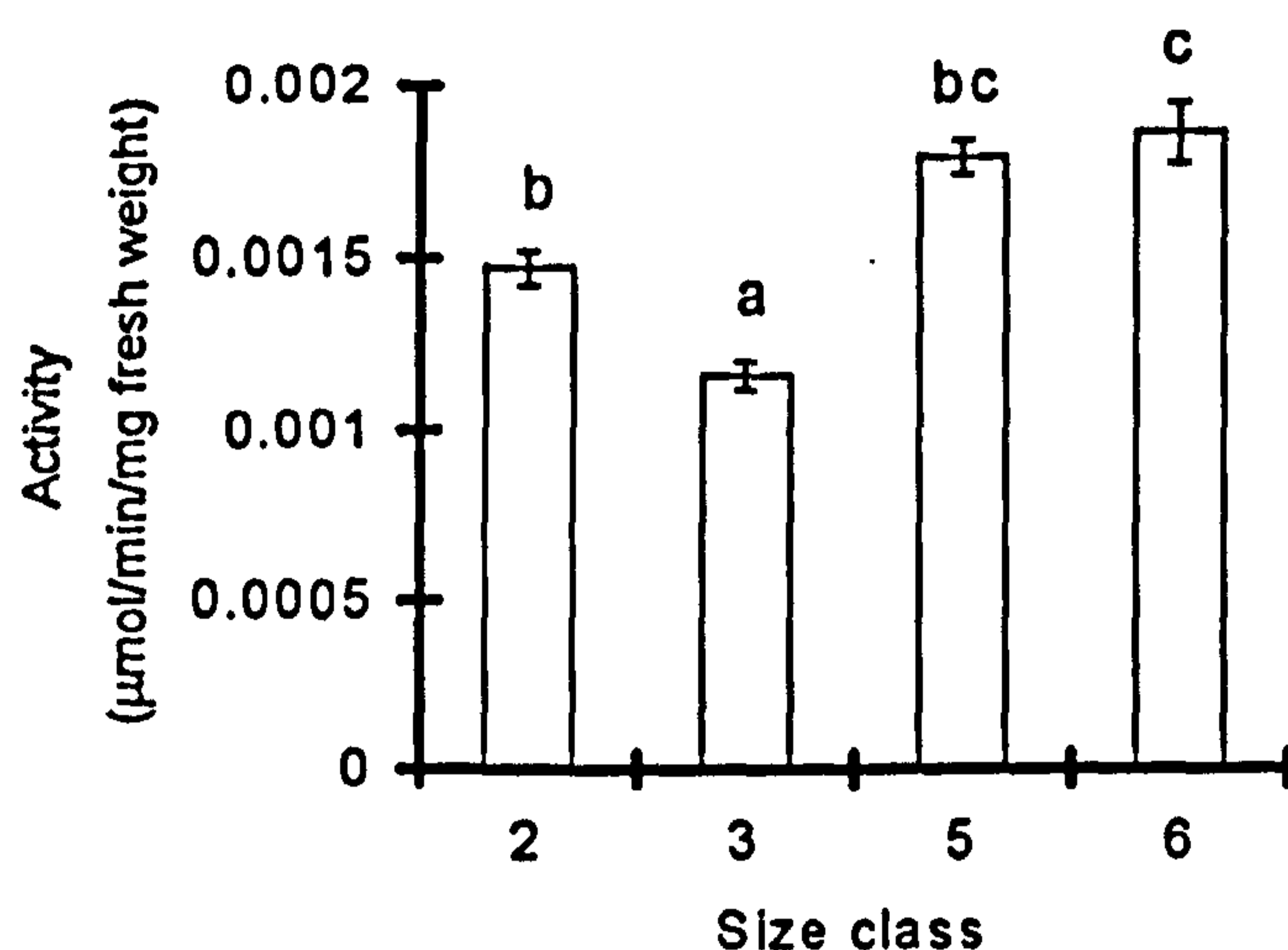


Figure. 2.21. GST activity is shown as weight specific GST activity. Values shown are the mean of triplicate readings of 20 animals. Error is standard error of the mean. Data sharing the same letter are not significantly different.

2.4. DISCUSSION.

The aim of the work set out in this chapter was to assess the activity of GST in *G. pulex* and to establish which factors affected activity. The first objective was the modification of the GST assay (Habig *et al.*, 1974) to enable a large number of individual *Gammarus pulex* to be assayed. The development of a simple method, using an extract that was not subject to protein purification, would then promote a rapid and simple method for measurement of GST activity in *G. pulex*. Once the assay had been developed, the second objective was to investigate variation in activity of unchallenged animals and the causes of variation. Both intrinsic, (e.g. body size) and extrinsic (e.g. environmental temperature) factors were investigated. The third objective was to minimise variation in order to assess whether a 'baseline' of activity could be observed and if so, to quantify it.

2.4.1 Measurement of GST activity in crude extracts of *G. pulex*.

GST activity was measured using either a dual beam spectrophotometer, a multicell spectrophotometer or a microplate reader. The assay used with the dual beam spectrophotometer was based on the method stated by Habig *et al.* (1974). The maximum absorbance wavelength was confirmed to be 340 nm. There was a linear increase in product formation for at least 3 minutes, adequate time for an estimation of the rate of product formation and thus activity.

The GST activity of crude extract of *G. pulex* was compared with a commercially available source of purified GST. This enabled verification that the sample matrix was not interfering with the measurement of GST activity. Furthermore, estimation of the optimum assay conditions and kinetic constants of a purified GST and *G. pulex* GST allowed comparison of the crude extract against a 'standard' of known specific activity. In order to be able to compare specific activities (i.e. activity per amount protein) of *G. pulex* GST, activity must be a linear function of protein concentration. The GST assay of *G. pulex* extract was linear over a range of protein concentrations from 0.3 to 2.6 mg/ml homogenate. This covered the range of protein concentrations encountered using *G. pulex* extract.

Optimum temperature and pH conditions for *G. pulex* activity were investigated and compared to those for a commercially available purified GST (equine). *G. pulex* extract GST activity was maximal at 37 °C whereas pure GST was maximal at 45 °C (Figure 2.7). This compared well with a maximum GST activity at 40 °C for the bivalve mollusc *Sphaerium corneum* (Beverley, 1994). It was therefore noted that assays measuring activity at 25 °C were at a sub-optimal temperature. However, for comparison with other work the standard temperature of 25 °C was used. Furthermore, there was no significant increase in *G. pulex* GST activity between 25 and 37 °C (Figure 2.7). Although assays were checked for a temperature of 25 °C, small increases in temperature would not have a large effect on *G. pulex* GST activity measurement.

Clark (1989) reviewed a range of pH optima for GST in a number of animals and found the optimal modal value was close to a pH of eight. The optimum for both *G. pulex* GST and pure equine GST was found to be pH 7.5 (Figure 2.8) which is in general agreement with the data of Clark (1989). The GST assay routinely performed at pH 6.5 which is sub-optimal. The conjugation of CDNB with GSH to form DNP-GSH is catalysed by GST but will also occur in the absence of GST. The GST assay measures the increase in DNP-GSH and therefore does not distinguish between enzymatic and non-enzymatic formation. At pH 7.5, DNP-GSH is produced by both reactions (Habig *et al.*, 1974). However, the rate of non-enzymatic reaction is decreased at 6.5 (Habig *et al.*, 1974). This lower pH is used in the assay to promote the formation of product via the GST-catalysed reaction. Furthermore, the rate of non-enzymic product formation was automatically subtracted from GST catalysed activity by the use of non-enzyme blanks.

Spectrophotometric assays.

The majority of work on invertebrate GST activity has utilised spectrophotometers (Tate and Herf, 1987; Beverley, 1994; Dierickx, 1984; Hazleton and Lang; 1983; Lee, 1988). However, the use of a microplate reader

enables a large number of individual samples to be analysed simultaneously. The microplate assay used in the present study was developed from the dual beam spectrophotometer method by the scaling down of reagents quantities from the 2.5 ml used in the dual beam spectrophotometer to the 200 μ l microplate wells (section 2.2.6). The use of 50 μ l samples of homogenate allowed the simultaneous assay of up to 96 samples per plate or 30 triplicate readings of 30 animal samples plus blanks. The microplate assay was based on a product absorbance coefficient of $8.9 \text{ mM}^{-1} \text{ cm}^{-1}$ for the microplate assay. The extinction coefficient (using the 200 μ l assay volume) in the microplate assay was found to differ from that of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ stated by Habig *et al.* (1974). The determination of the absorbance coefficient using a purified conjugation product has been reported (Grant *et al.*, 1989). However, a purified conjugation product, as determined by Hollingworth *et al.* (1973) was not used to determine the absorbance coefficient for the microplate assay. It was therefore assumed that all substrates had been conjugated to product for the determination of the extinction coefficient using the microplate reader. The microplate reader 'extinction coefficient' of 8.9 OD mM^{-1} (Appendix 2.4) was assumed to be correct for an assay volume of 200 μ l using phosphate buffer as the diluent.

The use of microplate enzymes assays readers is well documented. Grant *et al.* (1989) demonstrated the use of a microplate reader for the estimation of GST in individual mosquitoes and microplate enzyme assays have been used to assay acetylcholinesterase inhibition in insects (Brogdon and Barber, 1987; Brogdon *et al.*, 1988; Brogdon, 1988). Microplate methods have also been developed for protein assays (Brogdon, 1984). The main advantage of microplate assays are their rapidity and the small amount of animal homogenate needed. Between one tenth and one hundredth of that needed for a conventional spectrophotometer using a 1 ml cell volume. In this study the use of a microplate assay allowed one fifth of the *G. pulex* homogenate volume of the spectrophotometer method to be used.

There was no loss in accuracy in assaying *G. pulex* GST activity using the microplate method compared to using the dual-beam spectrophotometer. Indeed, the coefficient of variation of activity from 10 replicates of *G. pulex* was 14.3 % for the dual-beam spectrophotometer and 8.6 % for the microplate method. For purified equine GST activity the dual-beam spectrophotometer method had a coefficient of variation of 10.2 % whereas the microplate method had a coefficient of variation of 5.9 % (for data see Table 2.5).

In the experiment to assess the effect of feeding on *G. pulex* GST activity each of the treatment groups were analysed on a set of six microplates allowing a nested ANOVA to show the sources of variation. The majority of variability was attributable to variation of activity in individual animals and little variation was attributable to either the use of different microplates or replication of individuals (Table 2.6). These results were encouraging for the use of the microplate assay as they suggested that differences between groups could be detected using a number of different plates and the replication of individuals was precise. Grant *et al.* (1989) found that plate readers compared well to the spectrophotometric method they used, also finding that 85% of variability in an assay of pesticide resistant and susceptible mosquitoes was due to individual variation.

As with the dual beam spectrophotometer, there was a linear range of activity with total protein over the range 0.0065 to 0.034 μg (Figure 2.6). The microplate reader gave consistently lower rates of activity. This was due to a significant difference in the elevation of Lineweaver-Burke plots rather than a difference in slope. Thus, although the rate of activity increase was the same using both methods, the actual activity per unit of protein was lower.

Using the microplate method GST activity values ranged from 0.015 to 0.08 $\mu\text{mol}/\text{min}/\text{mg}$ protein. These values are low compared with those derived by other workers for other crustacean species, particularly *Gammarus* species. Reported specific activities for crustacean GST range from 0.2 to 40 $\mu\text{mol}/\text{min}/\text{mg}$ protein (Table 2.1). Dierickx (1984) reported a GST specific

activity of 0.21 $\mu\text{mol}/\text{min}/\text{mg}$ protein for *G. pulex* and Aceto *et al.* (1991) reported a value of 0.85 $\mu\text{mol}/\text{min}/\text{mg}$ protein for *G. italicus*. Both Dierickx (1984) and Aceto *et al.* (1991) determined GST activity using unpurified extracts; the method of extraction being similar to that used in the present study. Confidence in the methods used in the present study is gained from the results of assays run using pure enzyme. In all cases it was shown that derived GST specific activity was within 95 % of that given by the manufacturer. The activity of pure equine GST stated by the manufactures (Sigma) was 83 $\mu\text{mol}/\text{min}/\text{mg}$ protein at 25 °C with 1 mM CDNB and 2.5 mM GSH. The determined activities for dual beam and microplate were 84.4 ± 8.6 and 79.1 ± 4.7 $\mu\text{mol}/\text{min}/\text{mg}$ protein respectively. Therefore there was little loss in activity due to the assay method. The reason for the difference between the specific GST activity reported by Dierickx (1984) for *G. pulex* and specific GST activity for *G. pulex* in this study is not clear. Dierickx (1984) does not however indicate the variability of the GST activity given for *G. pulex*, this may have been high. Furthermore, it can be seen that there are interspecific differences in GST activity between *Gammarus* species (Table 2.1), there may also be differences in the GST activity of different populations of *G. pulex*.

Kinetic values.

Kinetic parameters, especially the Michaelis constant (K_m) allow the comparison of extract from *G. pulex* with data from pure GST and K_m values derived by other workers. A difference in the maximum velocity (V_{max}) would indicate a quantitative difference in enzyme activity whereas a difference in K_m would be indicative of a qualitative difference. It is clear that V_{max} differences between pure GST and *G. pulex* extract are a consequence of the relative purity of the preparations. Deriving K_m allows comparison of a constant that is independent of the purity of the preparation. However, K_m will depend on the isoenzyme compliment of the sample which may be dependent upon preparation methods.

Most Michaelis constants have been determined by varying the concentration of one substrate at a fixed single concentration of the other substrate. However, it is

also possible to derive a single constant by variation of both substrates over a range of concentrations as shown in section 2.3.4. The K_{mCDNB} for *G. pulex* extract of 0.66 mM is similar to that of 0.44 mM derived by Tate and Herf (1978) for the blue crab (*Callinectes sapidus*). The results for K_{mGSTII} of *G. pulex* extract were not conclusive as Lineweaver-Burk plots were not linear. However, K_{mGSTII} of 0.19 mM (dual beam) and 0.32 mM (microplate) derived for the pure equine preparation were close to the value of 0.2 mM given for rat liver (Habig *et al.*, 1974). It should be noted that on comparison of the kinetic parameters for pure GST and *G. pulex* extracts in this study with those derived by other workers that these values have been derived by a number of differing experimental approaches. Enzyme preparations range from unpurified whole body homogenates to homogenous isoenzyme preparations. The kinetic values derived from both pure enzymes and *G. pulex* homogenate using either dual-beam or the microplate reader methods show that there was some agreement between methods. K_{mGSTII} measured of pure GST on dual beam spectrophotometer and microplate reader were not significantly different but values for K_{mCDNB} did differ significantly. K_{mCDNB} of pure GST and *G. pulex* GST measured by dual beam did differ significantly.

ELISA detection of GST.

A pilot study was performed to evaluate the possibility of using commercially available GST monoclonal antibodies for measuring *G. pulex* GST. The rationale for this approach was that the use of antibodies that recognise invertebrate GST may be more specific to particular isoenzymes of invertebrate GST (e.g. Aceto *et al.*, 1991) and therefore be more sensitive to the induction of GST. Monoclonal antibodies to *Schistosoma* GST recognised one or more of the GST isoenzymes in *G. pulex* extract but produced no signal with a pure preparation of equine GST. It may therefore be possible to use *Schistosoma* GST MAb for assaying *G. pulex* GST. However, before the method was developed further it would be necessary to check the recognition of *G. pulex* GST against a positive control of purified *G. pulex* GST to ensure that the *Schistosoma* GST MAb was not recognising another protein in the homogenate matrix. Furthermore, antibody

recognition should be checked with a positive control of purified *Schistosoma* GST. Unfortunately, *Schistosoma* GST was not commercially available and it was beyond the scope of this study to purify *G. pulex* GST.

Conclusions

- GST could be detected in individual *G. pulex*.
- Specific activity was 0.015 to 0.08 $\mu\text{mol}/\text{min}/\text{mg}$ protein lower than the value reported by Dierickx (1984).
- Use of a microplate reader caused no loss in accuracy in the detection of GST activity as compared with a conventional spectrophotometer.
- The microplate method allowed multiple replicate readings of individual *G. pulex* and rapid analysis of large numbers of samples.

2.4.2. Effects of intrinsic and extrinsic factors on GST activity.

The second objective addressed was the assessment of the factors effecting the GST activity of *G. pulex*. The factors affecting the activity of GST can be divided into those that affect the activity *in vitro* (e.g. assay and storage conditions) and those that affect activity *in vivo*. Factors affecting GST activity *in vivo* may be extrinsic, (i.e. those that are a result of environmental factors) or intrinsic factors (i.e. those that are dependent upon differences between individual animals).

The effects of assay conditions have been discussed above. The affect on GST activity of freezing and storage conditions on GST activity was investigated (Figure 2.8). There was a significant loss of GST activity in fresh samples or samples stored at $-10\text{ }^{\circ}\text{C}$ after freezing. It was therefore necessary to rapidly freeze *G. pulex* samples and store them in an ultra cold freezer below -70°C . The rapid freezing of samples prior to GST analysis has been demonstrated in a number of studies (e.g. Darby, 1973; Pabst *et al.*, 1974; Förlin and Andersson, 1985).

Extrinsic factors that affected the activity of GST could be controlled or minimised to a certain extent if animals were held and maintained in laboratory conditions. In the present study, the GST activity of field collected animals was reduced after 6 weeks acclimation under laboratory conditions (Figure 2.11). Temperature affected GST activity, at lower holding temperatures (4 °C) GST activity was significantly elevated. This effect of holding temperature on GST activity may help to explain difference found in the field population over the year. However, although GST activity of animals collected in December were the highest, GST activity in January was significantly lower than all other months (Figure 2.11). Other workers have reported the effect of holding temperature on GST activity, for example, at a holding temperature of 8 °C GST activity in the mussel *Sphaerium coreum* was higher than the GST activity of animals held at 12 °C (Beverley, 1994). In the present study, animals that were held in tubes (Figure 2.2) had a significantly lower GST activity than unretained animals and this may be due to stressful interaction between animals. It has been observed that *G. pulex* exhibit cannibalism (Sutcliff and Carrick, 1985) and aggressive behaviour especially between males and this may increase stress and injury between animals that are able to interact. Animals that were fed with alder leaf material exhibited higher GST activity than unfed animals. The increase of GST activity due to ingested plant materials is well documented (Clark, 1989; Egaas *et al.* 1991) and is reported to be due to the metabolism and detoxification of plant allelochemicals such as pinenes, limonenes and terpinenes (Brattsen *et al.*, 1984).

Increases in GST activity have been reported for animals held under laboratory conditions, activities returned to levels at or below those measured in animals directly from the field (Collier and Varnassi, 1991 and Beverley, 1994). GST activity in *G. pulex* collected from Craggs stream was not significantly different from February through to July although animals collected in October had a significantly lower activity and animals in January significantly lower still (Figure 2.11). Seasonal fluctuation of GST activity has been reported in fish (George *et al.*, 1990).

Several studies have reported that males and female differ in GST activity. For instance Chasseaud (1979) reported differences in hepatic and renal GST between male and female rats and Almar *et al.* (1987) reported sex differences in the GST activity of the crayfish, *Procambarus clarkii*. Differences attributed to sex maybe explained by reproductive effects; for instance, Almar *et al.*(1987) demonstrated a higher turnover of GSH in female animals. Many crustacean species are sexually dimorphic (Barnes, 1987). In *G. pulex* males are larger than females (Adams and Greenwood, 1983). The effect of body size on GST activity was investigated by separating animals according to length as detailed in section 2.2.9. An expected relationship of length to weight was described by a curvilinear relationship (Figure 2.16). Relationships for weight and protein content to GST activity were linear. However, there were differences between size classes on analysis of specific activity and weight specific activity when classes were analysed individually as illustrated in Figures 2.20 and 2.21. Higher activities in smaller animals is indicative of a positive allometric effect. The size classes analysed encompassed juvenile groups and some differences may be expected between juveniles and adults (section 2.1.4.). Higher activities in smaller animals is indicative of a positive allometric relationship, although this relationship was isometric when plotting data from all size classes and analysing with linear regression. Indeed if, logarithmically transformed data were to be plotted the straight line relationship would give a slope of 0.76 (section 2.3.7) which suggests a positive allometric relationship (Peters, 1983). However, a similar plot of animals from size class 2 gave a regression coefficient of 1.1, which was indicative of an isometric relationship. In a study of whole body maximal specific enzyme activity in three aquatic crustaceans, Berges and Ballentyne (1991) compared enzymes involved directly in the control of aerobic metabolism (i.e. citrate synthase) and enzymes considered not to have a regulatory role in aerobic metabolism (i.e. alanine and aspartate amino transferases). They reported values of the slope of the regression relating citrate synthase activity to body size as 0.87, for alanine and aspartate amino transferases regression coefficients were close unity. GST is in not involved in regulatory metabolism, therefore if the results of Berges and Ballentyne (1991) were generally applicable one would

expect a regression coefficient of unity for the relationship between GST activity and total protein which was in fact the case for the size class of animals used in the present study. Beverley (1994) demonstrated an effect of body size on GST activity in the bivalve mollusc *Sphaerium corneum*. Activity per unit mass and per unit protein decreased with mass and total protein content, respectively. Thus, smaller animals showed higher specific activity per protein and higher mass based activity per unit mass. As discussed above, the present study activity is shown to be linear with protein.

Conclusions.

- Conditions for the acclimation, laboratory holding and sample handling of *G. pulex* showed significant effects on GST activity.
- Constant conditions that minimise activity increase or loss were used in the laboratory and for sample storage conditions (i.e. at 15 °C, in holding tubes and unfed where possible).
- There was a positive allometric relationship of GST activity with protein concentration over a large range of size classes.
- GST activity had an isometric relationship with protein concentration in the size class used for routine experiments in this study.

2.4.3. Baseline activity.

The final objective of the work described in this chapter was to establish a baseline of GST activity in *G. pulex*. The assessment of GST activity in animals in which effects on GST activity had been minimised would then allow toxicant-induced activity increases to be assessed on a quantitative basis.

In terms of a 'baseline' of GST activity for *G. pulex* the value 0.081 ± 0.0007 $\mu\text{mol}/\text{min}/\text{mg}$ protein is high. Values of specific activity for GST from animals collected and measured directly from the field from February to July were not significantly different and had activities ranging from $0.0329 (\pm 0.0052)$ to $0.0294 (\pm 0.0053)$ $\mu\text{mol}/\text{min}/\text{mg}$ protein (Figure 2.11). The highest activity in February was over twice the lowest activity found of that in January of 0.014 ± 0.0028

$\mu\text{mol}/\text{min}/\text{mg}$ protein. The baseline of GST activity in animals directly from the field would appear to be in the range 0.014 to 0.033 $\mu\text{mol}/\text{min}/\text{mg}$ protein.

A value of between 0.02 and 0.04 $\mu\text{mol}/\text{min}/\text{mg}$ protein would seem to be a 'baseline' of activity in large (10 to 15 mm) male *G. pulex*. However, it is not possible to use this as a quantitative 'benchmark' above which activity can be said to have deviated from the 'norm' or 'baseline'. Animals presented with a change in conditions should therefore be compared to animals in reference or control conditions. This has been found in other work, for example, Beverley (1994) reports GST specific activities in controls under the same conditions of 0.362 (± 0.025) and 0.43 (± 0.016) $\mu\text{mol}/\text{min}/\text{mg}$ protein in the freshwater bivalve *Sphaerium corneum*. This author reported control activities in another experiment from 0.215 ± 0.016 to 0.321 ± 0.026 $\mu\text{mol}/\text{min}/\text{mg}$ protein. These values would have been significantly different if analysed in the same experiment.

The measurements of a baseline of activity, although important in terms of measuring large deviations from the normal expected activity in *G. pulex*, should be treated with caution as they do not provide an absolute level of activity under a given set of conditions. The comparison with controls under the same conditions is therefore essential to the measurement of change in GST activity in *G. pulex*.

CHAPTER THREE

The effect of xenobiotic exposure on glutathione s-transferase activity in *Gammarus pulex*.

3.1 INTRODUCTION.

The preceding chapter described the development of a method to measure GST activity in *Gammarus pulex* and demonstrated sources of variability. In this chapter the effect of exposure to xenobiotics on GST activity in *G. pulex* is considered. As GST is involved in the detoxification of electrophilic compounds (section 1.4.1) it is likely to be involved in the detoxification of lipophilic organic xenobiotics (Habig *et al.* 1974). Induction of GST by various classical inducers of drug metabolism (e.g. polychlorinated aromatic hydrocarbons (PAH), phenobarbitone (PB) and polychlorinated biphenyls (PCB)), has been demonstrated in mammals (Booth *et al.*, 1961; Chasseaud, 1979) and fish (Collier *et al.*, 1986). Moreover, several studies have demonstrated the induction of increased GST activity in aquatic invertebrates exposed to organic chemicals (e.g. Dierickx, 1984).

Isoenzymes of GST catalyse the conjugation of different substrates to GSH to a greater or lesser extent (section 2.2). Therefore, it is reasonable to assume that these isoenzymes will be induced to varying degrees by exposure to a particular xenobiotic. It follows that subsequent assay of a crude (unpurified) extract with the universal substrate CDNB (section 2.2.3) may fail to detect the elevation of one or a number of induced isoenzymes. Furthermore, not all isoenzymes may be induced by xenobiotic exposure, indeed some isoenzymes may be inhibited (Baturu and Lagadic, 1996). For the reasons above, Clark (1989) states that caution should be exercised when interpreting xenobiotic-induced increase in GST activity that is determined using crude extracts with CDNB as the artificial substrate. However, it has been demonstrated that most GST isoenzymes are active in the conjugation of CDNB (see section 2.2.1) and thus total GST activity

can be estimated using this substrate (Ketterer *et al.*, 1988). Beverley (1994) quantified GST activity in *Sphaerium corneum* which had been exposed to the organochlorine pesticides lindane and dieldrin. Activity was estimated in both crude and purified tissue extracts using CDNB and the specific substrates; 1,2-dichloro-4-nitrobenzene (DCNB), p-nitrophenylacetate (PNPA), p-nitrotrimethylphenylacetate (PNMPA), p-nitrobenzyl chloride (PNBC), ethacrynic acid (ETA), bromosulphophthalein (BSP), nitropyridine -N-oxide (NPO) and 1,2-epoxy-3-(p-nitrophenoxy)propane (ENPP). Beverley (1994) demonstrated activity with CDNB and ETA only. The ratio of GST activity determined using CDNB and ETA was similar in a number of different tissue extracts. Moreover, this ratio was similar in both purified and crude extracts. These results suggest that the concerns of Clark (1989) may perhaps be allayed and that the assay of a crude extract with CDNB will provide a useful estimate of GST in invertebrates exposed to organic xenobiotics.

Increased GST activity has been shown to follow a dose-response relationship by a number of workers. LeBlanc and Cochrane (1985) reported a dose and time dependent increase in GST activity on exposure to CDNB in the freshwater cladoceran, *Daphnia magna*. Baturu and Lagadic (1996) demonstrated significant increases in GST activity in a freshwater snail (*Lymnaea palustris*) exposed to the herbicide, atrazine, for 96 hours in the laboratory. Egass *et al.* (1993) reported increased GST activity in the Cabbage moth (*Mamestra brassica*), on exposure to atrazine. Using two exposure concentrations a significant increase was shown from control and there was evidence of a dose response relationship. Thaker and Hartios (1989) demonstrated dose dependent GST activity in the shrimp, *Callinassa tyrrhena*, in response to a range of exposure concentrations of cadmium ions.

The assessments of the effect of organic xenobiotics on GST activity in aquatic invertebrates have been carried out either under laboratory conditions (section 3.1.2.) and on field populations (e.g. Rodriguez-Ariza *et al.*, 1992; Sheehan *et al.*, 1991). It is possible that a toxicant-induced effect on GST activity which is

detectable in the laboratory under constant conditions may be masked in the environment due to increased variability in response (Chapter Two). The use of mesocosm experiments allows the exposure of animals subject to a fluctuating environment but to a known chemical exposure regime. Sublethal “environmentally relevant” concentrations may also be used. Baturu and Lagadic (1996) reported increased GST activity in the snail *Lymnaea palustris* exposed to atrazine both in the laboratory and mesocosms.

3.1.1. Test chemicals.

Lindane.

In the current study the organochlorine pesticide, lindane, and the non-ionic surfactant, alcohol ethoxylate, were used to investigate GST induction in *G. pulex*. Lindane is the γ isomer of 1,2,3,4,5,6-hexachlorocyclohexane, the structure of which is illustrated in Figure 3.1

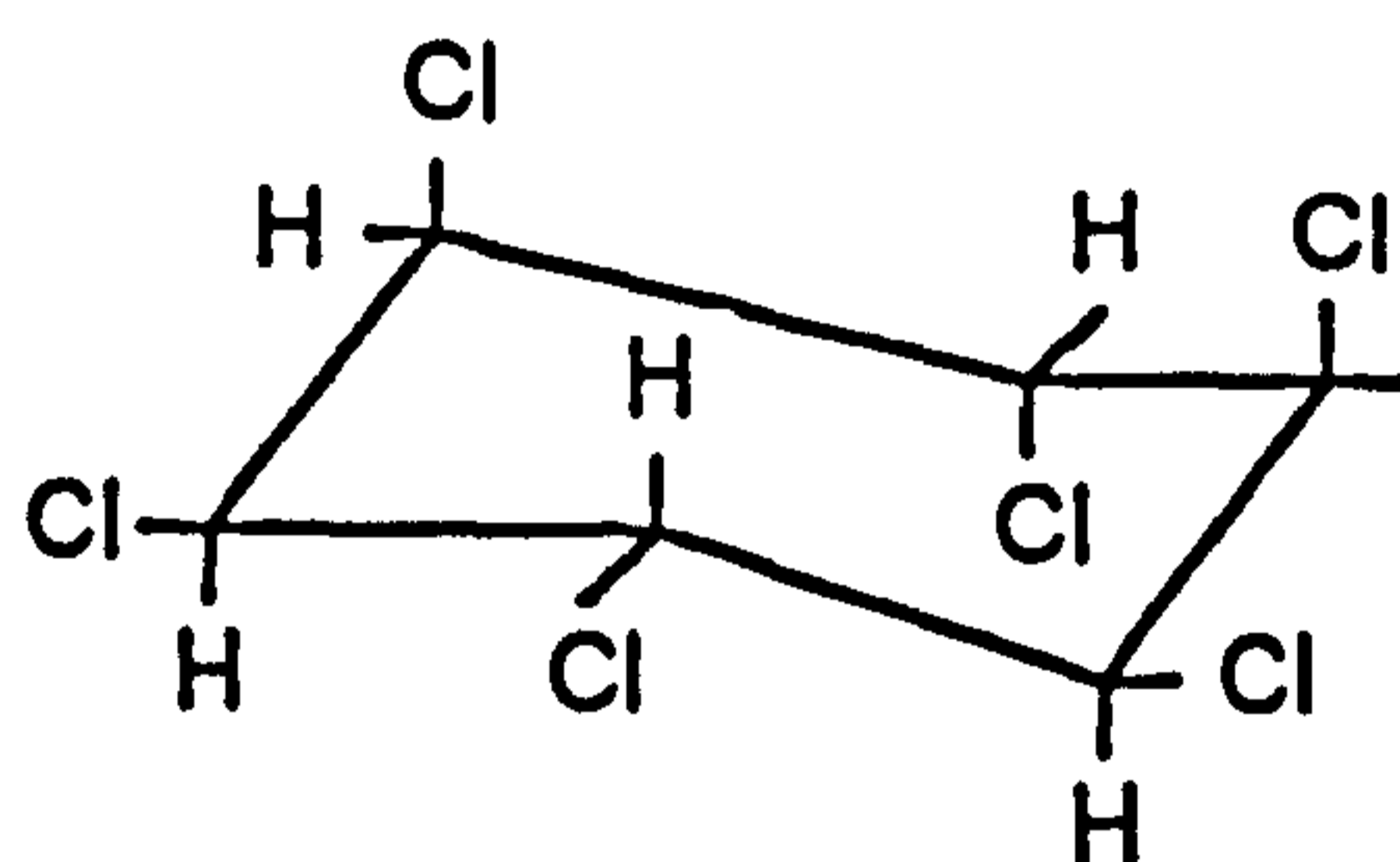


Figure 3.1 A diagram of the chemical structure of lindane.

Lindane has a large usage in agriculture so is likely to be found as a contaminant in water bodies (Crossland and Beasley, 1990). The main uses of lindane are as a soil and seed treatment (80 % of usage) and in veterinary science and timber protection (5 and 10 % of usage, respectively). Lindane (γ -HCH) is listed as a contact, ingested and fumigant organochlorine insecticide in the UK Pesticide Guide 1997. The criteria for inclusion in the UK Pesticide Guide are that the product has MAFF/HSE approval and that the product is on the UK market. Eleven products are listed as γ -HCH only, with another two containing γ -HCH in

combination with other pesticides. It is listed for use on aphids, capsids, leaf miners, leafhoppers, earwigs, apple blossom weevil, sawflies, leatherjackets, wireworms and other insect pests. Suggested crop use includes oil seed rape, brassica seed, oats, rye, spring and winter barley and wheat, orchard fruit, in forestry plantations and on cut timber. Maximum residue levels are quoted as 3 mg/kg for soft fruit, 2 mg/kg for vegetables and 0.1 mg/kg for cereals and eggs.

Lindane has a solubility in water of 10 mg/l at 20°C and has an log n-octanol/water partition coefficient of 3.2 to 3.7. It is highly active against the insect orders Isoptera (termites), Heteroptera (bugs), Homoptera (aphids and capricorn beetles), coleopterous larvae (wireworm and white grubs), Diptera (flies, leatherjackets), Hymenoptera (wasps, ants) and certain Lepidoptera. Lindane was selected as a test chemical in the present study as it is relatively toxic to invertebrates and its toxicity has been widely investigated. The toxicity of lindane to *Gammarus* species is shown in Table 3.1

Table 3.1. 96 hour LC₅₀ values for *Gammarus* species exposed to lindane. Brackets after LC₅₀ indicate 95% confidence limits. Test type; S = static and CF = continuous flow. Tests were conducted at differing water hardness * 100 mg/l CaCO₃ and + 250 mg/l CaCO₃, where there is no symbol water hardness was not given. ND = no data available.

Species	test type	96h LC ₅₀ (µg/l)	pH	temp. °C	Reference
<i>G. pulex</i>	S	24 (21-28)	ND	19.5 - 20.5	Bluzat & Seuge (1979)
	S	34 (25-46)	7-7.6	13 - 17	Abel (1980)
	CF	230	7.5-8	11	Green <i>et al.</i> (1986)
	S	13.6*	8.3	7 - 15	Stephenson (1983)
	S	5.9 ⁺	8.3	7 - 15	Stephenson (1983)
	S	79	6.8-7.2	11 - 13	Taylor <i>et al.</i> (1991)
<i>G. fasciatus</i>	S	39 (27-56)	ND	19 - 21	Macek <i>et al.</i> (1976)
	S	10-11	ND	ND	Sanders (1972)
<i>G. lacustris</i>	S	48	ND	ND	Sanders (1969)

From Table 3.1 it can be seen that the 96 h LC₅₀ ranges from 5.6 to 79 µg/l for static tests whereas a value of 230 µg/l is reported for a flow-through test. It appears that water hardness is important in the lethality of lindane; harder water giving a lower LC₅₀ (Stephenson, 1983). Indeed the lowest value for the 96 hour LC₅₀ is 5.9 reported by Stephenson (1983) for a study conducted in water with a hardness of 250 mg CaCO₃/l.

It is likely that GST is involved in the metabolism of lindane by conjugation of the metabolites of lindane with GSH (Sternburg and Kearns, 1956; Clark *et al.*, 1966; Kurihara *et al.*, 1979; Tanaka *et al.*, 1981). Lindane has been shown to be metabolised by glutathione-dependent dechlorination as well as by simultaneous reduction and oxidation processes of dechlorination (Kurihara *et al.*, 1979). A high level of resistance to lindane in house flies has been shown to be due to the combined effects of increased metabolism (including NADPH-dependent microsomal oxidation, glutathione-dependent dehydrochlorination, and glutathione conjugation) and decreased sensitivity of the central nervous system (Tanaka *et al.*, 1981).

The increased activity of GST in response to lindane exposure has been reported in oligochaetes, insects, mites and molluscs. Hans *et al.* (1993) reported an increase in GST activity in earthworms exposed to 1 mg /l lindane. Whereas GST activity in the freshwater mussel (*Sphaerium corneum*) was increase by exposure to 2 µg/l lindane (Garrood *et al.*, 1989; Boryslawskyj *et al.*, 1988). GST activity in cotton leaf worm (*Spodoptera littoralis*) was shown to peak after 8 hours exposure to a topically applied dose of 0.2 µg lindane/lavae by Lagadic *et al.* (1993), and Capua *et al.* (1991) reported a 31 % increase in GST activity in the bulb mite (*Rhizoglyphus robini*) on exposure to an unspecified but sublethal concentration of lindane.

Alcohol ethoxylate.

The alcohol ethoxylate used in this study contained a mixture of C12, C13, C14 and C15, predominantly linear, primary alcohols, with an average chain length of 13.5 and an average ethylene oxide number (EO) of 8.2. It is a non-ionic surfactant used in high performance washing powders and other cleaning products. Field monitoring showed that the average composition of alcohol ethoxylates in effluent from Dutch municipal sewage plants was similar to that used in this study. The alcohol ethoxylates were found to have an alkyl chain length of 13.3 and an average ethylene oxide number of 8.2. (Feijtel and van de Plassche, 1995).

The toxicity of alcohol ethoxylate surfactants is a function of their molecular structure (Talmage, 1994). Dorn *et al.* (1993) suggested that toxicity increases as the alkyl chain length increases or as the average ethylene oxide distribution decreases. Dorn *et al.* (1996) reported NOEC (No Observed Effect Concentration) for chronic (30 day) exposure survival for exposure of the fathead minnow (*Pimephales promelas*) and freshwater cladoceran *Daphnia magna* to C₁₄₋₁₅, EO 7, C₁₂₋₁₃, EO 6.5 and C₉₋₁₁, EO 6 alcohol ethoxylates (Table 3.2.).

Table 3.2 Chronic exposure (30d) no observed effect concentrations for a freshwater fish (*P. promelas*) and a freshwater cladoceran (*D. magna*) to three alcohol ethoxylates. After Dorn *et al.* (1996)

Surfactant	<i>P. promelas</i> NOEC (mg/l)	<i>D. magna</i> NOEC (mg/l)
C ₁₄₋₁₅ EO 7	0.74	1.02
C ₁₂₋₁₃ EO 6.5	1.76	2.69
C ₉₋₁₁ EO 6	1.01	5.57

The 10 day median lethal concentration of a C₉₋₁₁ EO 6 surfactant is reported by Dorn *et al.* (1996) as 13.7 mg/l (95 % CL, 12.1, 15.5) for the freshwater amphipod *Hyalella azteca*. Lower values were reported for larvae of the insect

Chironomus tentans (5.7 mg/l, 95 % CL, 4.6, 7.0) and for the fathead minnow *Pimephales promelas* (2.65 mg/l, 95 % CL 1.8, 3.8).

3.1.2. Objectives.

The primary objective of the work described in this chapter was to detect a GST response in *G. pulex* exposed to sublethal concentrations of two organic xenobiotics; lindane and alcohol ethoxylate. Lindane is known to induce GST activity in a wide range of species. It was therefore chosen as a model compound to assess the magnitude and persistence of the GST response of *G. pulex* under controlled laboratory conditions. The generality of the response was investigated by exposing *G. pulex* to a different class of organic xenobiotic, namely the non-ionic surfactant alcohol ethoxylate. A second objective was to assess the GST response in animals exposed in semi-natural conditions. This was achieved by exposing animals to alcohol ethoxylate in an artificial stream system. The use of caged animals in the stream system would provide data on GST activity which could then be compared with other effects of exposure in the streams.

It was hypothesised that short-term exposure to sublethal concentrations of organic xenobiotic will result in significant increases in GST activity in *G. pulex* exposed under controlled laboratory conditions and in semi-natural conditions. It was predicted that GST activity would be induced by both lindane and alcohol ethoxylate and the magnitude of response would increase with increasing concentration of exposure chemical. Furthermore, induction of increased GST activity would be detected on exposure to xenobiotic under semi-natural conditions.

3.2. MATERIALS AND METHODS.

3.2.1. Reagents.

Organic solvents were supplied by Fisons Scientific Ltd. UK. Acetone was 99 % pure and of AnalAR™ Grade and the n-hexane of Distol™ or pesticide grade. Lindane (1,2,3,4,5,6-hexachlorocyclohexane, γ isomer) and aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-endo,endo-1,4:5,8-dimethanonaphthalene) were supplied by Sigma Chemicals Ltd and were at least 99 % pure. Alcohol ethoxylate (C_{13.5} EO 8) was provided by Shell Research Ltd laboratories. All reagents used for GST and protein assays were as stated in section 2.2.1.

3.2.2. Experimental animals.

All *G. pulex* used in experiments were collected from Craggs Stream and acclimated to laboratory conditions (section 2.2.2). Only large males (10 to 15 mm in length) were used in tests and animals assayed for GST activity were killed and stored as detailed in section 2.2.3.

3.2.3. Preparation of test solutions.

To ensure all glassware was free of organic contamination, glassware was acetone and n-hexane rinsed after washing in Decon™ detergent and air dried. A 1000 mg/l lindane stock solution was prepared by weighing out 100 mg pure solid lindane on a Mettler AT261 Deltarange, digital balance (accuracy ± 0.0001 mg) and dissolving it into 100 ml acetone. Stock solution was kept in a sealed glass container in darkness at 4 °C for no longer than four weeks. All solutions were measured using appropriate volume glass pipettes and glass volumetric flasks. Intermediate solutions of 100 mg/l and 10 mg/l were prepared by dilution of the stock solution with acetone. For lethality test solutions, volumes of 200 μ l and 100 μ l stock solution were made up to 5 l with APW in glass volumetrics to give solutions of 40 and 20 μ g/l, respectively. Volumes of 500 and 100 μ l of 100 mg/l intermediate were made up to 5 l with APW to give solutions of 10 and 2 μ g/l, respectively. For each test concentration two batches of 5 l were mixed before adding 3 l to each of three test tanks. A litre of each test concentration was kept for chemical analysis (section 3.2.5).

For sublethal exposure tests, intermediate solutions were used to make up test solutions in two, 5-l batches for each concentration. Volumes of 650 µl and 150 µl of 100 mg/l intermediate were each made up to 5 l with APW (Appendix 1.2) to give solutions of 13 and 3 µg/l lindane, respectively. Volumes of 400 µl and 100 µl of 10 mg/l intermediate were made up to 5 l with APW to give solutions of 0.8 and 0.2 µg/l, respectively. Intermediate solutions were used such that acetone concentration in test solutions were between 0.22 ppm and 0.03 ppm acetone. For each test concentration two batches of 5 l were mixed before adding 3 l to each of three test tanks. A litre of each test concentration was kept for chemical analysis.

Pure solid alcohol ethoxylate (100 mg) was weighed out on a Mettler AE160 balance (accuracy ± 0.0001 mg) and dissolved in 100 ml of autoclaved APW. A stock solution of 1000 mg/l was kept in a sealed glass container in darkness at 4 °C. New stock was made up for each test. For lethality test solutions, volumes of 0.5, 1.5, 5 and 50 ml stock were made up to 5 l with APW to give solutions of 0.1, 0.3, 1.0 and 10 mg/l, respectively. For sublethal tests 1.5, 0.5 and 0.05 ml of 1000 mg/l stock were made up to 5 l with autoclaved APW give test solutions of 0.3, 0.1 and 0.01 mg/l, respectively. Two batches of 5 l of each solution were combined before adding 3 l to each tank. Chemical analysis was not performed on alcohol ethoxylate solutions used in laboratory tests.

Stock solutions of alcohol ethoxylate used to treat the artificial streams were prepared by employees of Shell Research Ltd. using deionised water sterilised using a 'UVAC' ultra violet light water sterilising unit. Stock solutions were stored for up to four days at room temperature. Watson-Marlow peristaltic pumps (model 505S), fitted with eight-roller pump heads (model 508 MC2) were used to continuously dispense stock solutions of alcohol ethoxylate into pipes supplying filtered water to the artificial streams. The tubing used to deliver the alcohol ethoxylate to the artificial streams was autoclaved prior to use and changed twice weekly.

3.2.4 Short-term lethality tests.

The 96 hour median lethal concentration (LC₅₀) of lindane to *G. pulex* was determined by exposing animals to nominal concentrations of 2, 10, 20 and 40 µg/l lindane. The LC₅₀ for alcohol ethoxylate was determined by exposing animals to nominal concentrations of 0.1, 0.3, 1 and 10 mg/l in autoclaved APW. APW was autoclaved in test solutions for determining the LC₅₀ for alcohol ethoxylate only, this was to prevent microbial breakdown of alcohol ethoxylate. APW served as a control in both experiments, for each treatment and control, ten animals were placed in individual holding tubes in three replicate 3-l glass tanks, (Figure 2.1) giving a total of thirty animals per concentration. All tanks were supplied with a continuous supply of air, via an air stone. Animals were observed daily, when dead animals were recorded and removed. Animals were considered dead if no appendage movement was visible during a 20 second period of observation. Animals were not fed during the test. The LC₅₀ values were calculated by probit analysis using log-transformed measured concentration values (Finney, 1971). Ninety five per cent confidence limits were also calculated using the method detailed by Wardlaw (1985).

3.2.5 Sublethal exposure.

Animals were exposed to lindane concentrations of 0, 0.2, 0.8, 3 and 13 µg/l. Twenty large male animals were placed in individual holding tubes in each of three replicate tanks at each concentration. Fifteen animals were removed from each concentration (five from each replicate tank) at the start of the exposure period and after 24, 96 and 336 hours of exposure. Animals were weighed, frozen and stored (section 2.2.3) prior to GST analysis (section 2.2.6). Test solution was changed weekly, a litre of each concentration being kept for chemical analysis (section 3.2.5).

Induction of GST activity was measured in animals exposed to alcohol ethoxylate concentrations of 0, 0.01, 0.1 and 0.3 mg/l in the laboratory. Five large male animals were placed in individual holding tubes in each of three replicate tanks at each concentration (a total of fifteen animals per concentration). The experiment

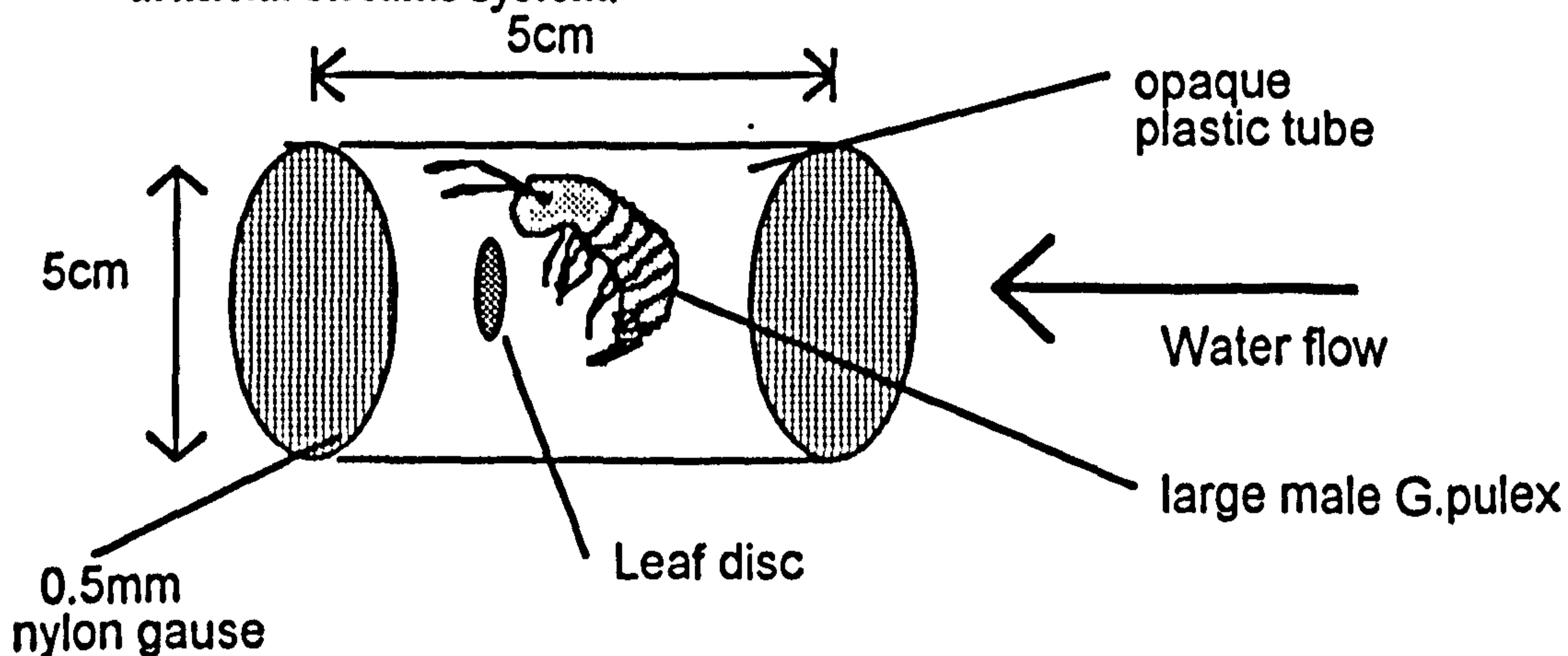
was terminated at 96 hours when all animals were removed, weighed and frozen for GST analysis (section 2.2.6). Test solutions were changed daily.

Exposure to alcohol ethoxylate under semi-natural conditions.

A long term exposure of *G. pulex* was carried out using the outdoor artificial stream system owned by Shell Research Ltd. and located at Leeds, Kent, UK. Seven nominal exposure concentrations (25, 50, 100, 200, 300, 400 and 800 µg/l) and an untreated control were randomly allocated to the eight artificial streams.

The animals used were collected from Craggs Stream (section 2.2.2) and transported to Kent in continuously aerated APW. They were allowed to acclimate under laboratory conditions for three days, in 5-l glass tanks of dechlorinated mains water (hardness: 250 mg/l as CaCO₃). Laboratory temperature was maintained at 15°C ± 1 °C. Animals were provided with conditioned alder leaves as food during this period. A total of 600 large male animals (10 - 15 mm) were prepared for placement in each of the eight treatments of the streams system. Animals were each placed into a holding chamber consisting of a polypropylene tube with 0.5-mm mesh nylon gauze (Figure 3.1).

Figure 3.2. A diagram of individual holding cages for *G. pulex* exposure in the artificial streams system.



Each animal was provided with five 12-mm pre-weighed discs of conditioned alder leaf as food (Appendix 1.3). In addition 40 chambers contained five 12-mm leaf discs but no animal were also deployed. These chambers acted as controls for changes in leaf disc weight due to physical and/ or microbial processes.

Animals to be removed after 12 hours exposure were not provided with leaf discs. All chambers were transported to the stream system in aerated tanks of dechlorinated tap water. A total of 75 experimental chambers plus five control chambers, were placed into the pool section of each stream (Figure 3.3).

Fifteen experimental chambers were removed from each stream at 12, 24 and 96 hours and at 7 and 14 days after deployment. One control chamber per stream was removed at each time period. Chambers were transported to the laboratory in stream water where animals and leaf material were removed. Animals were wet weighed on a Mettler AE 240 balance (accuracy $\pm 0.0001\text{g}$) and immediately frozen in liquid nitrogen and stored in a deep freeze at $-70\text{ }^{\circ}\text{C}$ (section 2.2.3). Leaf material was placed in numbered plastic cell trays and dried at $60\text{ }^{\circ}\text{C}$ for 48 hours before being weighed. Frozen animals were packed in solid carbon dioxide pellets ($-78\text{ }^{\circ}\text{C}$), transported to Sheffield University and stored in a deep freeze at $-70\text{ }^{\circ}\text{C}$ (section 2.2.3) until analysed for GST activity (section 2.2.6).

The artificial stream system.

The artificial streams were constructed of stainless-steel and each stream consisted of a trough 5 m long, 0.35 m wide and 0.25 m deep. Each stream was divided into a slow flowing section (0.2 m water depth, 5 - 10 cm/s flow velocity) and a faster flowing riffle section (0 - 0.02 m water depth, 20-25 cm/s flow velocity). The total volume of water in each stream system was 240 litres (Figure 3.3).

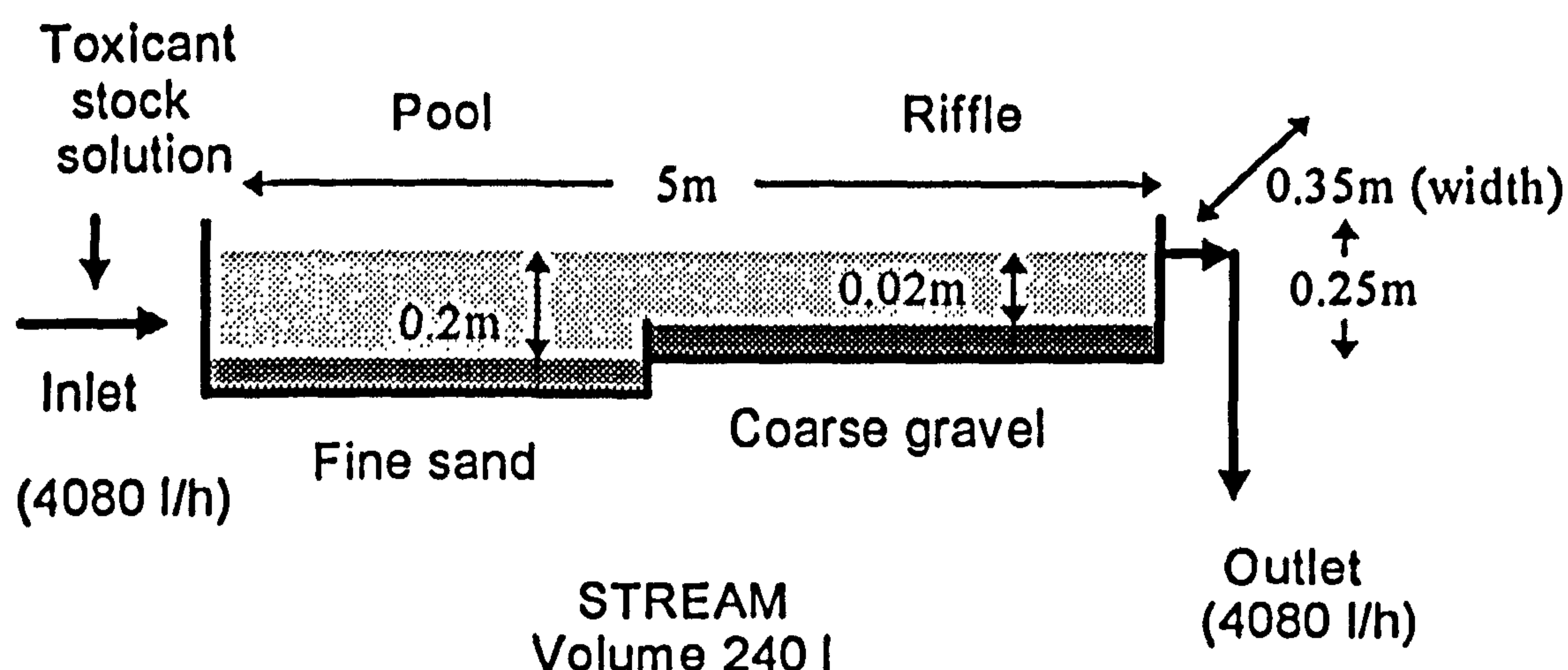


Figure 3.3. Diagrammatic representation of one of the artificial streams.

Water supplied to the streams was pumped from an adjacent natural calcareous stream at a rate of 68 litres/min. The water was first passed through a sand filter and then through 30- μm and 10- μm pore size cartridge filters. Water was then pumped through a 40 x 2.54 cm hydrocyclone assembly to reduce particulate matter in the water.

3.2.6. Chemical analysis of test solutions.

Liquid/liquid extraction of lindane solutions.

A volume of 200 ml of test solution was decanted into a clean glass 250-ml separating flask to which was added 50 ml of hexane. The flask was stoppered and the mixture shaken vigorously by hand for three minutes. The pressurised vapour was allowed to escape and the mixture was shaken again for two minutes. The aqueous solution was discarded and the hexane collected in a clean glass container. Samples were concentrated by evaporation of solvent under a stream of oxygen-free nitrogen. Sample volumes were made up to 5 or 10 ml in glass volumetrics by dilution with hexane.

Solid phase extraction of lindane from test solutions.

Three millilitre capacity solid phase extraction cartridges with a C18 bonded solid phase were obtained from Varian Ltd. The bonded phase was prepared for extraction (i.e. conditioned) by passing the following solutions through each

cartridge: 5 ml of acetyl acetate followed by 5 ml acetone and 15 ml distilled water. The test solutions were passed through the conditioned cartridges once before going to waste. Liquids were passed through cartridges under negative pressure using a Varian "Vac Elut" SPS 24 vacuum manifold connected to a vacuum pump with a vacuum pressure set to 25 inches Hg.

Lindane was extracted from the cartridges with hexane under gravity, applying a positive pressure of nitrogen gas to collect the last of the sample from the cartridge. Volumes of hexane sample were adjusted by "blowing down" with nitrogen and dilution to either 5 or 10 ml with hexane. Cartridges were used once and discarded. Recovery from extraction cartridges was assessed by the extraction and analysis of triplicate solutions of 0.5, 5.0 and 50 µg/l lindane in APW. Solutions were passed through conditioned cartridges and extracted as detailed above. The recovery was calculated as the percentage of the solution concentration that was extracted by cartridges as analysed by gas chromatography (section 3.3.1). Recoveries decreased with increasing lindane concentration and were greater than or equal to 74 %.

Aldrin was used as an internal standard as it is also an organochlorine pesticide with similar chemical properties. An internal standard of known concentration of aldrin enabled the concentration analyte of interest (lindane) to be estimated accounting for experimental error such as variation in injection sample size. Aldrin solutions (100 µg/l) were prepared in hexane as described in section 3.2.3. One millilitre of aldrin solution was added to 9 ml of lindane samples and standards such that all analysed solution contained an internal standard of 10 µg/l aldrin.

Analysis of extracted lindane solutions.

Lindane extracted into hexane was analysed by gas chromatography using a Varian 3500 capillary GC gas chromatograph fitted with a DB5 30 m capillary column with a 0.25 mm internal diameter with 0.25 µm solid phase. The gas chromatograph was operated using an electron capture detector with a nickel 63

^{63}Ni) source set to a temperature of 300 °C. A Varian 8035 autosampler was fitted to the GC enabling the automatic injection of 1 µl of sample. The injected sample was split to a 1:10 ratio with the injection port temperature set to 280 °C. The gas phase used was helium with a 1.5 ml/min flow rate. The oven temperature was set to an initial value of 210 °C and held for two minutes. The temperature rise was programmed to 5 °C/min to reach a final value of 250 °C for two minutes. The gas chromatograph unit was fitted with an integrator and printer that gave a graphical illustration of analyte peaks and a digital readout of detected peak areas.

Lindane was analysed using the same method at both Shell Research and Sheffield University. However, at Shell Research, the gas chromatograph used was a Hewlett Packard 5890 fitted to a HP 7673A controller and a HP 7673A autosampler with a HP 3390A integrator. The injector temperature was set to 250 °C and the detector temperature was set to 270 °C. The oven profile was for an initial temperature of 70 °C with a 20 °C/min increase to a final temperature of 230 °C. Each standard and sample was run three times and peak area determined. Unknown sample concentrations were calculated by plotting a linear regression of peak area values of lindane standard against lindane concentration. The retention time of lindane and aldrin was determined by running standards of aldrin and lindane in hexane and was found to be 10.31 minutes for lindane and 12.29 minutes for aldrin. This information enabled the attenuation of the integrator to be set such that the expected concentration of the samples would be shown on the read out. Variation in the peak area of internal standard (aldrin) was used to automatically correct for variability in sample concentration.

3.2.7 Statistical analysis.

Statistical analyses were performed using the statistical software package Minitab, version 9.2 for Windows. All data were checked for a normality using normal probability plots and Anderson-Darling normality tests. Oneway analysis of variance (ANOVA) was used to analyse data from short-term exposure tests and data from the artificial streams. The significance of pair-wise differences in

ANOVA was assessed by the multiple comparison Tukey's test q values stated are derived by the method of Zar (1996). Significance was at $p = 0.05$ unless otherwise stated.

3.3. RESULTS.

3.3.1. Short-term lethal tests.

The actual lindane exposure concentrations measured using GC/ECD (section 3.2.5) are shown in Table 3.3. Actual concentrations were between 74 and 94 % of nominals. Alcohol ethoxylate solutions used in laboratory tests were not analysed.

Table 3.3. Analysed lindane concentrations for lindane LC₅₀ test.

Nominal µg/l	Actual µg/l	sd
0	<0.01	-
2	1.87	0.021
10	8.43	0.056
20	15.9	0.31
40	29.6	0.56

The median lethal concentrations (LC₅₀) for *G. pulex* exposed to lindane or alcohol ethoxylate for 72 and 96 hours are presented in Table 3.4. LC₅₀ values are based on measured lindane concentrations and nominal alcohol ethoxylate concentration.

Table 3.4. Median lethal concentrations (LC₅₀) and exposure time for *Gammarus pulex* exposed to lindane and alcohol ethoxylate in the laboratory.

Exposure time (hours)	LC ₅₀	95% confidence limits
lindane		
72	32.4 µg/l	27.5, 38.6 µg/l
96	7.8 µg/l	6.76, 8.91 µg/l
alcohol ethoxylate		
72	13.5 mg/l	10.2, 17.8 mg/l
96	3.9 mg/l	3, 5 mg/l

Lindane was approximately three orders of magnitude more toxic to *G. pulex* than alcohol ethoxylate. However, for both chemicals, increasing the exposure

period from 72 to 96 hours reduced the LC₅₀ value by approximately 75 % (76 % and 71 % for lindane and alcohol ethoxylate, respectively).

3.3.2. Sublethal exposure.

The concentration of lindane measured in one-week old solutions by GC/ECD are given in Table 3.5 for samples taken after 168 hours and 336 hours exposure. Actual concentrations of lindane were low, being between 22 and 26 % of nominal values.

Table 3.5. Chemical analysis of lindane solutions for sublethal exposure experiment.

Nominal µg/l	actual µg/l	sd	% nominal
168 hour sample			
0	<0.01	-	-
0.2	0.044	0.0053	22
0.8	0.171	0.0167	21.4
3	0.71	0.046	23.7
13	3.38	0.13	26
336 hour sample			
0	<0.01	-	-
0.2	0.05	0.002	25
0.8	0.176	0.042	22
3	0.73	0.062	24
13	3.42	0.2	26

Although the aim of this experiment was to expose *G. pulex* to sublethal concentrations of lindane, mortalities of 40 % were observed at the highest concentration of lindane at 96 hours and 53 and 73 % mortalities were observed at the highest two concentrations of lindane at 336-hour exposure. These treatments were therefore omitted from subsequent analysis. GST activity of *G. pulex* exposed to lindane solutions for 24, 96 or 336 hours is shown in Figure 3.4.

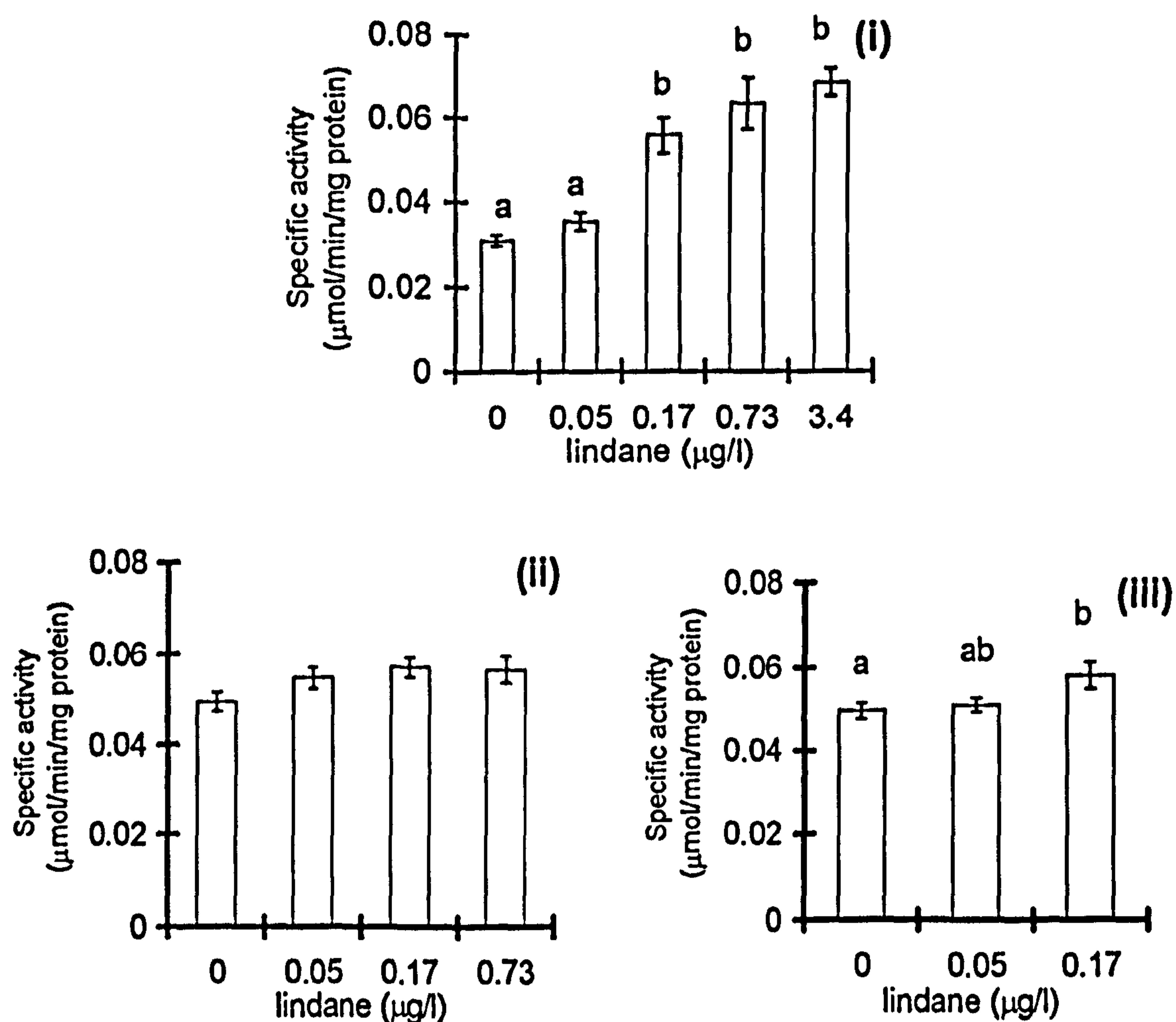


Figure 3.4. GST activity in *G. pulex* exposed to lindane. Animals sampled at i) 24 hours, ii) 96 hours, iii) 336 hours. Error shown is the standard error of the mean. One way ANOVA indicated significant treatment effects after 24 hours ($F_{4,107} = 24.48$) and 336 hours ($F_{2,78} = 3.93$) exposure but not after 96 hours. The significance of pairwise comparisons was assessed using Tukey's multiple comparison test. Within each time period treatments sharing the same letter were not significantly different ($q < 3.93$)

After 24 hours exposure there was a concentration-response relationship between GST activity and lindane concentration. The EC_{50} for specific GST activity of $0.53 \mu\text{g/l}$ at 24 hours was 61 times less than the 72-hour LC_{50} and 15 times less than the 96-hour LC_{50} . Specific GST activity at $0.17 \mu\text{g/l}$ (i.e. 46 times less than the 96 hour LC_{50} lindane concentration) was 80 % greater than that of control animals. At the highest concentration tested (i.e. $3.4 \mu\text{g/l}$) GST activity was 120 % greater than that of control animals. After 336 hours exposure the increase in GST activity of animals exposed to $0.17 \mu\text{g/l}$ over control animals was reduced to 17 %.

The effect of sublethal exposure to alcohol ethoxylate on GST activity of *G. pulex* is illustrated in Figure 3.5. Animals were exposed for 96 hours after which there was a significant effect of exposure concentration on GST activity ($F_{3,151} = 8.88$). Each treatment was replicated three times. Although there was significant within treatment variation ($F_{8,151} = 2.14$) this did not mask the between treatment variation.

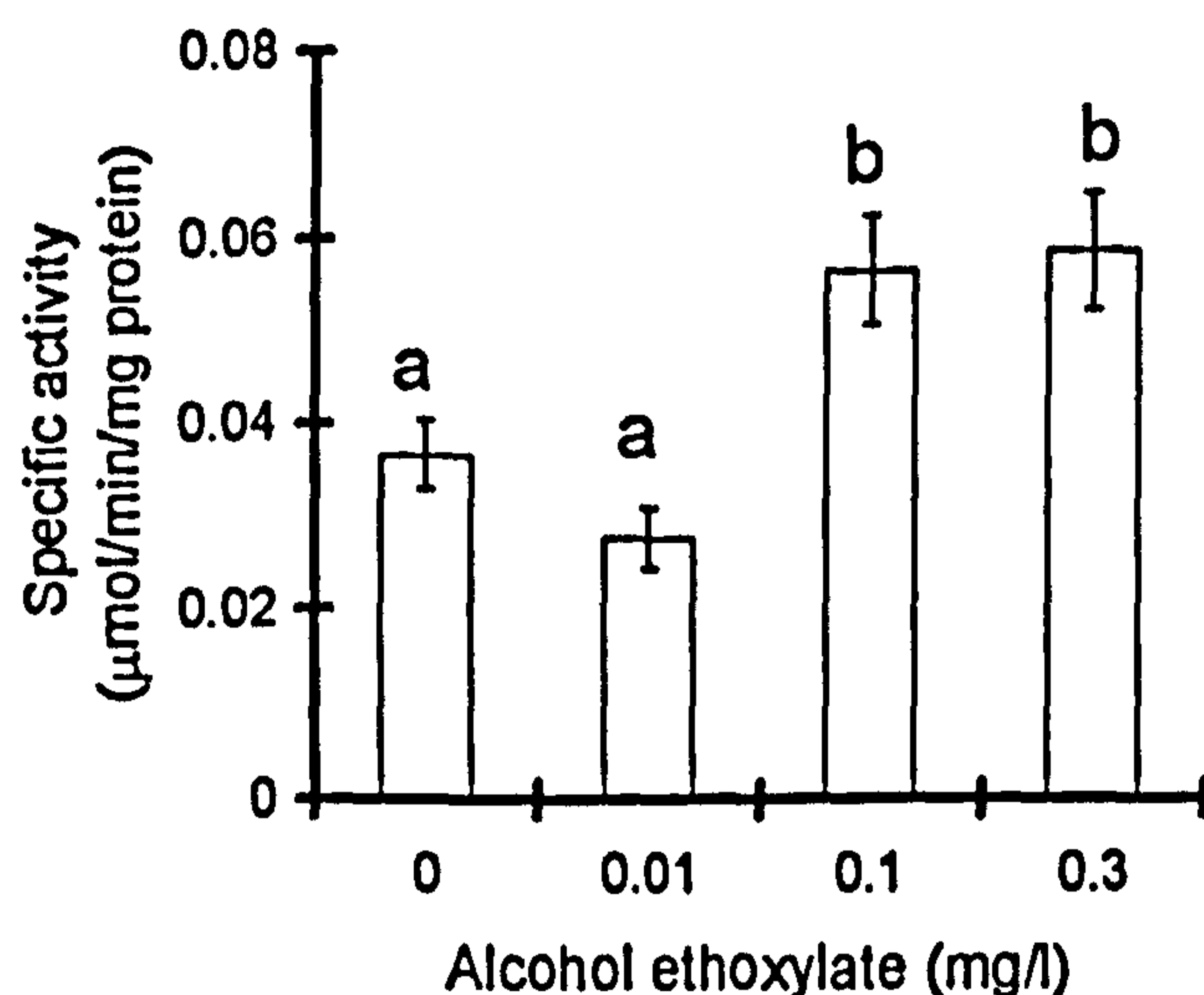


Figure 3.5. GST activity in *G. pulex* exposed to alcohol ethoxylate. Replicates are pooled for each treatment and error is the standard error of the mean. The significance of pairwise comparisons was assessed using Tukey's multiple comparison test. Treatments sharing the same letter were not significantly different ($q < 3.67$).

Significant differences in specific GST activity compared to control were demonstrated at 0.1 mg/l (i.e. 36 times less than the 96-hour LC_{50}). The increase in specific activity at 0.1 mg/l was 55.4 % greater than that of control animals.

3.3.3. Exposure of *G. pulex* to alcohol ethoxylate in semi-natural conditions.

Alcohol ethoxylate concentrations in stream water were analysed by chemists at Shell Research Ltd. Values were found to be at least 68 % of nominals over the period in which the animals were in the streams. A copy of actual analysis is shown in Appendix 2.1.

G. pulex were assayed for GST activity after 24, 96, 168 and 336 hours exposure to alcohol ethoxylate in artificial streams (Figure 3.6).

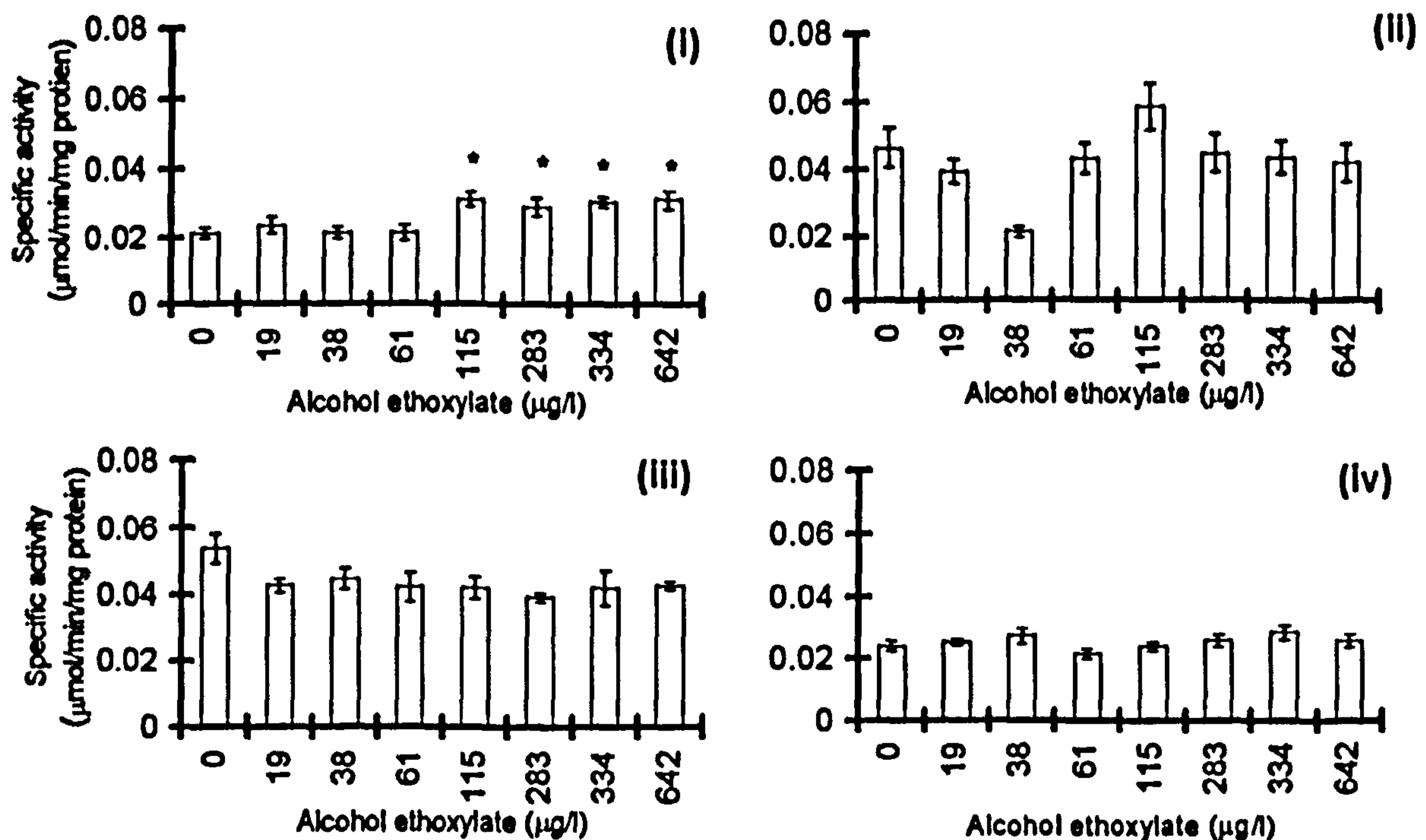


Figure 3.6. GST activity in *G. pulex* exposed to alcohol ethoxylate for i) 24 hours, ii) 96 hours, iii) 168 hours and iv) 336 hours. Error shown is the standard error of the mean. Asterisks represent significant differences from controls (Dunnett's test).

GST activity varied between treatments, and the effect was significant after 24, 96 and 168 hours exposure ($F_{>7,71} > 3.24$). Treatments were compared to controls using a Dunnett's test. The GST activity of animals exposed to alcohol ethoxylate exposure only differed from controls at 24 hours exposure ($q > 2.67$). At this time there was a clear threshold at 115 µg/l; GST activity of animals exposed to 115, 283, 334 and 642 µg/l being significantly greater than that of control animals. Twenty-four hour exposure to alcohol ethoxylate resulted in a 42.7 % increase in GST activity at a concentration which was 19.5 times less than the 96-hour LC_{50} .

3.4. DISCUSSION.

3.4.1. Laboratory exposures.

The objective of the work described in this chapter was to assess the change in *G. pulex* GST activity on exposure to xenobiotics. Two chemicals were used, lindane and alcohol ethoxylate. Lindane is an organochlorine insecticide known to induce GST activity in invertebrates. Alcohol ethoxylate is a non-ionic surfactant and was used to assess the specificity of the GST response. Animals were exposed under controlled laboratory conditions and in outdoor artificial streams. The artificial stream experiment also provided an opportunity to compare the GST response to other end-points.

Increased GST activities of animals exposed to lindane or alcohol ethoxylate were above 'baseline' activity (0.02 - 0.04 $\mu\text{mol}/\text{min}/\text{mg}$ protein) tentatively suggested in Chapter Two (section 2.4.3). Twenty-four hour control activities were 0.031 ± 0.007 $\mu\text{mol}/\text{min}/\text{mg}$ protein for laboratory exposed animals and 0.021 ± 0.006 $\mu\text{mol}/\text{min}/\text{mg}$ protein for animals exposed in the artificial streams. Increases in control activities occurred in both exposure regimes; maximum control activities being 0.05 ± 0.01 $\mu\text{mol}/\text{min}/\text{mg}$ protein at 336 hours.

Lindane.

The median lethal toxicity of lindane to *G. pulex* calculated in the present study was 32.4 (95 % C.L. 27.5, 38.6) $\mu\text{g}/\text{l}$ and 7.8 (95 % C.L. 6.8, 8.9) $\mu\text{g}/\text{l}$ for 72 and 96 hour exposures, respectively. Previous studies have reported 96-hour LC_{50} values ranging from 5.9 to 79 $\mu\text{g}/\text{l}$ in static tests. Results in the present study compare well with those reported by Stephenson (1983); 96-hour LC_{50} of 5.9 $\mu\text{g}/\text{l}$ lindane (250 mg CaCO_3/l) and 13.6 $\mu\text{g}/\text{l}$ lindane (100 mg CaCO_3/l). The test regime adopted in the present study was similar to that of Stephenson (1983). In particular, water hardness which is apparently important in determining the toxicity of lindane to *G. pulex* was similar (i.e. 92 mg CaCO_3/l) compared to the lower value for hardness of Stephenson (1983) (i.e. 100 mg CaCO_3/l).

The 96-hour LC₅₀ value was used to determine sublethal exposure concentrations to be used in subsequent experiments investigating lindane-induced effects on GST activity. The actual concentrations of lindane measured in the sublethal study ranged from 0.05 to 3.4 µg/l. Despite this, there were mortalities of 40 % on exposure to 3.4 µg/l at 96 hours and 53 and 66 % on exposure to 0.73 and 3.4 µg/l, respectively after 336 hours.

A significant increase in GST activity was apparent after 24 hours exposure to 0.17 µg/l; exposed animals having 80 % more activity than controls. This increase in activity was not apparent after 96 hours exposure and after 336 hours was only 17 %. A non-monotonic relationship between contaminant-induced GST activity and exposure time has been reported in previous studies. Hans *et al.* (1993) reported a 75 % increase in GST activity of the earthworm *Pheretima pothuma* exposed to lindane (1 µg/g soil) for seven days. This increased to 105.3 % of control activity, after 14 days, but then decreased to 16.5 % after 28 days exposure. In contrast, GST induction in the freshwater mollusc, *Sphaerium corneum* was sustained over 198 hours. For instance Boryslawskyj *et al.* (1988) reported control values of 0.022 µmol/min/mg protein for the *Sphaerium corneum*, (*c.f.* 0.02 to 0.05 µmol/min/mg protein for *G. pulex*) which increased by 263 % for mussels exposed to 2 µg/l lindane for 24 hours and increased by 1060 % after 44 hours and 1000 % after 198 hours exposure.

Data for the induction of GST on exposure to lindane in the present study show that there was an increase in control GST activity over time. The GST activity at 24 hours in control animals was 0.031 µmol/min/mg protein and increased to a value of 0.049 µmol/min/mg protein at 96 hours. This elevated level was sustained and at 336 hours GST activity was 0.05 µmol/min/mg protein. Activity of animals exposed to the lowest lindane exposure concentration (*i.e.* 0.05 µg/l) for 96 and 336 hours was 0.055 and 0.051 µmol/min/mg protein, respectively. These activities were significantly greater than the 24 hour control activity (*i.e.* 0.031 µmol/min/mg protein) but were not significantly different from corresponding control values. Garrood *et al.* (1989) report that control GST

activity for the freshwater mollusc *Sphaerium corneum*, was constant over an 8-day period. However, Beverley (1994) reports over a 100 % increase in control GST activity in digestive gland extract from *Sphaerium corneum* over a 72 hour period (i.e. 24 hour versus 96 hour exposure). Indeed, at 96 hours exposure, control GST activity was higher than that of animals exposed to 1, 1.5 and 5 µg/l lindane. Although, as in the present study, control GST activities were lower than lindane-exposed GST activities at 24 hours exposure.

Alcohol ethoxylate.

The second xenobiotic investigated was alcohol ethoxylate. Alcohol ethoxylate was much less toxic than lindane and had a 96-hour LC₅₀ value of 3.9 mg/l. This result compares with a 10-day LC₅₀ of 13.7 (12 to 15.5, 95 % CL) for *Hyaella azteca* reported by Dorn *et al.* (1996) for a C₉₋₁₁ EO 6 alcohol ethoxylate. Although the LC₅₀ value in the present study is based on nominal concentrations, it is consistent with the observation that surfactants generally show low toxicity to *Gammarus* species. Aceto *et al.* (1991) examined the lethal toxicity of a number of chemicals including metal ions, pesticides (organochlorine and organophosphate) and surfactants (anionic and non-ionic) to *Gammarus italicus*. The non-ionic surfactants Triton X100 and Brij 30 had 96-hour LC₅₀ values (with 95 % confidence limits) of 73.3 (70, 77) and 9.5 (9, 10) mg/l, respectively. These compared with values for the pesticides methyl azinphos and 4,4 -DDT of 0.99 (0.885, 10.87) µg/l and 4,4 -DDT of 10 (9.52, 13.0) µg/l.

In the present study a 55.4 % increase of GST activity was observed in *G. pulex* on exposure to 0.1 mg/l alcohol ethoxylate for 96 hours. This exposure concentration was 36 times less than the 96 hour LC₅₀. This compares well with the concentration at which GST activity is significantly increased on exposure to lindane which is 46 times less than the LC₅₀ value. The increase in GST activity upon exposure to alcohol ethoxylate demonstrates a dose dependent increase showing a further increase at 0.3 mg/l of 61.6% over the control value. This pattern is similar to that for *G. pulex* exposed to lindane.

It is apparent from the results described in this chapter that sublethal exposure to both lindane and alcohol ethoxylate induce significant increases in GST activity under laboratory conditions. Moreover, this induction occurs at concentrations which are approximately 3 % of the 96-hour LC₅₀. The extent to which GST activity is induced is toxicant specific. In the present study, 24-hour exposure to lindane resulted in a 80 % increase in activity whereas 96-hour exposure to alcohol ethoxylate resulted in a 55 % increase.

Baturo and Lagadic (1996) reported a 115 % increase in GST activity of freshwater snails (*Lymnaea palustris*) exposed to atrazine, but failed to detect induction in snails exposed to high concentrations of the organochlorine fungicide hexachlorobenzene. Similarly, Boryslawskyj *et al.* (1988) have reported differences in the extent to which GST is induced in the mussel *Sphaerium corneum* by the organochlorine pesticides lindane and dieldrin. At 24 hours exposure, animals exposed to 2 µg/l lindane showed a greater increase in GST activity relative to controls than animals exposed to 2 µg/l dieldrin (0.08 and 0.044 µmol/min/mg protein, respectively). However, at 44 and 198 hours exposure, animals exposed to 2 µg/l dieldrin had higher GST activities (0.36 and 0.35 µmol/min/mg protein at 44 and 198 hours, respectively) than animals exposed to 2 µg/l lindane (0.26 and 0.24 µmol/min/mg at 44 and 198 hours exposure, respectively). This may be suggestive of differential induction of GST in *Sphaerium corneum* by different organochlorine pesticides.

3.4.2. Exposure under semi-natural conditions.

Data presented in Chapter Two demonstrated that environmental variables such as temperature can, to some extent, have an effect on GST activity. It is clear that minimising the effects of environmental variables on GST activity under constant laboratory conditions maximises the detection of changes in GST activity due to xenobiotic challenge. However, exposure in a mesocosm ensures exposure to a range of known concentrations of xenobiotic, under conditions of environmental variability. Animals can then be assessed for changes in GST activity due to chemical exposure. This was done using the artificial stream

system where the exposure concentration is known but the test system is subject to environmental variables. This semi-natural system ensures all test animals are subject to the same environmental variability but the range of exposure concentrations is controlled.

There were significant differences in GST activity in *G. pulex* exposed to alcohol ethoxylate in the artificial streams. However, significant increases in GST activity from control were only demonstrated at 24 hours exposure. In contrast to sublethal laboratory exposure to alcohol ethoxylate, increases in GST activity were not dose dependent. Rather, at 24 hours exposure there was a threshold at 0.115 mg/l below which there was no alcohol ethoxylate-induced elevation in GST activity. At 0.115 mg/l GST activity was increased by 47.5 % relative to controls. This concentration compares with 0.1 mg/l at which GST activity was significantly increased by 55.4 % from control at 96 hours exposure in the laboratory.

The GST activity of control animals deployed in the artificial streams demonstrated a non-monotonic relationship with time. The control value at 24 hours of 0.021 $\mu\text{mol}/\text{min}/\text{mg}$ protein increased to 0.047 and 0.054 $\mu\text{mol}/\text{min}/\text{mg}$ protein at 96 and 168 hours respectively, finally decreasing to 0.024 $\mu\text{mol}/\text{min}/\text{mg}$ protein at 336 hours. Moreover, this non-monotonic relationship is also observed in the GST activity of animals exposed to alcohol ethoxylate in the artificial streams. For example, animals exposed to 200 $\mu\text{g}/\text{l}$, had a GST activity of 0.031 $\mu\text{mol}/\text{min}/\text{mg}$ at 24 hours exposure which increased to 0.059 $\mu\text{mol}/\text{min}/\text{mg}$ protein at 96 hours. Activity subsequently decreased to 0.042 and 0.024 $\mu\text{mol}/\text{min}/\text{mg}$ protein at 168 and 336 hours exposure respectively. A non-monotonic curvilinear relationship between GST activity and time was apparent at all exposure concentrations.

Monotonic increases in control GST activity with time were observed in the laboratory in the present study (section 3.5.1) and in other work (e.g. Beverley, 1994). Non-monotonic relationships in which GST activity is initially elevated

and then decreased have also been described. As discussed earlier the GST activity of earthworms exposed to 1 µg lindane/g soil was shown to initially increase relative to controls at 14 days then decrease at 28 days exposure (Hans *et al.*, 1993). The reason for subsequent decreases in activity after initial induction are not clear. However, Hans *et al.* (1993) suggest an initial increase may be a defensive adaptation to xenobiotic challenge to facilitate rapid biotransformation and that the decline in GST activity on prolonged exposure may be due to the depletion of reduced glutathione.

3.4.3. Sensitivity of the GST response.

As discussed previously, induction of GST activity was more sensitive to lindane and alcohol ethoxylate exposure than the 96-hour LC₅₀ values. However, comparisons can be made with other sublethal endpoints determined by other workers. The effect of lindane on the feeding and pre-copula pairing behaviour of *G. pulex* was investigated by Malbouisson *et al.* (1995). A significant decrease in feeding rate was observed after 48 hours exposure to 5.0 µg/l or 20 minutes exposure to 1 mg/l lindane. The re-pairing of disrupted precopula pairs was unaffected by 48 hour exposure to 5 µg/l but was affected by 2 minutes exposure to 0.5, 1.0 and 2.0 mg/l lindane. The GST response which was detected after 24 hours exposure to 0.17 µg/l was therefore more sensitive to lindane than either the feeding response or behavioural response studied by Malbouisson *et al.* (1995). A 14-day exposure to 6.1 µg/l lindane decreased the growth of juvenile *G. pulex* (Blockwell *et al.*, 1996b). Although this value seems high, Blockwell *et al.* (1996b) report a water hardness of 141 mg CaCO₃/l (c.f. 92 mg CaCO₃/l in the present study). Thus it is difficult to explain the discrepancy between the estimated of toxicity for lethality in the present study and the data of Blockwell *et al.* (1996b). Confidence is given to the values derived in the present study as a similar method for determining LC₅₀ was use to Stephenson (1983) that gave comparable values. Furthermore, the value given by Blockwell *et al.* (1996b) is a LOEC (lowest observed effect concentration) and this was the highest concentration used by these workers. The NOEC (no observed effect concentration) was 2.8 µg/l lindane. The use of a NOEC/LOEC estimate of

toxicity is dependent on the concentrations that are used in the test as the NOEC value will be the concentration at which no effect was observed and the LOEC the lowest concentration at which effects were observed. The use of this type of estimate of toxicity has been criticised and the estimation of a regression design giving effect or lethal concentrations for percentages of the population (i.e. EC_x or LC_x) has been favoured because they give a more accurate estimation of experimental error (Laskowski, 1995). The 96-hour LC₅₀ value of 7.8 µg/l determined in the present study is 46 times greater than the concentration of lindane resulting in a significant increase in GST activity at 24 hours. The enhanced sensitivity of GST induction compared to feeding, growth or reproductive behaviour might be expected as GST is involved in the detoxification of organic xenobiotics such as lindane and will therefore respond rapidly to exposure. Furthermore, as growth is the sum total of all the anabolic and catabolic processes in an animal then it might be expected to be less sensitive to exposure and to respond less rapidly.

The significant increase in alcohol ethoxylate-induced GST activity in *G. pulex* was at a concentration 36 times less than the LC₅₀ at 96 hours. This demonstrates GST activity as a marker of sublethal exposure to alcohol ethoxylate in the laboratory. Dorn *et al.* (1996) report a survival NOEC for the freshwater cladoceran, *Daphnia magna*, of 1.75 mg/l to a C₉₋₁₁ EO-6 alcohol ethoxylate. This value is 17.5 times higher than the concentration at which GST activity is significantly increased from control in *G. pulex* (Figure 3.5). Furthermore, although precautions were taken to limit the loss of alcohol ethoxylate (e.g. autoclaving APW for test solutions, section 3.2.3) nominals were used for all estimates of alcohol ethoxylate exposure in this study. It is therefore assumed that exposure in the present study was under-estimated as some of the test chemical may be 'lost' from solution. This means that the conclusions drawn here are conservative.

The threshold of increased GST activity at 115 µg/l alcohol ethoxylate compares to a threshold in feeding rate inhibition (consumption of leaf material) of 115 µg/l

in the same animals at one week exposure (see Appendix 2.2 for data). Furthermore, other end-points measured during the same stream experiment by workers at Shell Research Ltd, showed the GST response to be among the most sensitive. For example, the NOEC for *G. pulex* juvenile and mayfly nymph (*Baetis* species.) population density at 28-day exposure was 160 µg/l alcohol ethoxylate, whereas the NOEC value for community leaf processing (invertebrate consumption and microbial breakdown of leaf material) was 300 µg/l at 28 days. The only endpoint more sensitive than GST activity was the swimming activity of *G. pulex* observed after a 96-hour exposure period, a NOEC of 40 µg/l being reported (Tattersfield *et al.*, 1996).

3.4.4 Conclusions.

The objective of this chapter was to detect a GST response in *G. pulex* on sublethal exposure to lindane and alcohol ethoxylate. Furthermore, to detect the GST response in exposure under fluctuating environmental conditions, with known xenobiotic exposure, provided by the artificial stream system.

- GST activity was increased in response to short-term xenobiotic exposure.
- The GST response to organic xenobiotics was general, in that both lindane and alcohol ethoxylate induced increased GST activity.
- The response was rapid; increased activity was detected at 24 hours exposure.
- The response was sensitive; significant increases in GST activity at 24 hours were at concentrations of 36 (alcohol ethoxylate) and 46 (lindane) times less than 96-hour LC₅₀ concentrations.
- Increases were not always sustained, and this may be due to the increases in control GST activity. This is true both in the laboratory with lindane exposure and in semi-natural conditions with alcohol ethoxylate exposure.

CHAPTER FOUR

Glutathione s-transferase activity in contaminated and non-contaminated populations of *Gammarus pulex*.

4.1. INTRODUCTION.

The work detailed in Chapter Three demonstrated the induction of elevated GST activity in *Gammarus pulex* exposed to sub-lethal concentrations of lindane in the laboratory. Furthermore, an increase in GST activity was reported in *G. pulex* after short-term exposure to alcohol ethoxylate in a mesocosm. The aim of the work described in this chapter was to assess the effect of long-term exposure to organochlorine pesticides on GST activity in natural populations of *G. pulex*. The comparison of GST activity of *G. pulex* from contaminated and non-contaminated populations was used to assess the validity of GST as a marker of long term exposure to xenobiotics. Furthermore, interpopulation variation in the induction of GST after prolonged exposure to field concentrations of pesticide was assessed in the laboratory.

4.1.1 GST activity in contaminated and non-contaminated populations.

Interpopulation variation in GST activity has been demonstrated in several studies. The role of GST in insecticide-resistant populations of insects is well documented (Oppenoorth, 1984). Several hundred arthropod species have acquired resistance to all major classes of insecticide, including organochlorines, organophosphates, carbamates and pyrethroids (Georghiou, 1990). Furthermore, resistance to pesticides in invertebrates can be directly correlated with enzyme biomarkers such as MFO and GST (Lagadic *et al.*, 1994). The development of resistance depends on genetic viability already present in a population or arising during the period of selection. This results from the evolution of mutations that gives rise to resistance alleles in the natural populations of the species (Oppenoorth, 1985). Widespread applications of pesticides propagate resistance alleles through preferential survivals and they become dispersed throughout the

population (Metcalf, 1989). Detoxification systems such as GST display a great flexibility and many organisms are capable of genetic adaptation to most toxicants when under long-term selection pressure (Metcalf, 1989). However, correlations of enzyme activity with resistance are complicated by the existence of multiple molecular forms of GST that may have specificities for individual pesticides (Hodgson, 1985; Clark, 1989).

Pascual *et al.* (1991) reported increased GST activity in fish (*Mugil* sp.) in polluted coastal sites compared with GST activity of members of the same species from clean reference sites. A number of studies on mollusc populations have reported variation in GST activity associated with increased contamination. The bivalve molluscs *Chamaea gallina* (striped venus), *Ruditapes decussatus* (grooved carpet shell) and *Crassostrea gigas* (Pacific oyster) from metal contaminated sites had elevated GST activity compared to individuals from the same species collected from non-contaminated sites (Rodriguez-Ariza *et al.*, 1992). The GST activity of the marine mussel, *Mytilus edulis*, from several sites in a "polluted" harbour was significantly higher than the activity of mussels from a population found in a "clean" reference site (Sheehan *et al.*, 1991). Similarly, GST activity in the freshwater mussel (*Sphaerium corneum*) obtained from a site contaminated with organochlorine pesticide was greater than that of *S. corneum* collected from non-contaminated sites (Boryslawskyj *et al.*, 1988; Garrod *et al.*, 1989; Beverley, 1994).

4.1.2 Lindane contamination of freshwater systems.

Lindane is applied to a wide range of crops (section 3.1.2) and may enter freshwater either by direct over-spray, surface run-off or leaching through the soil column. In the present study, GST activity was investigated in *G. pulex* from populations that were subject to contamination by several pesticides, including lindane, the source of which was agricultural run-off.

Run-off is dependent on the volume of rainfall and the topography of the catchment area. Pesticides in run-off may be dissolved or adsorbed to associated

particulate material. Pesticides such as lindane that are poorly soluble in water are likely to be adsorbed to fine particulate organic material. The adsorption of lindane to organic material has been demonstrated by Huggenburger (1972). It has been reported that the concentration of lindane in catchment rivers is affected by run-off, caused directly by increases in rainfall close to the time of application (Clark *et al.*, 1991). Lindane run-off from an agricultural catchment sprayed with a known amount of lindane was studied by Clark *et al.* (1991). These workers report that from a normal loading application (9.5 kg/km²) to the catchment area a resulting maximum of 0.05 µg/l was found in the catchment stream.

The water solubility of a pesticide will influence its mobility through the soil. Pesticides in solution may move through the soil profile until they reach an impermeable layer and enter surface or ground water. This process is heavily dependent on the amount of rainfall and the physico-chemical properties of the pesticide. Poorly water soluble pesticides such as lindane will have a poor mobility. Indeed, Huggenburger (1972) demonstrated that lindane did not penetrate deeper than 8 cm through experimental soil columns.

The fate and behaviour of lindane in freshwater are influenced by its relative stability and insolubility (Crossland and Beasley, 1990). Lindane has a half-life of 123 days in freshwater (Hamelink and Waybrant, 1976). The high octanol-water partition coefficient of lindane (log. P_{ow} 3.2 to 3.7) suggests that it is likely to be bioaccumulated. A bioaccumulation factor of 50 was reported for the freshwater crustacean *Asellus aquaticus* exposed for 48 hours to 2 µg/l lindane (Thybaud *et al.*, 1987). Lindane may also be accumulated as a result of the ingestion of contaminated food. Hansen (1980) reported that the freshwater cladoceran *Daphnia magna* exposed to aqueous lindane had a bioconcentration factor of 37.5 whereas animals exposed to contaminated food and water had a bioaccumulation factor of 80.

4.1.3 Objectives.

The major objective of the work described in this chapter was to investigate interpopulation differences in the induction of GST activity in *G. pulex* collected from lindane-contaminated and reference sites. It was hypothesised that long-term exposure to lindane would select for *G. pulex* with elevated GST activity as they would be better adapted for detoxifying the chemical. It was therefore predicted that GST activity in *G. pulex* from a lindane-contaminated site would be significantly greater than GST activity in *G. pulex* from a non-contaminated site. Lindane exposure via run-off will be episodic and maximal soon after application. If GST activity is a measure of exposure, activity levels should also be maximal soon after pesticide application. Lindane is applied in March, it was therefore predicted that GST activity of animals from lindane-contaminated site would be greatest in late spring/early summer. This prediction was tested by collecting *G. pulex* from contaminated and non-contaminated sites during spring, summer and autumn. Even though contaminated-site animals may have had higher 'baseline' activities they should still increase GST activity when challenged with lindane. A third prediction was therefore, that short-term exposure to a sublethal concentration of lindane would result in increases in GST activity in *G. pulex* from contaminated sites. This prediction was tested by exposing *G. pulex* from a contaminated and non-contaminated site to a sublethal concentration of lindane for 48 hours and measuring the change in GST activity.

If the elevated 'baseline' GST activity of *G. pulex* from a contaminated site was due to acclimation, then it would be predicted that maintaining animals from contaminated and non-contaminated sites under either control conditions or low lindane concentrations should reduce or remove interpopulation differences in GST activity. Alternatively, if the elevated GST activity of contaminated site animals was due to genetic adaptation, interpopulation differences should persist for animals maintained in the low lindane treatment. The 3 predictions were tested by pre-exposing animals for 40 days before assessing their response to short-term lindane exposure.

4.2. MATERIALS AND METHODS.

4.2.1. *Gammarus pulex* populations.

G. pulex were obtained from four sites near Braunschweig, Lower Saxony in Germany (Figure 4.1).

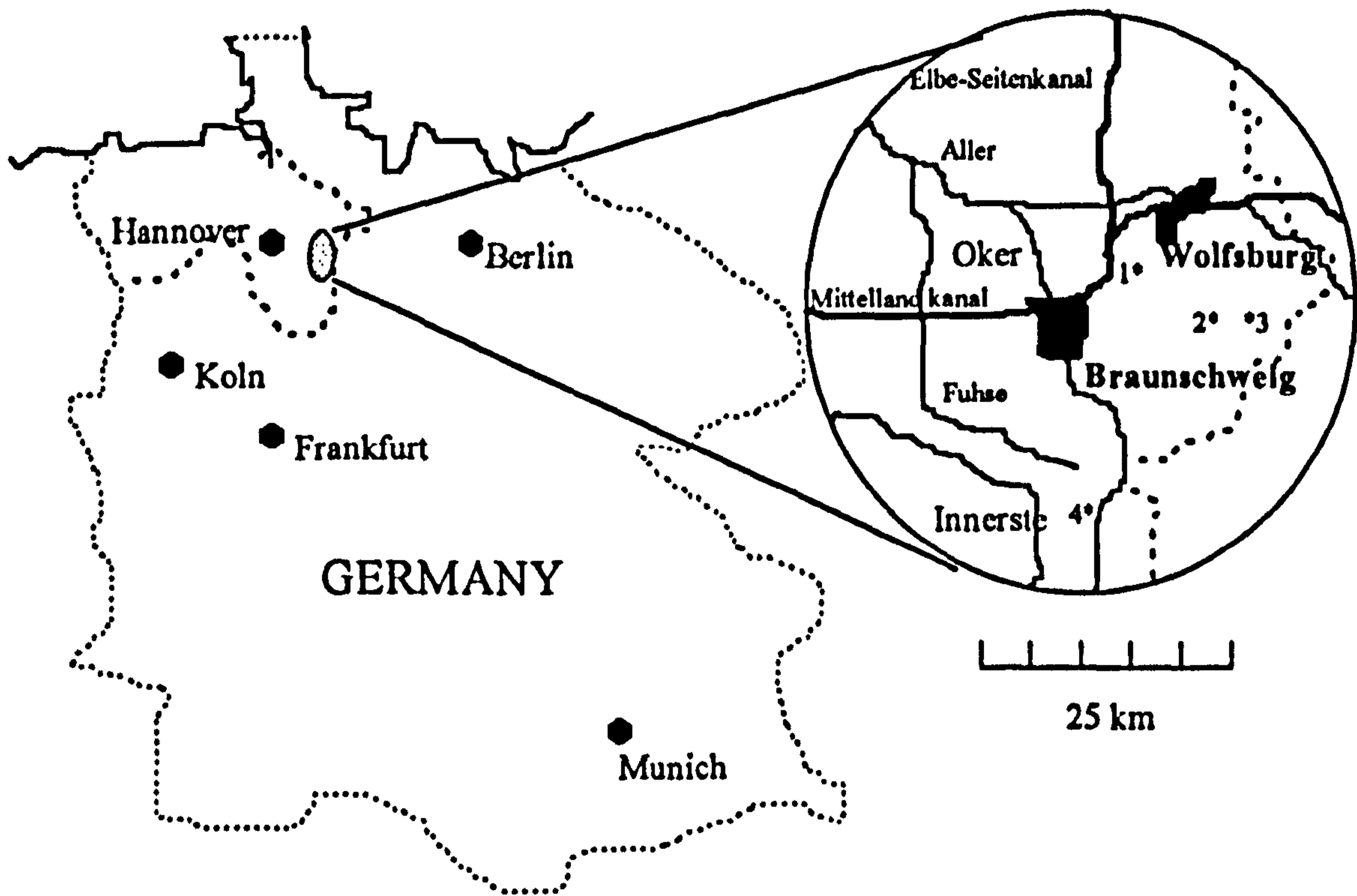
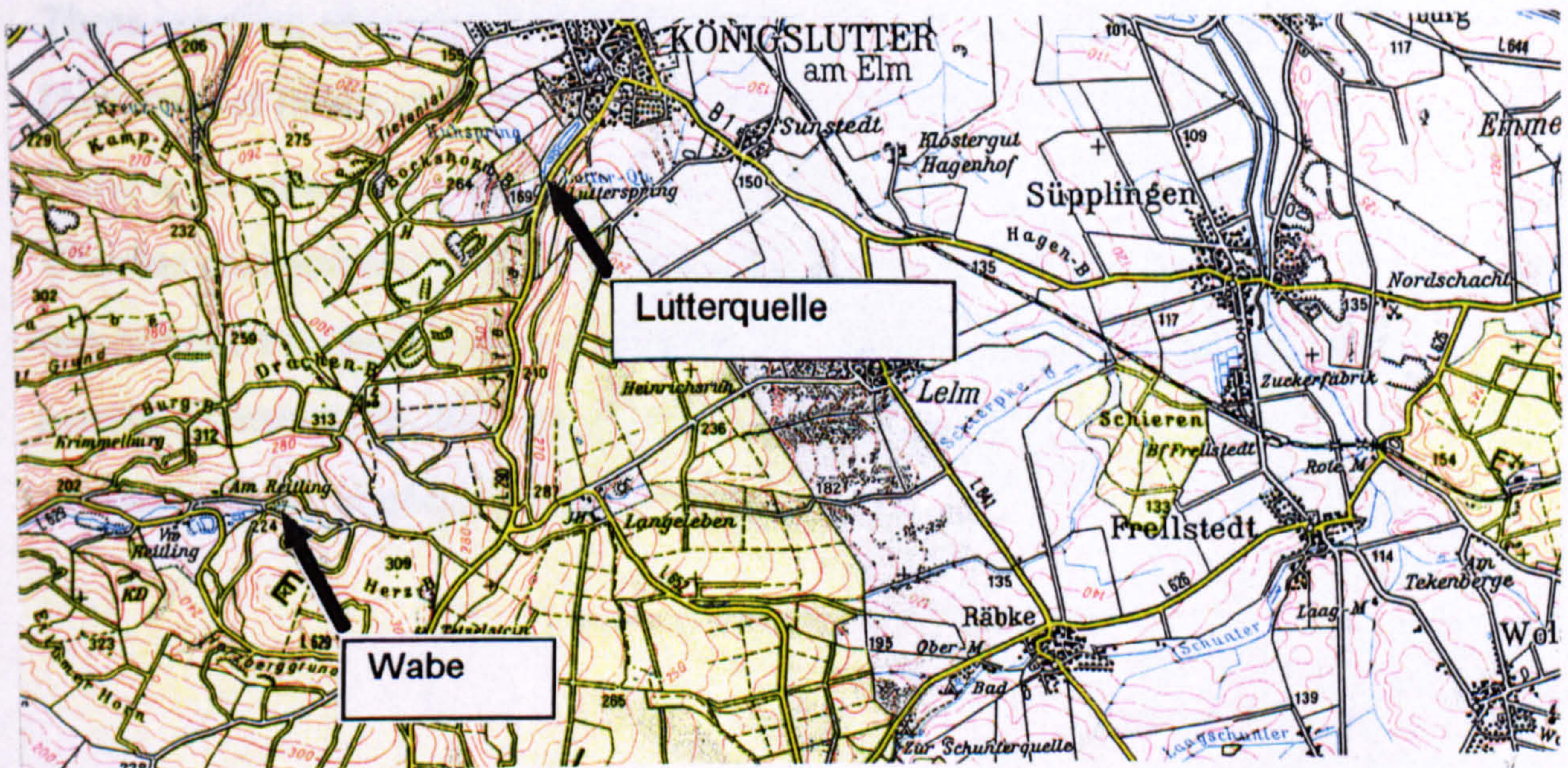
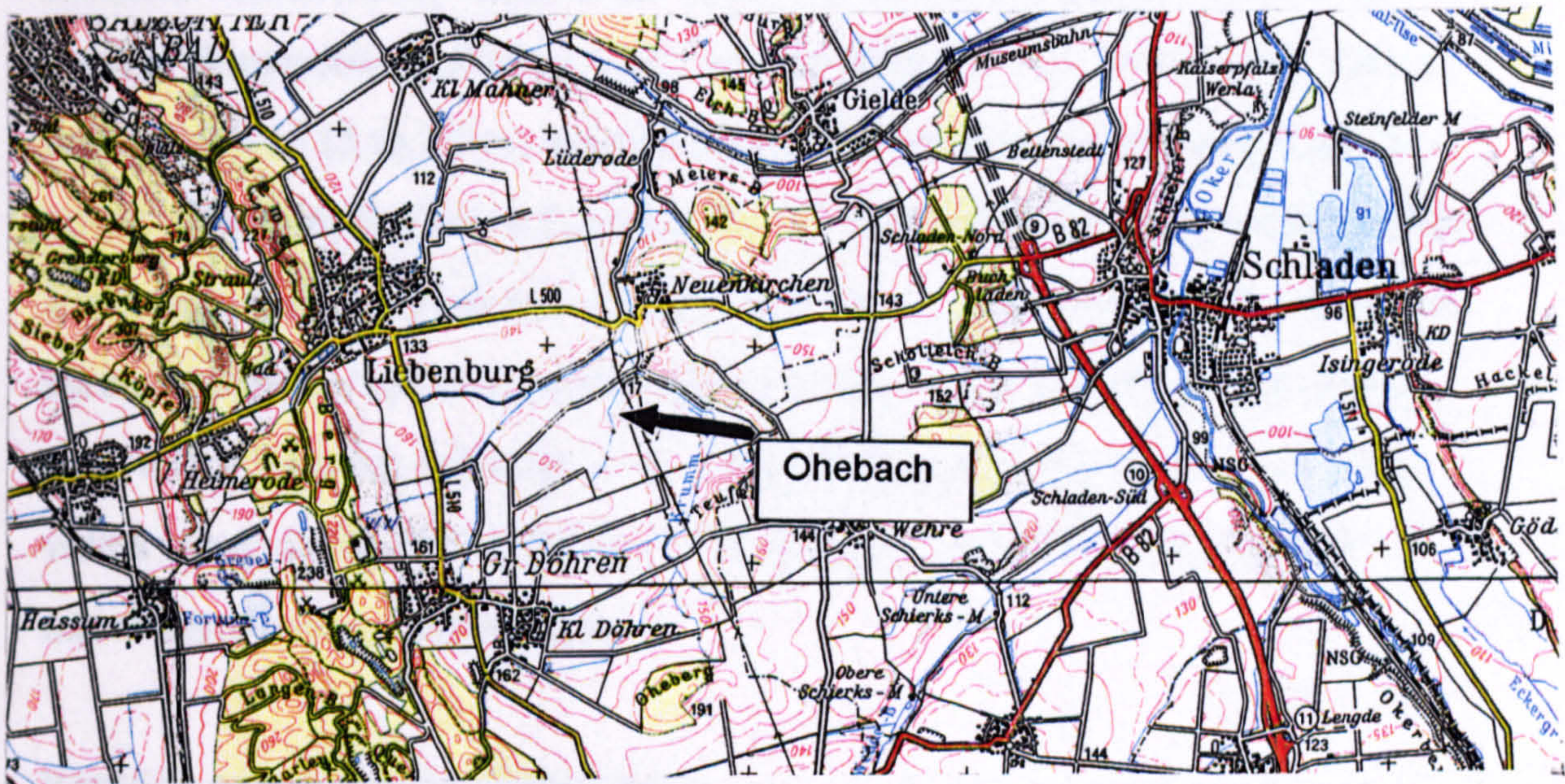
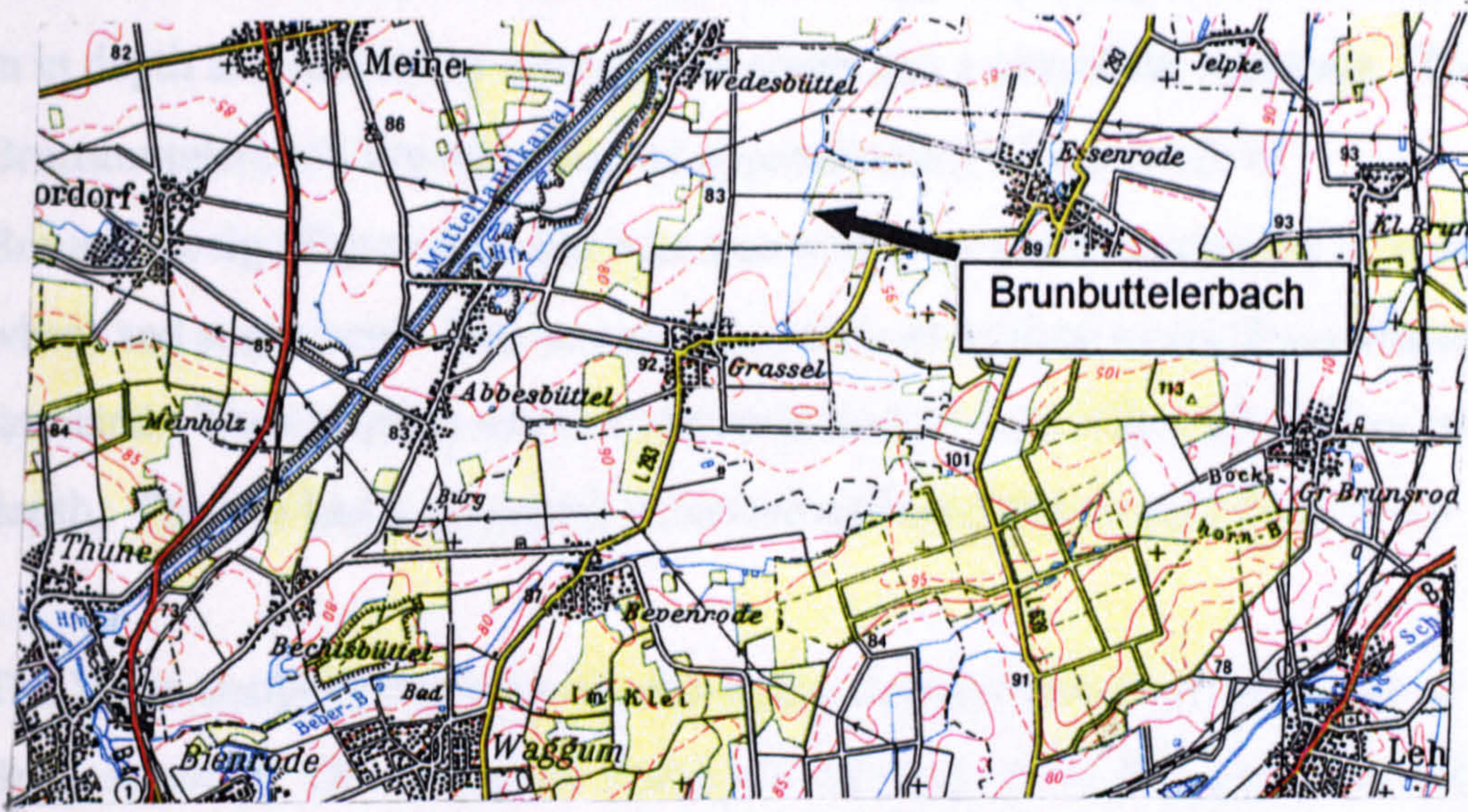


Figure 4.1. The location of field sites in the Lower Saxony region of Germany. Major rivers in the region are shown. Field sampling sites are indicated at Brunsbuttelerbach (1), Lutterquelle (2), Wabe (3) and Ohebach (4).

Two sites, Ohebach and Brunsbuttelerbach, were surrounded by arable farmland, whereas two sites, Wabe and Lutterquelle were in a wooded area (Figure 4.2).

Figure 4.2 Detail of field sites in Lower Saxony. Brunsbüttelerbach, Ohebach Lutterquelle and Wabe. Reproduced from: Topographische Karte, Regionalkarte 14; Großraum Braunschweig, scale 1:100,000.



The Ohebach catchment was 0.9 km² and contained crops of sugar beet, winter barley and winter wheat. The sampling site on this stream received surface water runoff from adjacent farmland. It was approximately 0.5 m wide and 0.25 m in depth and had heavy macrophyte cover and a gravel/silt substrate. The Brunsbuttelerbach site was situated approximately 15 km north of Braunschweig (Figure 4.2) and was also within an arable catchment of winter wheat and sugar beet. This stream also received surface runoff from adjacent farmland. The sampling site was approximately 0.5 m wide with 0.25 m water depth. The site had a gravel/silt substrate and moderate macrophyte cover.

The Wabe sampling site was situated in the wooded Elm area south east of Braunschweig. The spring fed Wabe is a tributary of the Schunter river. The Schunter feeds into the Oker river, which it joins approximately 10 km north west of Braunschweig (Figure 4.2). The sampling site was 40 cm wide, with a water depth of 15 to 20 cm and had a gravel substrate and heavy macrophyte cover. The sampling site at Lutterquelle was situated on the eastern edge of the Elm wooded area, 20 km east of Braunschweig. The Lutterquelle stream is also spring fed and served as a potable water source. It is a tributary of the Lutter river which joins the Schunter river. The sampling site was 1.0 to 1.5 m wide with a water depth of 15 to 20 cm. The substrate was a rocky gravel with macrophyte cover at the margins.

Pesticide contamination of field sites.

These sampling sites have been subject to study by workers at the Technical University of Braunschweig. The sites at Lutterquelle and Wabe have been found to be consistently free of pollutants (Liess and Schulz, pers. comm.). Whereas contaminated sites at Brunsbuttelerbach and Ohebach have been demonstrated to receive agricultural run-off containing lindane as well as the pyrethroid insecticide, fenvalerate, and the organophosphorus insecticide, parathion (Liess, in press; Schulz, in press).

During the years 1993 to 1995, the water and sediment of the Ohebach site had been routinely sampled for pesticides (e.g. Liess *et al.*, 1996). Parathion was associated with high run-off events in 1993. Concentrations increased from 0.5 µg/kg sediment dry weight in April to 13 – 15 µg/kg in late May and early June before decreasing again to 3 µg/kg in July (Liess *et al.*, 1996). In 1994, run-off events in May and June were associated with elevated parathion concentrations in sediments of between 40 and 50 µg/kg decreasing to between 5 and 10 µg/kg in mid June. Fenvalerate concentrations were shown to follow the same trend as parathion with values of between 40 and 70 µg/kg sediment dry weight associated with run-off events in late May/early June 1994 (Liess *et al.*, 1996). During 1994 samples were taken during run-off events and values for lindane and parathion were measured in the water column and in the sediment as shown in Table 4.1.

Table 4.1 Concentrations of lindane and parathion in Ohebach sampled during run-off incidents in 1994. Sediment concentrations are per kg dry weight, nd = not detected.
Data from: Liess (in press); Schulz (in press); Liess (unpublished).

Sample date	water concentration		sediment concentration	
	lindane µg/l	parathion µg/l	lindane µg/kg dwt	parathion µg/kg dwt
25.4.94	0.02	0.04	5.7	nd
19.5.94	0.05	6.0	2.5	50.8
25.5.94	0.05	0.9	3.9	50.8
8.6.94	0.07	0.2	2.2	19.4

During 1995, the pesticides lindane, parathion and fenvalerate were detected in samples from the Ohebach site (Table 4.2).

Table 4.2 Pesticide concentrations associated with run-off events for Ohebach in 1995. Data are given as concentration from sediment samples (S) and water samples (W). Detected but not quantifiable pesticides are denoted by nq. The limit of quantification was 1 µg/kg for lindane and parathion and 5 µg/kg for fenvalerate in sediments, 0.01 µg/l for lindane and parathion and 0.05 µg/l for fenvalerate in water. Data from Liess (unpublished).

Sample date	Lindane	Parathion	Fenvalerate
2.3.95	2.9 µg/kg (S)	nq	nq
2.7.95	nq	0.08 µg/l (W)	0.85 µg/l (W)
1-16.9.95	nq	nq	nq
1-16.10.95	nq	nq	1500 µg/kg (S)

The Brunsbuttelerbach site had detectable but not quantifiable levels of lindane in March and July of 1995 (Liess, unpublished data). Both the Ohebach and the Brunbuttelerbach sites had a macroinvertebrate community structure which was indicative of pesticide contamination (Liess, unpublished data). Liess *et al.* (1993) investigated the ecological effects of pesticide containing surface water run-off on the macroinvertebrate community in streams with agricultural catchments. Streams were classified on the basis of the effect of run-off on the macroinvertebrate community by obtaining information on species abundance, drift and emergence. This then was used as a biological indicator system, to link causal connections regarding the macroinvertebrate community and surface water run-off in streams (Liess *et al.*, 1993).

4.2.2 Sampling of *Gammarus pulex* populations.

At all sites, *G. pulex* were collected using a Surber sampler (base 0.062 m²) fitted with a 1-mm mesh net. Large male animals (10 to 15 mm) were selected and transported to the laboratory in stream water from the collection site.

Spatial variation in GST activity.

Forty large male animals were collected from the Wabe, Lutterquelle, Ohebach and Brunsbuttelerbach sites in October 1995 (section 4.2.2). Animals were weighed on a Sartorius 2434 balance (accuracy ± 0.01 mg), frozen in liquid nitrogen and stored at -80 °C prior to GST analysis (section 2.2.3).

Temporal variation in GST activity.

Fifty large male animals were collected from the Ohebach site in March, July, September and October 1995 and from the Wabe site in July, September and October 1995. Animals were weighed on a Sartorius 2434 balance (accuracy \pm 0.01 mg), frozen in liquid nitrogen and stored at -80 °C prior to GST analysis (section 2.2.3).

4.2.3 Interpopulation variation in response to short-term lindane exposure.

Sixty animals were collected from both the Wabe and Ohebach sites in September 1995 (section 4.4.2). Thirty animals from each site were frozen in liquid nitrogen immediately after collection and stored at -80 °C prior to GST analysis (section 2.2.3). The remaining 30 animals/site were exposed as a group, to a nominal concentration of 1 μ g/l lindane for 48 hours. Animals were then frozen and stored prior to GST analysis (section 2.2.3).

4.2.4. Effect of pre-exposure on the GST response to short-term exposure.

Large male *G. pulex* were collected from both Wabe and Ohebach site in September 1995 (section 4.2.2). Fifty animals from each site were placed in 10 l of filtered spring water (unexposed). A further fifty individuals/site from each site were placed in 10 l spring water with a nominal concentration of 0.02 μ g/l lindane (pre-exposed). Holding solutions were aerated and changed weekly. A 1-l sample was taken for analysis, prior to exposure (section 4.2.6). Animals were provided with conditioned alder leaves (Appendix 1.3) as food and maintained at 15 °C (\pm 1 °C) under an 12 hour/12 hour light/dark photoperiod laboratory for 42 days. Two days before the end of the experiment (i.e. day 40), a group of 24 animals was removed from each treatment and were each placed in 10 l of a nominal concentration of 1.0 μ g/l lindane. A 1-l sample was taken for analysis (section 4.2.6). After 42 days all animals were removed, weighed on a Sartorius 2434 balance (accuracy \pm 0.01 mg), frozen in liquid nitrogen and stored at -80 °C prior to GST analysis (section 2.2.3).

4.2.5 Transportation of frozen samples.

Animals were transported to Sheffield for GST analysis. Animals were frozen at -80 °C and were transported packed in solid carbon dioxide pellets (-78 °C) in expanded polystyrene boxes. A journey time of 6 hours ensured that samples remained at -78 °C until storage at -70 °C on arrival in Sheffield. Samples collected in March and July were analysed in July 1995, September and October samples were analysed in October 1995 ensuring storage was no longer than three months for any one sample.

4.2.6 Lindane analysis.

Lindane was extracted on to solid phase extraction cartridges (section 3.3.1). Cartridges were kept frozen until return to Sheffield for extraction. Test solution concentrations from laboratory experiments were analysed by GC/ECD as detailed in section 3.2.5. Field lindane concentrations were analysed at the Technical University of Braunschweig also using GC/ECD.

4.2.7 Statistical analysis.

Statistical analyses were performed using the statistical software package Minitab, version 9.2 for Windows. All data were checked for normality using normal probability plots and Anderson-Darling normality tests. Oneway analysis of variance (ANOVA) was used to analyse data from different sites in October, for animals sampled from Ohebach and Wabe over the year and for all experimental treatments of animals from the Wabe and Ohebach sites. The significance of pair-wise differences in ANOVA was assessed by the multiple comparison test Tukey's test; *q* values stated are derived by the method of Zar (1996). Differences between unexposed and exposed; unexposed and unexposed/exposed; pre-exposed and pre-exposed/exposed, were analysed using Student's t-test on Minitab. Significance was at $p = 0.05$ unless otherwise stated.

4.3. RESULTS.

Specific activities of GST in *G. pulex* sampled in October from two non-contaminated populations at Lutterquelle and Wabe and two lindane contaminated sites at Brunsbuttelerbach and Ohebach are shown in Figure 4.3.

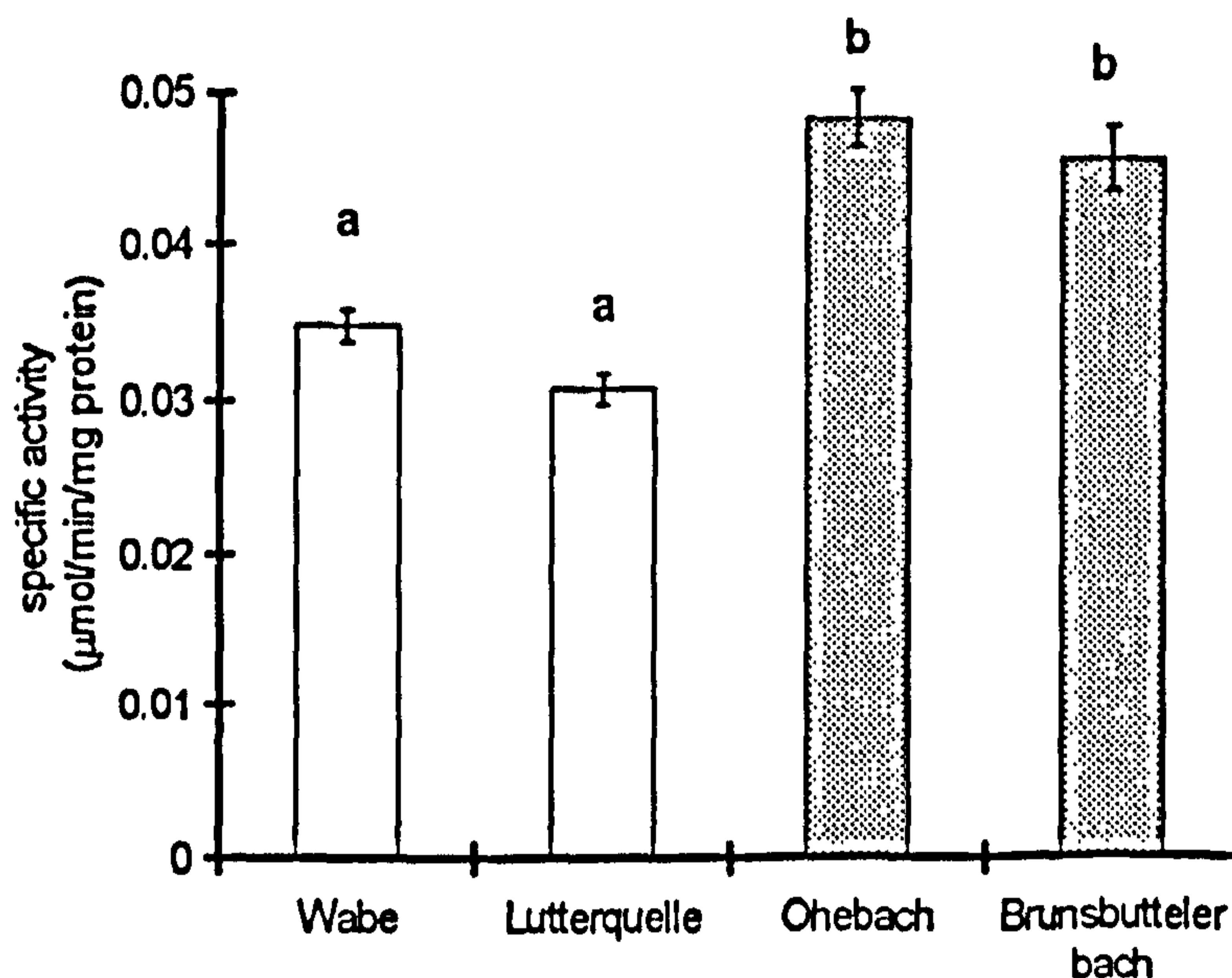


Figure 4.3 GST activity of *G. pulex* from uncontaminated sites (open bars) and contaminated sites (solid bars). Bars sharing the same letter are not significantly different analysed by Tukey's multiple comparison ($q > 3.68$). Data are presented as mean values of 40 animals. Error is the standard error of the mean.

There was significant interpopulation variation in specific GST activity ($F_{3,142} = 27.9$); with animals from contaminated sites (Ohebach and Brunsbuttelerbach) having a significantly higher activity than those from uncontaminated sites (Wabe and Lutterquelle). Animals from the Ohebach site had 39 % and 57 % higher activity than animals from the Wabe and Lutterquelle sites, respectively. Animals from Brunsbuttelerbach demonstrated 31 % higher activity than Wabe animals and 49 % higher activity than Lutterquelle animals.

The specific GST activity of *G. pulex* from the Wabe non-contaminated and Ohebach contaminated site is shown in Figure 4.4.

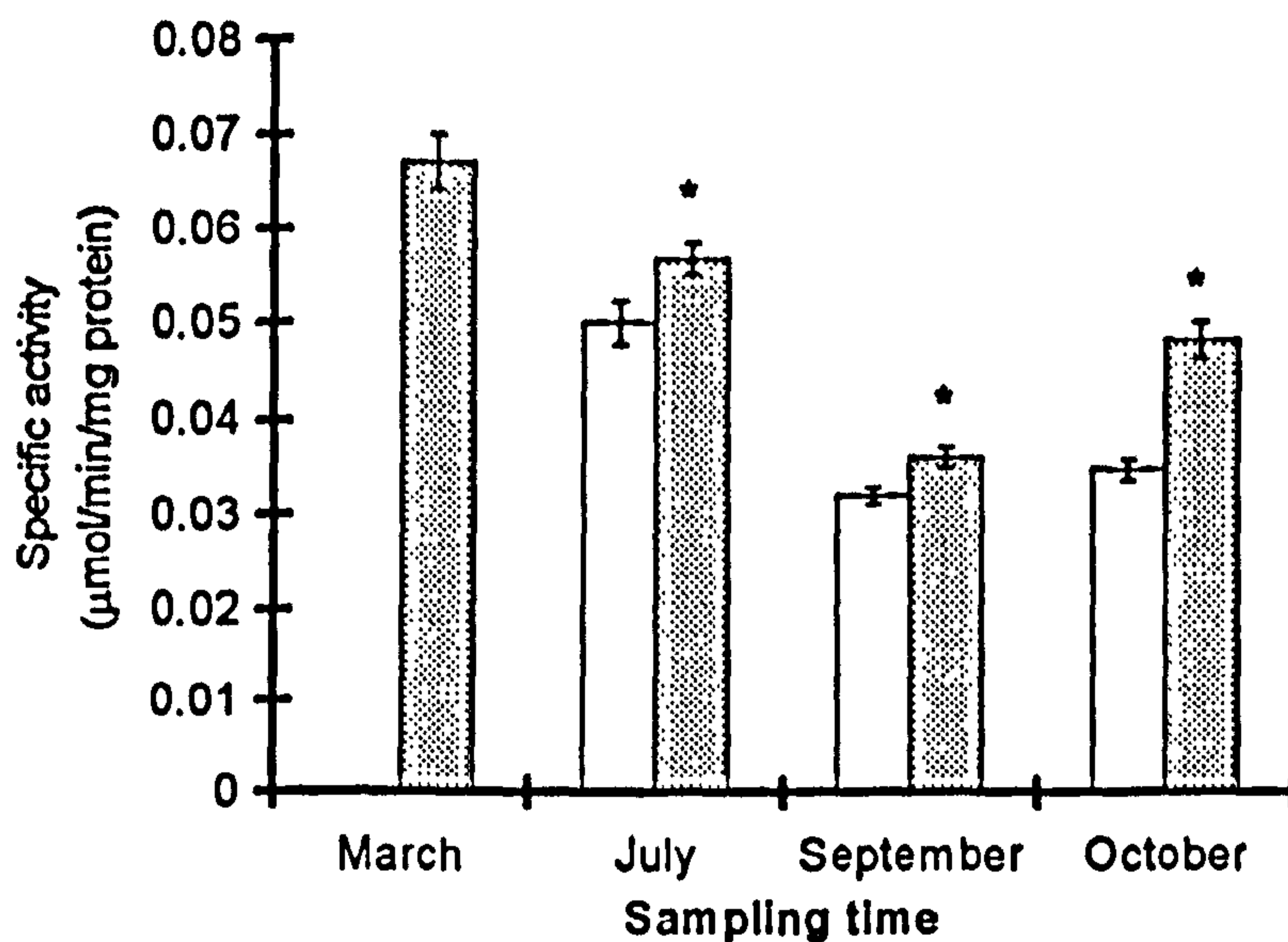


Figure 4.4. GST activity of animals collected from Ohebach (solid bars) and Wabe (open bars). Significant interpopulation differences are indicated with an asterisk. Data are presented as mean values of 50 animals. Error bars are the standard error of the mean.

There was significant temporal variation in the specific GST activity of *G. pulex* from Wabe ($F_{2,112} = 33.2$) and Ohebach ($F_{3,160} = 39.4$). Activity was greatest in March and lowest in September. However, on all sampling occasions the activity of animals from the Ohebach was significantly higher than that of animals from Wabe ($F_{6,278} = 44.8$, $q > 4.17$). Interpopulation differences in GST activity varied from 39 % (October) to 13 % (July).

The specific GST activity of animals from both Wabe and Ohebach was elevated after exposure to 1 µg/l lindane (Figure 4.5).

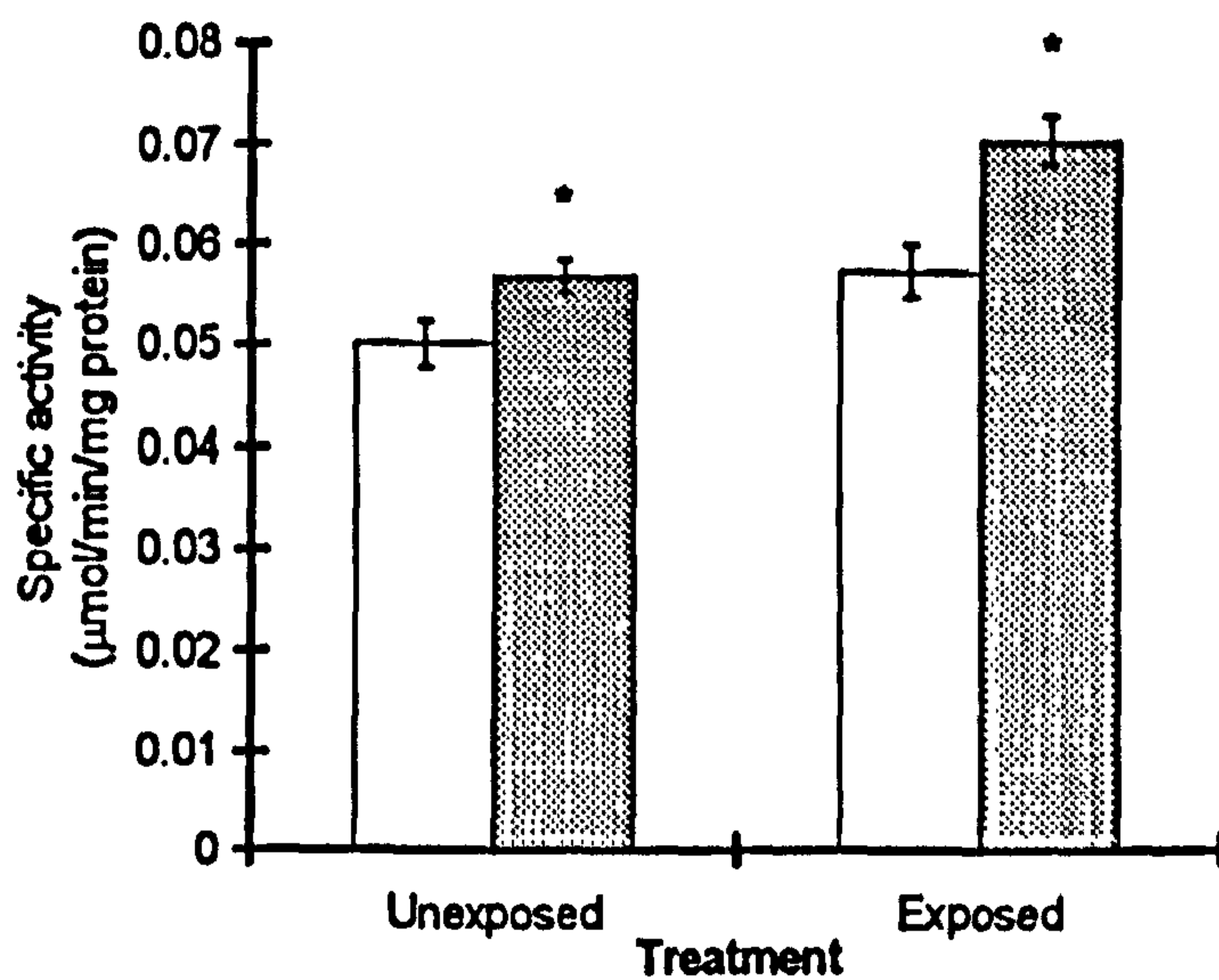


Figure 4.5. GST activity of *G. pulex* from Wabe (open bars) Ohebach (solid bars), unexposed or exposed to 1 µg/l lindane. Significant interpopulation differences are indicated by asterisks. Data are presented as mean values. Error bars are the standard error of the mean.

The GST activity of Ohebach animals was increased by 24 % on exposure to lindane compared to a 14 % increase in the GST activity of Wabe animals. Interpopulation differences apparent in unexposed animals ($t_{28} = 24$) were maintained after lindane exposure ($t_{75} = 3.67$). Furthermore, the magnitude of this difference increased from 14 % prior to exposure to 23 % post exposure.

It is clear from Figure 4.5 that animals from Ohebach and Wabe have a similar response to short-term lindane exposure. The interpopulation differences observed in the field-collected animals may be due to acclimation to environmental concentrations of pesticide. If this is the case, then interpopulation differences may be reduced or removed by maintaining animals from both sites under similar conditions in the laboratory.

Animals from both Wabe and Ohebach were held in clean conditions (filtered spring water) in the laboratory for 40 days. A group from each site were then exposed to 1 µg/l for the final two days of treatment. The remaining animals were maintained in filtered spring water.

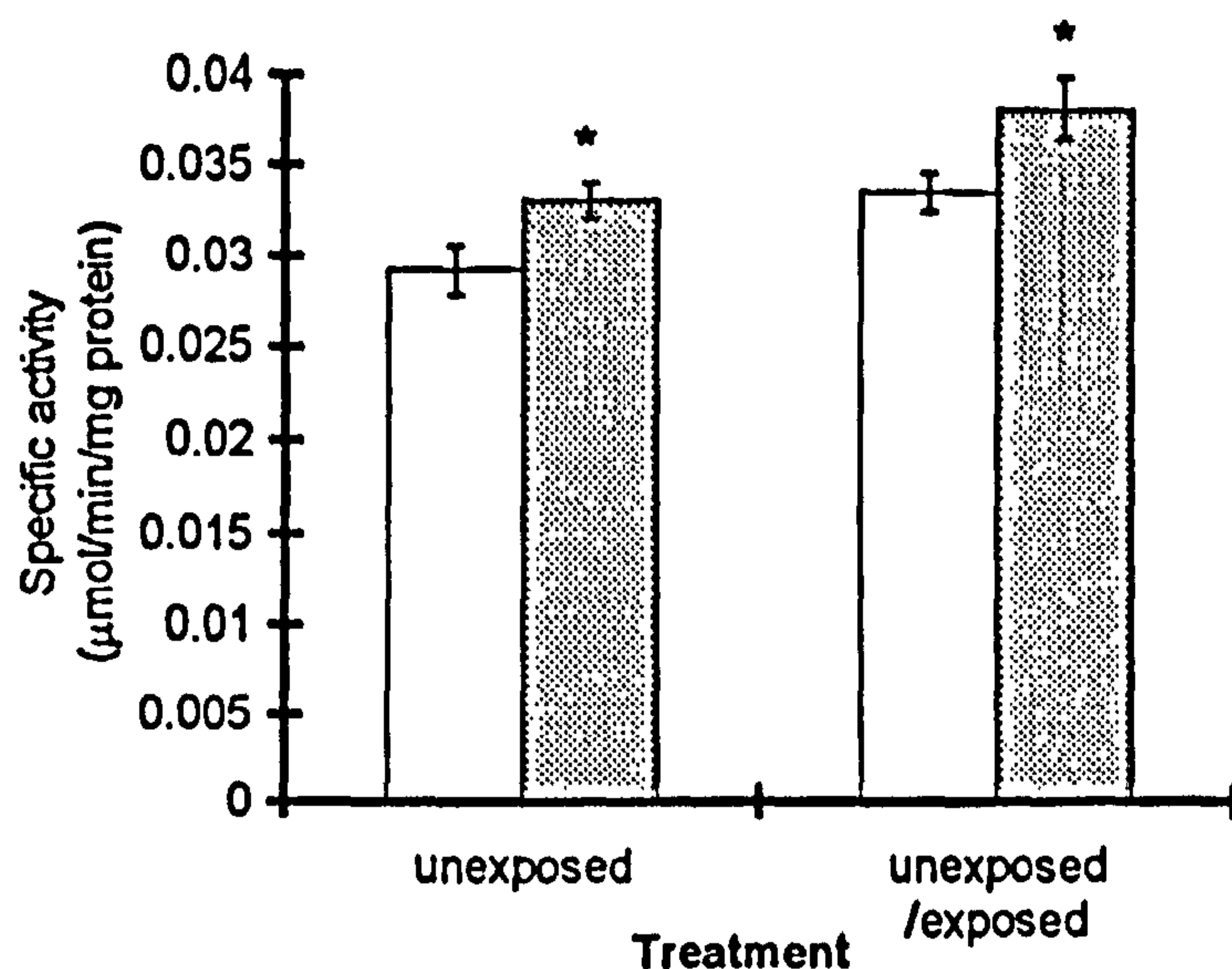


Figure 4.6. Specific GST activity of animals from Wabe non-contaminated site (open bars) and Ohebach contaminated site (solid bars). Animals were either held unexposed in clean filtered spring water for 42 days (unexposed) or held in spring water for 40 days and exposed to 1.0 μg/l lindane for a further 2 days (unexposed/exposed). Significant interpopulation differences in GST activity are indicated by an asterisk. Data are presented as mean values ± one standard error.

Interpopulation differences in specific GST activity were still apparent after 42 days maintenance in clean water in the laboratory (Figure 4.4, $t_{61} = 2.31$). Moreover, the relative difference in GST activity of animals from Wabe and Ohebach was maintained after 40 days in clean water followed by 48 hours exposure to 1 μg/l lindane ($t_{36} = 2.33$). On exposure to lindane, the specific GST activity of Wabe and Ohebach animals increased by 15 and 16 %, respectively.

Animals from both Wabe and Ohebach were maintained in 0.02 μg lindane/l for 40 days after which, a group of animals from each site were exposed to 1 μg/l lindane for two days. The remaining animals were kept in 0.02 μg/l lindane. Pre-exposure solutions were changed weekly and chemical analyses were performed on samples of the new test solution prior to use. Chemical analyses of exposure test solutions (i.e. 1 μg/l) was also performed.

Table 4.3. Chemical analysis of pre-exposure solutions. Solutions were analysed before exposure in test tanks. A single measurement was taken for each sample time.

Day	Ohebach animals pre-exposure	Wabe animals pre-exposure
	Lindane concentration µg/l	Lindane concentration µg/l
0	0.025	0.023
7	0.023	0.022
14	0.024	0.024
21	0.022	0.021
28	0.023	0.025
35	0.021	0.023

Table 4.4. Chemical analysis of test solutions for exposure of test animals. Solutions were analysed before exposure in test tanks.

New test solution	mean lindane concentration µg/l	standard deviation
Ohebach exposure	1.07	0.03
Wabe exposure	1.05	0.01

Chemical analysis demonstrated that pre-exposure and exposure test solutions were close to nominal concentrations (Table 4.3; 4.4).

After 42-day exposure to 0.02 µg/l lindane, specific GST activity in animals from both contaminated and non-contaminated populations was not significantly different (Figure 4.7, $t_{64} = 0.76$). However, after 40 days pre-exposure to 0.02 µg/l lindane and subsequent exposure to 1.0 µg/l lindane the GST activity of Wabe animals was significantly greater than the GST activity of Ohebach animals ($t_{62} = 2.51$). The specific GST activity of pre-exposed Wabe animals increased by 22 % on exposure to 1.0 µg/l lindane, this compared to a 4 % increase by Ohebach animals.

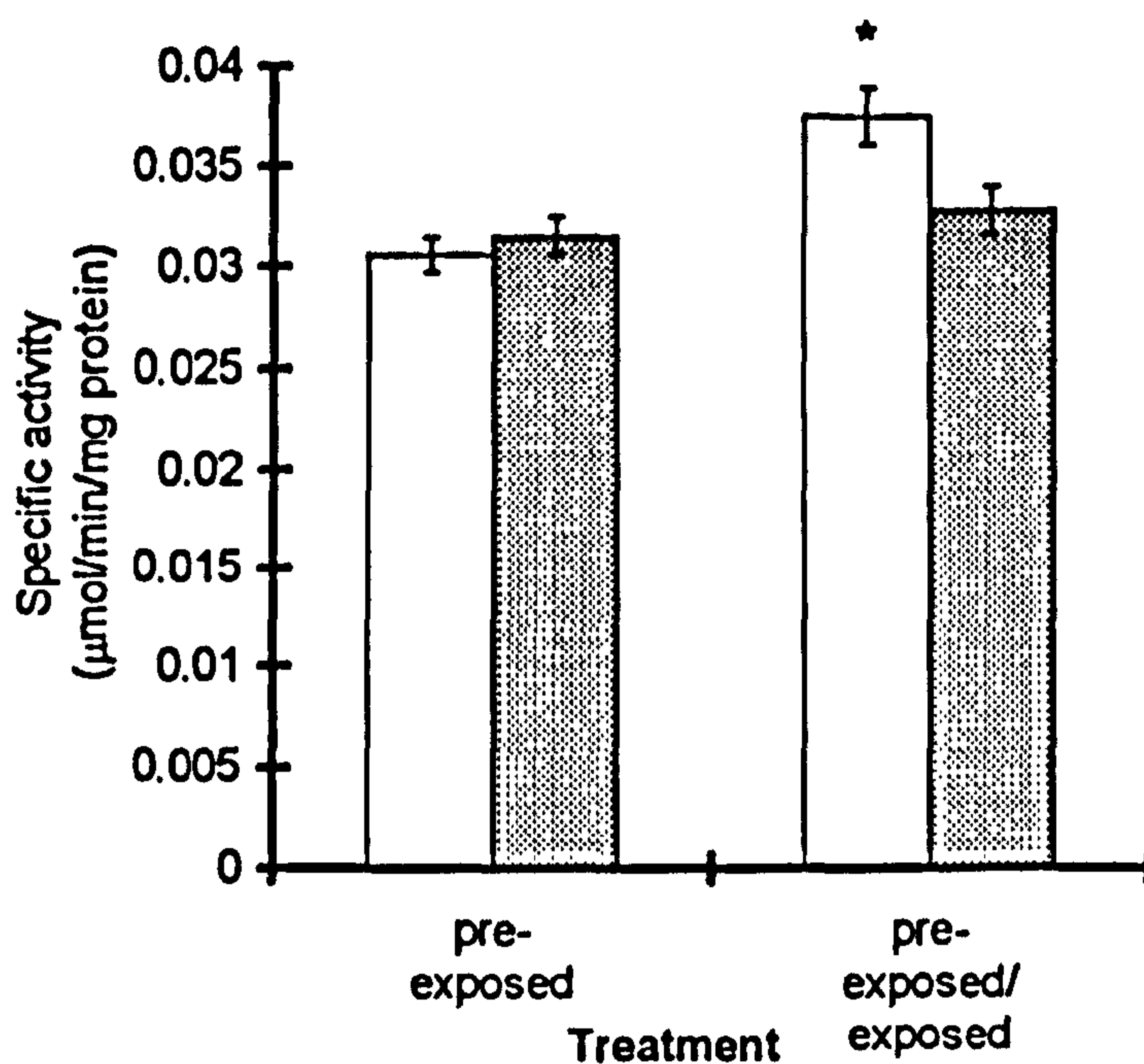


Figure 4.7. Specific GST activity of animals from the non-contaminated Wabe site (open bars) and the contaminated Ohebach site (solid bars). Pre-exposed animals were held for 42 days in 0.02 µg/l lindane. Pre-exposed/exposed animals were held in 0.02 µg/l lindane for 40 days and exposed to 1.0 µg/l for a further two days. Significant interpopulation differences are indicated with an asterisk. Data are presented as mean values, error bars are the standard error of the mean.

Analysis of all treatment groups shown in Figure 4.6 and 4.7 showed that there was significant differences between treatments ($F_{7,256} = 6.43$). However, there was no significant increase in GST activity between Wabe unexposed and Wabe pre-exposed animals ($q < 2.62$). Neither was there a significant increase in unexposed Ohebach animals on pre-exposure ($q < 2.62$). The further exposure of pre-exposed Wabe animals (pre-exposed/exposed) resulted in a significant increase in GST activity over pre-exposed and unexposed animals ($q > 2.62$).

4.4. DISCUSSION.

The main objective of the work described in this chapter was to investigate interpopulation differences in GST activity in *G. pulex* collected from pesticide contaminated and non-contaminated (reference) sites. It was predicted that the difference in GST activity would be due to long term exposure to low concentrations of pesticide in the field. Because pesticides such as lindane enter freshwaters via run-off it was predicted that GST activity would be greatest soon after pesticide application (late spring/early summer). Furthermore, it was predicted that interpopulation differences in GST activity would be lost on 'acclimation' to 'clean' or low concentration of pesticide (lindane) in the laboratory.

4.4.1 Interpopulation differences in GST activity.

It was clear there was a significant difference in GST specific activity between *Gammarus pulex* collected from Wabe and Lutterquelle and those collected from Ohebach and Brunsbuttelerbach (Figures 4.1 and 4.2). The Ohebach and Brunsbuttelerbach were contaminated with pesticides, whereas the Wabe and Lutterquelle were known to be free of pollution (section 4.2.1). Increased GST activity of *G. pulex* collected from the contaminated sites was therefore assumed to be the result of pesticide input from agricultural run-off.

The data presented in Figure 4.3 was from animals collected in October 1995. Pesticide loading would normally be low at this time of year as most pesticides detected are applied in spring. There was evidence of fenvalerate contamination of sediments in October (Table 4.2) and it is therefore possible that this was the cause of the increase in GST activity. The use of fenvalerate is recommended for insect pest control in winter cereal crops (*c.f.* crops at Ohebach catchment section 4.2.1), for which a spray in mid October and repeat spray at the end of October/early November is recommended (UK Pesticide Guide, 1997). Thus an increase concentration of fenvalerate in the Ohebach site in October is expected and was observed (Table 4.2).

Increased GST activity is known to be induced by organochlorine pesticides, but fenvalerate is a pyrethroid. Induction of GST activity in invertebrates exposed to non-organochlorine pesticides has been reported by other workers. For example, 72-hour exposure of fall armyworm (*Spodoptera frugiperda*) larvae to either 100 µg/g animal fresh weight of fenvalerate or cypermethrin (both pyrethroid insecticides) both resulted in significantly increased GST activity (Punzo, 1993). The organophosphate insecticide parathion was also detected in the Ohebach site (Table 4.2). Parathion has been reported to increase GST activity in the lacewing (*Micromus tasmaniae*) (Rumpf *et al.*, 1994). Thus pyrethroid and organophosphate insecticides and may also induce increased GST activity in *G. pulex*.

Episodic run-off events would have the effect of a pulse exposure of a higher pesticide concentration in the short term. The specific GST activity of *G. pulex* collected from Ohebach was maximum in March, a time when lindane was at its highest in the sediment following a run-off event. The next highest activity was in July when both parathion and fenvalerate were found in the water column and in October when fenvalerate was found in the sediment. The lowest activity in September was when there was least pesticide detected in the Ohebach (Table 4.2).

The elevated GST activity of *G. pulex* from the contaminated Ohebach site was sustained in July, September and October (Figure 4.4). The GST activity of *G. pulex* from the Wabe site also varied seasonally but was always less than that of the Ohebach animals. The same type of seasonal pattern in GST activity was observed in animals collected from Craggs Stream (Chapter Two, Figure 2.11). In all cases activity was greatest in spring and summer decreased in autumn and increased again towards the end of the year.

Short term exposure.

On exposure to lindane in the laboratory, animals from both Wabe and Ohebach had increased GST activity. However, Ohebach animals maintained a

significantly higher activity than Wabe animals (Figure 4.5). It appeared that animals subject to pesticide contamination for long periods were able to exhibit a greater increase in GST activity on exposure to a relatively high concentration of lindane (1 $\mu\text{g/l}$, that was 13% of the 96 hour LC_{50}) than animals not subject to long-term contamination. This response was in agreement with the prediction that population difference in GST activity would be sustained on further exposure to lindane in the laboratory. Increased GST activity on exposure to relatively high, but sublethal, concentrations of xenobiotic has been reported in animals previously exposed to low levels of xenobiotic (Hayaoka and Dauterman, 1982; Le Blanc and Cochrane, 1985). Moreover, the phenomenon of induction of increase detoxification enzyme due to pre-exposure is well documented in toxicology (Timbrell, 1991).

4.4.2 Effect on GST activity of pre-exposure to lindane.

Animals collected from both the Ohebach and Wabe sites were held in clean conditions in the laboratory for a period of six weeks and exposed to 1 $\mu\text{g/l}$ lindane for the final two days of treatment. By maintaining animals under clean conditions for 42 days significant interpopulation differences in GST activity were observed. The interpopulation difference in GST activity was maintained even after short-term exposure to 1 $\mu\text{g/l}$ lindane. Thus, although it was expected that GST activity in animals from the two sites would not be significantly different as GST activity levels would decrease to clean-site levels due to no induction by pesticides. After six weeks, the 'acclimation' to clean conditions appeared to have no effect on the difference between GST activity in animals from the two sites. It is possible that the acclimation period may not have been long enough to allow for GST activity to return to uncontaminated levels. This, however, seems unlikely as the rapid return of GST activity to pre-treatment levels after an initial dose has been reported in previous studies. For example, significantly increased GST activity in larvae of the lepidopteran insect *Spodoptera littoralis* was observed 8 hours after a single non-lethal dose of lindane. However, GST activity returned to control levels 32 hours post treatment (Lagadic *et al.*, 1993). It is possible that Ohebach *G. pulex* accumulated pesticide which was still

inducing GST activity. However, lindane appears to be rapidly eliminated in crustacean species. For example, 24 hours post-exposure, 40 % of initially bioaccumulated lindane was eliminated by the freshwater isopod, *Asellus aquaticus* (Thybaud and Le Bras, 1988). A similar pattern of elimination was observed in the freshwater cladoceran, *Daphnia magna*, with the organochlorine insecticide, chlordane (Moore *et al.*, 1977).

It is difficult to explain the maintenance of higher GST activity in animals from Ohebach compared with Wabe after 'acclimation' to clean conditions. Ohebach animals have a higher GST activity when there is no apparent inducer of activity suggesting a fixed higher baseline of activity. The maintenance of a permanently increased GST activity would suggest that there is no extra energetic cost associated with this increased enzyme production. However, the maintenance or possession of processes or genes in environments where they are not beneficial is often disadvantageous. For example, in the marine mussel *Mytilus edulis*, genetic variation at the leucine aminopeptidase (Lap) locus is associated with levels of environmental salinity (Koehn, 1978). The Lap⁹⁴ allele shows dominance for high enzyme activity, and genotypes with this enzyme accumulate free amino acids at a greater rate than other genotypes in a hyperosmotic solution. This counters the high salinities found in marine environments but causes wasteful nitrogen excretion at low salinity levels, resulting in a reduced growth rate and increased mortality (Hilbish and Koehn, 1985). In the present study, it is not known whether *G. pulex* from Ohebach encounter higher energetic costs or have reduced fitness under clean conditions. However, the energetic cost of long-term exposure of *G. pulex* to lindane is considered in Chapter Five.

It was predicted that exposure of animals to low 'environmental' concentrations of lindane in the long-term would result in similar activity in animals from both Wabe and Ohebach sites. This would be due to pre-exposure causing induction of GST activity in Wabe animals. Because these low levels are encountered by Ohebach animals in the field it was predicted that long-term exposure to low concentrations of lindane would result in no change in the GST activity of these

animals. Thus, animals from both sites would have similar GST activity after long-term exposure to 0.02 µg/l lindane in the laboratory. Animals from both Wabe and Ohebach, were pre-exposed to 0.02 µg/l lindane for six weeks in the laboratory. Pre-exposed animals from each site were then further exposed to 1 µg/l lindane for the final two days of treatment. After pre-exposure GST activity in animals from both sites was similar. However, on further exposure to 1 µg/l lindane, pre-exposed animals from the Wabe site had significantly higher GST activity than Ohebach animals subject to the same treatment. Pre-exposure had removed the differences in GST activity between animals from different sites and on further exposure had reversed the differences that were observed on exposure of unexposed and clean acclimated animals.

In the present study, although pre-exposure did not significantly increase the GST activity of animals from either site, it did remove the differences in GST activity between sites. Furthermore, pre-exposed animals from the Wabe site exhibited significantly higher GST activity on further exposure to 1 µg/l lindane. This may be indicative of tolerance to further exposure. Tolerance due to pre-exposure has been reported by other workers. For example, induced GST activity by pre-exposure to phenobarbital was observed to increase the tolerance of houseflies to pesticides (Hayaoka and Dauterman, 1982). Le Blanc and Cochrane (1985) reported that pre-exposure of *Daphnia magna* to CDNB and to pentachlorophenate (PCP) for 16 to 19 days resulted in elevated GST activity in the offspring of pre-exposed animals. GST activity was elevated by 25, 29 and 35 % among daphids pre-exposed to 75, 150 and 220 µg/l CDNB respectively. Moreover, exposed animals were more tolerant in terms of survival to further lethal exposure to PCP. As well as pre-exposure resulting in removal of differences in GST activity in animals between Wabe and Ohebach sites, *G. pulex* from the Ohebach site studied by Werner (1993) had higher median lethal exposure times (LT₅₀) on exposure to fenvalerate than animals from a clean site. Le Blanc and Cochrane (1985) concluded that the observed increase in tolerance and the associated increase in GST activity through PCP exposure, coupled with

the probable direct susceptibility of PCP to GST metabolism, suggests that GST may be a likely source of the tolerance.

4.4.3 Conclusions.

- **There were clear differences in GST activity of *G. pulex* from clean and contaminated sites; both for animals measured directly from the field and on further exposure of these animals to lindane in the laboratory.**
- **Higher relative GST activity in animals from the contaminated site, Ohebach, appeared to be the consequence of pre-exposure to pesticide in the laboratory.**
- **It is therefore hypothesised that higher GST in contaminated site animals is a result of prolonged pesticide exposure in the field.**
- **It should be noted that the higher GST activity of contaminated site animals compared with non-contaminated site animals was relative.**
- **GST activity was seen to fluctuate over the year in animals analysed directly from the field.**
- **Animals held in clean conditions did not show any lower GST activity than animals that were pre-exposed, although the consequences of pre-exposure to GST activity on further exposure were clearly different in animals from the different sites.**

CHAPTER FIVE

Physiological responses of *Gammarus pulex* to lindane exposure.

5.1. INTRODUCTION.

The work described in Chapter Three demonstrated that increase in GST activity was a sensitive and rapid marker of exposure to lindane and alcohol ethoxylate both in the laboratory and in semi-natural conditions. Moreover, GST activity was a marker of xenobiotic exposure in *Gammarus pulex* collected from pesticide contaminated sites (Chapter Four). It is therefore clear that GST activity can be useful as a biomarker of exposure to organic xenobiotics. What these studies do not indicate, however, is the relationship between GST response and toxicant-induced effects.

Exposure concentrations that are detrimental to organisms are not indicated by an increase in GST activity *per se*. Although, detoxification enzymes such as GST may be rapid to respond and sensitive to low concentrations of xenobiotic, they are not an indication of actual harm. Indeed, detoxification enzymes systems have been implicated as mechanisms of tolerance to potentially lethal xenobiotic exposure (Le Blanc and Cochrane, 1985) and the role of GST in insecticide resistance is well documented in insects (Oppenoorth, 1985). Thus, the levels of exposure that cause an increase in activity of GST may be of no consequence at the physiological and individual levels.

5.1.1 Levels of xenobiotic effect.

The effects of exposure to organic xenobiotics can be manifest at all levels of biological organisation (Figure 5.1). Changes at the biochemical level may give little information to predict changes that may affect the functioning of the organism at an individual level (e.g. growth and reproduction). However, an understanding of effects at the organism and population level may be gained by

linking changes in the biochemistry and physiology of an organism mechanistically to changes at the organism level. For instance, it has been suggested that toxicant-induced changes in physiological energetics can be linked to changes at the organism (i.e. growth and reproduction) and population (i.e. population size) level (Maltby and Calow, 1989).

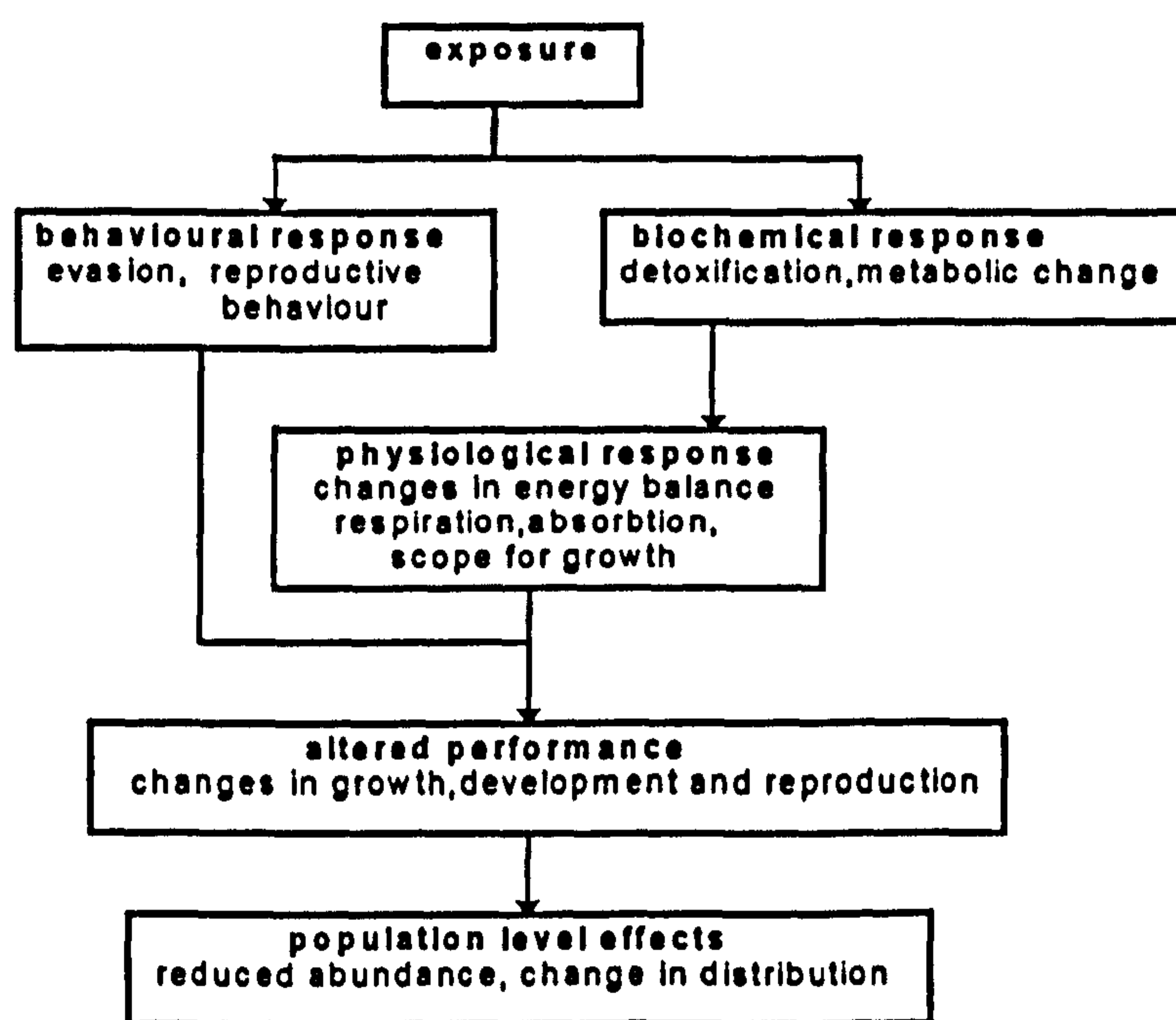


Figure 5.1. A flow diagram of effects that are expected to be induced by exposure to organic xenobiotics. After Maltby (1994).

In order to link exposure-induced changes in GST activity with changes in physiological energetics of *G. pulex*, the energetic cost of enzyme production would have to be estimated. This, however, would be difficult as the direct energetic cost of GST synthesis would have to be separated from the other energetic costs of metabolism. In contrast, measurement of decreases in the energy available for growth and reproduction at the same exposure levels, are relatively straight forward (Maltby *et al.*, 1990). In response to xenobiotic challenge there is an increase in the production of detoxification enzymes such as GST and other protective systems (e.g. heat shock proteins and other enzyme systems (Hayes and Wolf, 1988)) in attempt to prevent damage caused by xenobiotic exposure. Continued and increased exposure will exhaust the enzyme

turn, lead to tissue damage. Tissue damage will then have to be repaired by the organism, or consequently disease and death will follow. As toxicant exposure increases there is a progression from homeostasis to a compensation phase and then to non-compensation, with a consequent affect on the health of the organism (Hatch, 1962; Figure 1.4). The compensation phase, and return from non-compensation phase, will involve the production of protective proteins and detoxification enzyme systems and the repair of damaged tissue, respectively. All these processes will have an energetic cost to the organism that will increase energy demand. The resources acquired by an organism can be allocated to a number of processes (Figure 5.2).

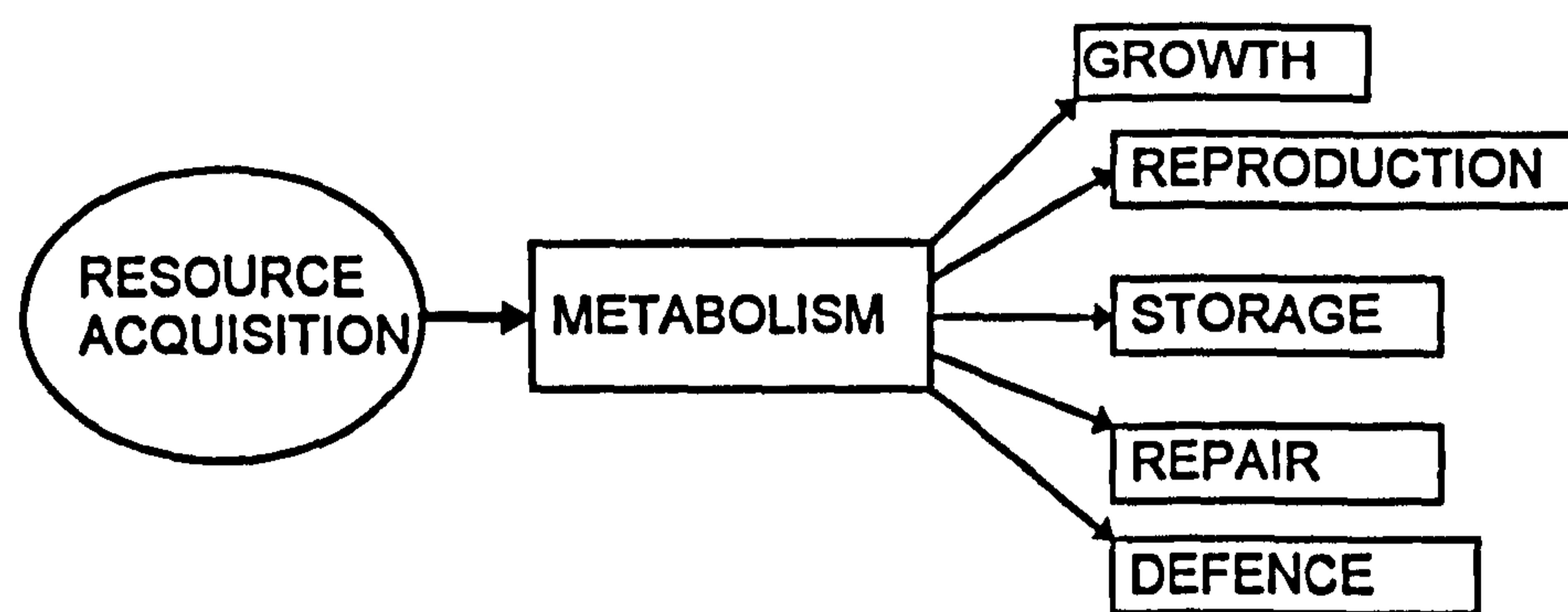


Figure 5.2. Resource allocation of acquired energy to biological processes. After Calow and Townsend (1981).

The physiological processes involved in counteracting xenobiotic exposure are active (e.g. transport against concentration gradients, cell division, protein synthesis) and require energy (ATP). For example, protein synthesis has been reported to comprise a major element of maintenance metabolism in the marine mussel *Mytilus edulis*, carrying a metabolic cost that was at least 20 % of the total energy expenditure (Hawkins *et al.*, 1986). Toxicant exposure will cause an increase in the energy demand of metabolism, defence (e.g. detoxification enzymes) and repair and therefore less energy will be available for the processes of growth, reproduction and storage. To maintain processes at a normal level of functioning (i.e. homeostasis) there must be an increase in the acquisition of energy (resources) to satisfy the increased energy expenditure. If resource energy is not available, there must be a trade-off between processes. Using the

above model of resource allocation, the increased cost of defence and repair are incorporated into the energetic 'cost' of toxicant exposure.

5.1.2. Scope for growth.

A measure of the energy available for production can be used as a measure of the individuals potential (or scope) for growth and/or reproduction. Scope for growth (SfG) is estimated by the measurement of energy gain by absorption of food material and energy loss via respiration and excretion (Warren and Davis, 1967). Scope for growth has been estimated in aquatic invertebrates, notably in the marine mussel *Mytilus edulis*, in both the laboratory (e.g. Widdows and Bayne, 1971) and in the field (e.g. Bayne and Widdows, 1978). The effect of natural and anthropogenic stressors on the SfG of *Mytilus edulis* has been extensively studied (Donkin and Widdows, 1986; Widdows *et al.*, 1987; Widdows and Page, 1993). Other invertebrates investigated include gastropod molluscs (Stickle *et al.*, 1984), echinoderms (Shirley and Stickle, 1982), cnidarians (Zamer and Shick, 1987) and copepods (Capuzzo, 1985). Most relevant to this present study is the SfG work on *G. pulex*. Changes in *G. pulex* SfG have been measured in response to exposure to oxygen and ammonia (Maltby *et al.*, 1990), metal ions (Maltby and Naylor, 1990, Stulbacher and Maltby, 1992), hydrogen ion concentration (Naylor *et al.*, 1989) and organics (Maltby *et al.*, 1990; Maltby, 1992). In *G. pulex* only the amount of energy gained by absorption and loss, via respiration is measured, energy loss via excretion is assumed to be minimal and relatively constant (Naylor *et al.*, 1989) and consequently is not measured.

It has been noted that in the majority of cases, exposure of *G. pulex* to toxic chemicals results in a reduction in energy intake before any change in energy expenditure (Maltby, 1994). Whether energy intake or energy expenditure is the most sensitive component may depend on the chemical to which the organism is exposed. Willows (1994) states that components of SfG may be differentially affected in *Mytilus edulis* depending on the type of chemical exposure. Many organic chemicals such as aromatic hydrocarbons have a narcotic effect, only affecting energy intake (i.e. feeding rate). In contrast, chemicals exhibiting a non-

specific metabolic uncoupling mode of action (e.g. pentachlorophenol) affect both feeding and respiration (Willows, 1994). Lindane is a known neurotoxin (Antunes-Madeira and Madeira, 1985), thus might be expected to affect energy intake. As well as its neurotoxic affects, lindane has been reported to function as a respiratory poison (Ulmann, 1972). Thus it might be expected for lindane to have an effect both energy absorption and energy loss via respiration. *G. pulex* feeding rate (Maltby, 1992) has been used to assess the effect of xenobiotic exposure to pesticides (Matthiessen *et al.*, 1995 and Malbouisson *et al.*, 1995), metals (Roddie *et al.*, 1992) and agricultural slurry (Veerasingham and Crane, 1992). Whereas oxygen consumption has been used as a measure physiological change in response to lindane exposure in marine shrimp larvae, *Penaeus vannamei* (Galindo *et al.*, 1996) and the freshwater isopod, *Asellus aquaticus* (Le Bras, 1987).

5.1.3 Stored energy.

If exposure to xenobiotics increases energy expenditure and/or decreases energy intake, then one consequence may be a decrease in stored energy. The amount of stored energy has previously been used as a measure of the energetic status of the animals. Work on insects has demonstrated that the carbohydrate, glycogen, is the typical storage chemical; lipids being used for longer term storage (Beenackers, 1969; Van Handel, 1974; Lee *et al.*, 1975; Downer and Matthews, 1976). Glycogen has also been used as an estimate of energy reserves in the freshwater isopod *Lirceus fontinalis*. Sparkes *et al.* (1996) reported that the glycogen concentration of the isopod was reduced in response to increased activity associated with male mate-guarding behaviour and short-term (2 -3 days) food deprivation. Exposure of crustaceans to xenobiotics has been shown to reduce the concentration and synthesis of glycogen. Glycogen concentration in woodlice (*Porcellio scaber*) from a site polluted with cadmium and zinc was significantly lower than that of reference (uncontaminated) site animals (Donker, 1992). Seven-day exposure to sublethal lindane concentrations significantly decreased the rate of glycogen synthesis in shrimp (*Penaeus vannamei*) larvae (Galindo *et al.*, 1996). Depleted glycogen concentrations may be replenished

rapidly, for instance, *Lirceus fontinalis* fully replenished glycogen reserves within 36 hours of depletion to 50 % of total reserves after cessation of increased energy expending behaviour, due to mate-guarding (Sparkes *et al.*, 1996).

5.1.4 Effects of short-term and long-term exposure.

The measurement of SfG and energy reserves (i.e. glycogen) may enable the assessment of the energetic cost of exposure to a xenobiotic. Changes in energy intake and expenditure are considered in response to short-term exposure to xenobiotics. A decrease in available energy may lead to a reduced SfG in the short-term, but extended exposure may lead to lethal effects through the lack of energy available to maintain normal functioning or direct toxic effect on the organism. However, long-term exposure to lower exposure concentrations may lead to an increased energetic cost of the maintenance of normal metabolic functioning which may be compensated for by increased energy intake. An assessment of the energetic cost of long-term exposure can be made by measuring energy intake, expenditure and energy reserves. The energy available to counter further increased exposure in the short-term may be affected by previous exposure. Animals that counter the detrimental effects of a stress (here, xenobiotic exposure) with partial or complete recovery are considered to show acclimation, in which case the animal has re-established homeostasis and therefore acquired increased stress resistance (Hoffman and Parsons, 1991). Measurements of SfG and glycogen reserves after extended periods of exposure to sublethal levels of organic xenobiotic may elucidate the ability of *G. pulex* to acclimate to organic xenobiotic contamination. If this is the case the energetic cost of exposure will be reflected in an increase in respiratory energy loss, but this would be counteracted by an increase in energy absorption.

5.1.5 Objectives.

The objective of the work described in this chapter was to measure the effect of lindane exposure on the uptake and loss of energy by *G. pulex*. Measurement of energy intake, via consumption and absorption of food material and energy lost via respiration allowed assessment of the changes in the acquisition and

expenditure of energy. Furthermore, the effect of short-term and long-term exposure to lindane was to be assessed by estimation of energy loss, uptake and the concentration of energy reserves.

It was hypothesised that lindane exposure causes an increase in the production of protective proteins and an increase in tissue damage. More energy would therefore be required for maintenance of normal functioning and repair.

Furthermore, the narcotic affect of lindane exposure would decrease feeding rate with increasing concentration. Thus, as a consequence of the decrease in energy acquisition and the increase in energy expenditure, energy stores will be depleted. The specific predictions tested were that lindane exposure would result in a :

1. decrease in feeding rate
2. decrease in energy absorbed
3. increase in respiration rate
4. decrease in glycogen concentration.

On long-term exposure to low 'environmental' concentrations of lindane the hypothesis was similar to that stated above except that it was hypothesised that potential tissue damage from exposure to the low concentrations of lindane would be countered by protective proteins and enzyme systems. Thus, the increase in energy expenditure would be due to protective protein production, with-out the added energetic cost of repair and therefore not as great as on exposure to a higher concentration in the short-term. Decrease in absorbance due to narcotic effect would be not as great as for exposure to a higher concentration short-term exposure. Thus, the predictions made for long-term exposure were qualitatively similar to those for short-term exposure but quantitatively different. It was predicted that long-term exposure to a low 'environmental' concentration of lindane would increase respiration rate and decrease absorption, but the respective energy loss and gain would be less than on short-term exposure.

5.2. MATERIALS AND METHODS.

Large (length 10 to 15 mm) male *Gammarus pulex* were collected from Craggs stream and acclimated to laboratory conditions (section 2.2.2) for one week prior to use in experiments. Lindane test solutions (0.02, 0.2, 1.0, 3 and 10 µg/l) were made up from stock solutions as needed (section 3.2.3). All experiments were conducted under laboratory conditions of 15 °C (±1 °C) and 12/12 hours light/dark, in artificial pond water (APW, Appendix 1.2) unless otherwise stated.

5.2.1. Short-term lindane exposure.

Energy intake.

Large male *G. pulex* were exposed to 0.2, 1.0, 3 and 10 µg/l lindane in individual glass holding tubes (section 2.2.2, Figure 2.1). There were three replicates per treatment and 12 animals per replicate. Holding tubes were fitted with a fine (0.2 mm) mesh base and contained a 1-mm mesh bottomed polythene inner tube. Animals and food were retained in the inner tube, faecal material would pass through the 1 mm mesh and be retained by the 0.2 mm mesh. Each animal was provided with five conditioned 12-mm diameter alder leaf discs (Appendix 1.3) that had been pre-weighed and rehydrated in the appropriate test solution for 2 days. Five control leaf discs (i.e. no animal) were placed in each replicate tank, in the control and 10 µg/l treatments.

After 96, 168 and 336 hours exposure, twelve animals and associated leaf material were removed from each treatment (four from each replicate). One set of five control leaves were removed at each time interval to check for weight loss due to microbial breakdown and leaching of soluble organics. Animals removed at 168 hours were placed into respirometry chambers (see below). All other animals and all leaf material was dried to a constant weight at 60 °C in a drying oven and weighed on a Mettler ME30 balance (accuracy ± 0.0001 mg). Faecal material from 168-hour exposed animals was collected onto preweighed filter paper (Whatman 5.5 cm). Faeces-free water was passed through five filter papers to control for weight change due to processing. Filter papers were dried to a

constant weight at 60 °C in a drying oven and weighed on a Mettler ME30 balance (accuracy ± 0.0001 mg).

The consumption of each individual (C , J/mg dry weight animal/d) was calculated by determining the dry weight of leaf material eaten and converting to energy using a joule equivalent (Equation 5.1).

$$C = \frac{[(L_1 \times C_1) - L_2] \times E_1}{W \times t} \quad \text{Equation 5.1.}$$

Where L_1 and L_2 are the initial and final dry leaf material weights (mg) respectively, E_1 is the energy equivalent of the food determined by bomb calorimetry (i.e. 21.551 J/mg, Naylor *et al.*, 1989), W is the dry weight (mg) of the animal and t is the exposure time in days. C_1 is a correction factor to account for weight loss from control leaves and was determined using Equation 5.2.

$$C_1 = \frac{\sum (A_2 / A_1)}{5} \quad \text{Equation 5.2.}$$

Where A_1 is the initial leaf material dry weight (mg) and A_2 is the leaf material dry weight (mg) after treatment.

The amount of energy lost in faeces in Joules per mg dry weight per animal per day (F) was calculated using Equation 5.3.

$$F = \frac{[F_2 - (F_1 \times C_f)] \times E_f}{W \times t} \quad \text{Equation 5.3.}$$

Where F_1 and F_2 are the initial and final weight (mg) of experimental filter papers and E_f is the energy equivalent of the faeces obtained by bomb calorimetry (18.737 J/mg Naylor *et al.*, 1989).

Weight change due to filter processing (C_f) was calculated using Equation 5.4.

$$C_f = \frac{\sum (B_2 / B_1)}{5} \quad \text{Equation 5.4.}$$

Where B_1 and B_2 are the initial and final weights of control filter weights respectively.

The amount of energy absorbed (A) in joules per mg dry weight animal per day was given by the difference between C and F .

Measurement of respired energy.

Animals removed from the feeding chambers after 168 hours exposure were placed into a flow-through respirometer (Figure 5.1, Wrona and Davies, 1984). Glass reservoirs with a compressed air supply were filled with 10 l of test solutions with nominal concentrations of 0, 0.2, 1.0, 3.0 and 10 $\mu\text{g/l}$ lindane. The reservoir was connected to a glass distribution chamber with a silicon rubber tube (internal diameter 5 mm). Water flowed from the distribution chamber to respirometry chambers (2-ml glass syringes) via 0.38 mm internal diameter PVC tubes (Watson-Marlow). Each syringe was capped with a hypodermic needle (40 mm, 0.8 mm bore, Becton Dickinson) with a small length of PVC (0.38 mm bore) tubing on the end. Water was pumped through the system using a Watson-Marlow (202U) peristaltic pump set at 50 rpm. The flow rate through each chamber was determined by measuring the time taken for 50 μl to flow out by attaching a "microlitre" (Hamilton TM) syringe with the plunger removed to the end of the chambers. This procedure was repeated twice and the average flow rate calculated. There were twelve experimental respirometry chambers, each containing a single animal, and one control chamber (no animal) per run. Animals were not fed and were left in the respirometry chambers for a period of 15 hours prior to measurement to allow them to acclimate to test conditions (15 $^{\circ}\text{C} \pm 1^{\circ}\text{C}$, 12/12 hours light/dark).

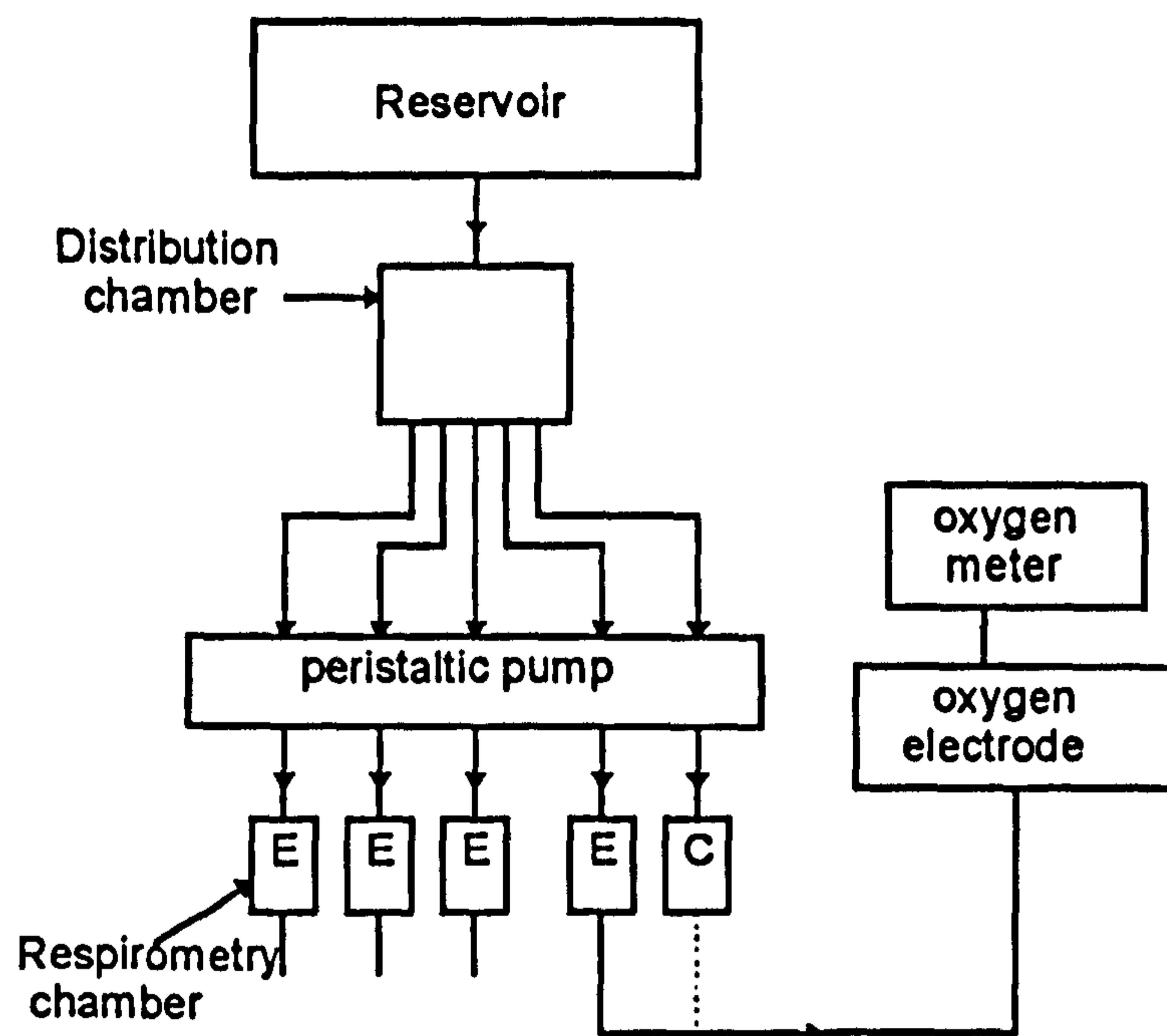


Figure 5.3. Respirometer experimental equipment. Test solution flows from a reservoir to a distribution chamber from which it is pumped via a peristaltic pump to up to 16 respirometry chambers (only five shown). The 2-ml respiratory chambers (glass syringes) either housed an individual *Gammarus pulex* (E) or contained no animal and was used as a control (C).

A sample of water was taken from each chamber by allowing water to flow in to an attached 50- μ l Hamilton syringe. The sample from each respiratory chamber was then injected into a Radiometer oxygen electrode (for calibration and zeroing procedures see Appendix 3.1). This procedure was repeated to ensure only sample water was in the electrode chamber. The electrode was allowed to equilibrate for 2 minutes before percentage oxygen saturation was read from a Strathkelvin Instruments (Model 781) oxygen meter. For each experimental chamber reading, a control chamber reading was also taken to determine the oxygen content of water entering the chamber. Each chamber was sampled twice and an average was calculated. After each sample, the electrode chamber was filled with 100 % oxygen saturated APW and the meter reading checked and adjusted to 100 % saturation if necessary.

The atmospheric pressure was read from a barometer (Casella, standard conditions $g = 980.665 \text{ cm/s}^2$, Temperature 0°C) on the day of measurement and the water vapour pressure at that pressure (at 15°C) was taken from Weast, Selby and Hodgman (1964). The partial pressure in Torr of 100 % saturation of oxygen in test solution (PO_2) was calculated using Equation 5.5

$$PO_2 = 0.2095 \times (Pa - Pw) \quad \text{Equation 5.5.}$$

where 0.2095 is the proportion of the atmosphere that is oxygen (20.95 %), Pa is the atmospheric pressure in Torr and Pw is the water vapour pressure in Torr. The oxygen partial pressure in Torr of sample solution entering (PEO_2) and leaving (PLO_2) the chambers was calculated using Equation 5.6

$$PEO_2 = \frac{\%O_{2c}}{100} \times PO_2$$

$$\text{or, } PLO_2 = \frac{\%O_{2e}}{100} \times PO_2 \quad \text{Equation 5.6.}$$

where $\% O_{2c}$ is the percentage oxygen saturation of water leaving the control chamber and $\% O_{2e}$ is the percentage oxygen saturation of water leaving the experimental chamber.

The weight-specific oxygen uptake of each animal (MO_2 , $\mu\text{mol/mg/h}$) was given by Equation 5.7.

$$MO_2 = \frac{(PEO_2 - PLO_2) \times AO_2 \times Fl}{W} \quad \text{Equation 5.7.}$$

where AO_2 is the solubility coefficient of oxygen in water at 15°C (i.e. $2.01 \mu\text{mol/l/Torr}$) and Fl is the flow rate of water through the chambers (l/h). Oxygen uptake was corrected from an hourly rate to a rate per day ($\times 24$), and from μmol to litre ($\times 22.41 \times 10^{-6}$). The conversion of oxygen consumption to

energy assumes that all respiration is oxidative and that there is consequently a direct relationship between oxygen consumption and heat loss. The oxyjoule equivalent varies with the substrate being metabolised and for carbohydrate it is 21×10^3 J/l O₂ (Elliott and Davison, 1975). Energy respired per mg animal dry weight per day (*R*) was calculated using Equation 5.8.

$$R = MO_2 \times 24 \times (22.41 \times 10^{-6}) \times (21 \times 10^3) \quad \text{Equation 5.8.}$$

Scope for growth.

Scope for growth (SfG) measurements were calculated as the difference between energy absorbed from food estimated from the energy consumed as food (*C*) and egested as faeces (*F*) and the energy expended in metabolism measured as respiration (*R*) (Equation 5.9).

$$C - F - R = \text{SfG} \quad \text{Equation 5.9.}$$

5.2.2. Long-term lindane exposure experiments.

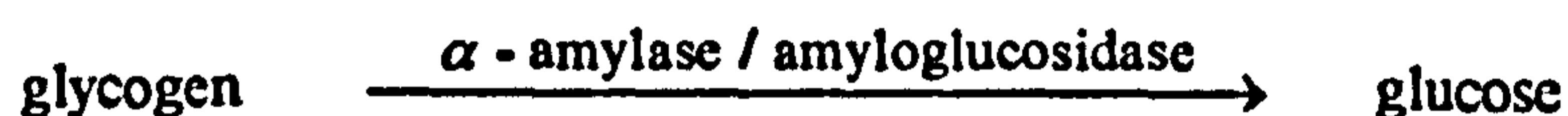
Large male *G. pulex* were maintained at 15 °C (±1°C) under 12/12 hours light/dark for 35 days in either APW (control) or APW containing 0.02 µg/l lindane (pre-exposed). Animals were held in individual holding tubes, thirty in APW and a further thirty in lindane solution. For each treatment there were three replicate 3-l tanks each holding ten animals. Solutions were replaced weekly, old solution being analysed for lindane (section 3.3.1). All animals were fed *ad libitum* on conditioned alder leaves (Appendix 1.3.).

After 35 days exposure animals were transferred to two-chambered holding tubes to enable faecal material to be collected (section 5.2.1). Fifteen animals from each treatment were placed into three, 3-l tanks containing a nominal concentration of 1 µg/l lindane (“exposed”) for a further seven days exposure. The other 15 (5 animals per tank) were left in their original solution (i.e. APW or 0.02 µg/l lindane). During the final 7 days of exposure each animal was provided with five pre-weighed conditioned alder leaf discs (Appendix 1.3), re-hydrated in the

appropriate test solution for 48 hours. After 7 days, leaf material and faecal material was collected, dried and weighed (section 5.2.1). The oxygen uptake of twelve animals per treatment was measured using the methods described in section 5.2.1. All animals were dried and weighed after respirometry measurements were completed. Energy consumed (C), egested (F), absorbed (A) and respired (R) were calculated using equations 5.1 to 5.8. Scope for growth was calculated using Equation 5.9.

5.2.3. Effect of lindane exposure on glycogen concentration.

Glycogen was estimated in *Gammarus pulex* using an enzymatic method adapted from Stitt *et al.* (1978) utilising amyloglucosidase and α -amylase to catalyse the breakdown of glycogen polymer molecules into glucose.



Hexokinase catalyses the phosphorylation of glucose with ATP to glucose -6-phosphate.



Glucose is then measured by absorbance increase at 340 nm in an assay with glucose-6-phosphate dehydrogenase with NAD^+ .



Exposure to lindane.

Animals were exposed for 168 hours to nominal concentrations of 0, 0.2, 1, 3 and 10 $\mu\text{g/l}$ lindane using the methods described in section 5.2.1. There were five animals per treatment. At the end of the exposure period animals were blotted dry, weighed (Mettler ME 30; accuracy ± 0.001 mg) and immediately frozen in liquid nitrogen and stored at -70°C (section 2.2.3). Animals were then processed and assayed for glycogen (see below).

Animals exposed in the long term exposure experiment (section 5.2.4) were also analysed for glycogen concentration in the same way. The animals analysed for glycogen were those that had been used for respirometry. Ten dried animals from each treatment (control, acclimated to 0.02 µg/l lindane, control/exposed to 1 µg/l lindane and acclimated /exposed to 1 µg/l lindane) were analysed. Each sample consisted of two animals.

Preparation of enzymes, reagents and standards.

All enzymes (amyloglucosidase, α -amylase, hexokinase and glucose-6-phosphate dehydrogenase) were obtained from Boehringer and kept in cold storage (4°C) until use. Adenosine triphosphate (ATP) and nicotinimide adenine dinucleotide (NAD) were obtained from Sigma and kept at -20°C until use. Glucose (D-glucose) and glycogen (type II from oyster) were obtained from Sigma as was HEPES (N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid]) buffer. Sodium acetate and magnesium chloride were obtained from BDH Ltd.

Forty microlitres of α -amylase (10 U/ml) were centrifuged for 2 minutes at 14,000 rpm. The supernatant was discarded and the pellet resuspended in 1 ml 100 mM sodium acetate buffer at pH 7.4. A quantity of 100 mg of amyloglucosidase was weighed out on a Mettler AJ100 balance (accuracy \pm 0.1 mg) and added to 9 ml of sodium acetate buffer at pH 7.4. The α -amylase and amyloglucosidase solutions were mixed using a "Whirlymix" mixer. The resulting mixture provided enough enzyme solution for 100 samples.

For the glucose assay, 0.0404 g ATP and 0.0265 g NAD were weighed out (Mettler AJ100 balance, accuracy \pm 0.1mg) and added to 1 ml 100 mM HEPES buffer with 5 mM magnesium chloride (pH 7) to give a 100 mM solution of each. Enzymes were prepared by centrifuging 50 µl glucose-6-phosphate dehydrogenase was centrifuged at 14,000 rpm for 2 minutes and resuspending the pellet in 1 ml 100 mM HEPES/ 5 mM MgCl₂ buffer. Hexokinase was prepared by centrifuging 30 µl of hexokinase at 14, 000 rpm for 2 minutes and resuspending the pellet in 1 ml 100 mM HEPES/ 5 mM MgCl₂ buffer.

Glucose and glycogen standards were prepared as follows. A 0.432 g sample of glucose was weighed out and dissolved in 10 ml of 100 mM sodium acetate buffer (pH 4.7) to give a 0.24 M solution. This was further diluted to a 0.08 M solution with 100 mM sodium acetate buffer (pH 4.7). Serial dilutions were prepared to give 4 (1:20), 2 (1:40), 1 (1:80), 0.5 (1:160), 0.25 (1:320), 0.125 (1:640) and 0.0625 (1:1280) mM solutions. Glycogen standards were prepared by weighing out 0.05 g glycogen and mixing with 10 ml, 100 mM sodium acetate buffer. A serial dilution was made with buffer to give 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 dilutions, giving 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078 mg/ml solutions respectively. Each glycogen standard was divided into four 400- μ l samples and placed in polythene Eppendorf tubes which were then autoclaved for 40 minutes. Glycogen standards were cooled and 100 μ l α -amylase/amyloglucosidase enzyme mixture added and incubated for three hours at 37 °C. Standards were then centrifuged (14,000 rpm for 5 minutes) and the supernatant used in the assay.

Preparation of animal material.

Individual *G. pulex* (whole body) were homogenised for two minutes in 1 ml of 50 mM HEPES (N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid]) buffer with 5 mM magnesium chloride in an 80 % solution of ethanol at pH 7.0. The pH was checked with a calibrated Corning pH (general purpose) probe and Corning 140 pH meter and adjusted if necessary with a 10 % potassium hydroxide solution. The homogenised sample was decanted into a 1.5-ml screw-cap polythene "Eppendorf" tube and placed in a Teche DB-3 heating block at 75 °C for 30 minutes. Samples were then centrifuged in an Eppendorf model 5417C centrifuge at 14,000 rpm for 5 minutes. The supernatant was removed and labelled and the pellet resuspended in 1 ml of 50 mM HEPES buffer with 5 mM magnesium chloride in an 80 % solution of ethanol at a pH of 7.0. The sample was heated (30 minutes at 75 °C), centrifuged (5 minutes at 14,000 rpm) and the supernatant removed and labelled. The pellet was resuspended in 400 μ l of 100 mM sodium acetate buffer (pH 4.7) and autoclaved at 2 bar at 120 °C for 40 minutes. The sample was left to cool to room temperature. A volume of 100 μ l

of α -amylase/amyloglucosidase enzyme mixture was added to the sample. The sample was then incubated at 37 °C in a water bath for 3 hours, after which the sample was then centrifuged at 14, 000 rpm for five minutes and the supernatant was removed for glucose analysis of insoluble carbohydrate (see below).

Labelled supernatants were pooled for each sample and placed in a clean glass test-tube. The test-tube was in a vacuum oven at 80 °C for 12 hours after which the residue was rehydrated in 400 μ l sodium acetate buffer. A 10 μ l sample was analysed directly for free glucose (see below). A volume of 100 μ l α -amylase/amyloglucosidase mixture was added and the sample was incubated at 37 °C for three hours. The sample was then centrifuged (14,000 rpm for 5 minutes) and the supernatant was removed for analysis of soluble carbohydrate (see below).

Glycogen assay.

Two hundred microlitres of 100 mM HEPES/ 5 mM MgCl₂ was added to each well of a 96 well microplate (Falcon Microtest III), with an 8 channel automatic pipette (Anachem). Ten microlitres of NAD, 10 μ l ATP and 10 μ l of glucose-6-phosphate dehydrogenase solutions was then added to each well with an Eppendorf multi-pipette. Finally 10 μ l of sample or standard was added to each well, each standard or sample being run in triplicate. Each microplate had glucose and glycogen standards. The microplate assay was run in an Anthos HTII microplate reader at a wavelength of 340 nm with a reference wavelength of 405 nm. An initial reading was taken and 10 μ l of hexokinase enzyme solution was added to each well using an Eppendorf multipipette. The microplate was left to incubate at room temperature for 30 minutes before being read three times at 1 minute intervals. This procedure was repeated after 30 minutes to check that the enzymatic reaction was completed. Absorption was given as a readout in optical density units (OD), initial OD (OD_i) subtracted from final OD (OD_f) gave the change in OD (Δ OD). The Δ OD was plotted against the mM concentration of glucose in the standards the resulting linear regression being used to calculate the glucose concentration (mM) of samples assayed on the same microplate. The amount of glucose (g) in the sample assay volume was calculated using the

molecular weight of glucose (i.e. 180.16). The amount of glucose in the sample was calculated from the total assay volume in the assay (i.e. 240 µl) and the volume sample used in the assay (10 µl). The amount of glucose in glycogen standards was calculated in the same way. The amount of glucose in glycogen standards calculated from assay, was then plotted against the actual amount of glycogen in standards. A regression of this plot allowed the calculation of the amount of glycogen in samples from the amount of glucose in the assay. The glycogen in soluble and insoluble fractions was combined to give the total glycogen for the samples (Equation 5.10).

$$\text{glycogen}_{\text{total}} = \text{glycogen}_{\text{soluble}} + \text{glycogen}_{\text{insoluble}} \quad \text{Equation 5.10}$$

The effect of using dried animals on glycogen content was determined by measuring the glycogen content of 20 large male animals maintained under laboratory conditions (section 2.2.2) for 1 week and fed *ad libitum* with conditioned alder leaves (Appendix 1.3). Ten animals were immediately frozen in liquid nitrogen and stored at -70 °C for one week the other group was dried at 60 °C for 48 hours and kept in a desiccator at room temperature for the remaining 120 hours. Animals were individually analysed for glycogen as stated above.

5.2.4. Statistical analysis.

Results of physiological measurements; feeding rate, absorption, respiration and scope for growth were analysed using statistical analyses performed using the statistical software package Minitab™, version 9.2 for Windows. All data were checked for a normality using normal probability plots and Anderson-Darling normality tests. Oneway analysis of variance (ANOVA) tests were used to compare data for feeding rate energy absorption and respiration energy loss and SfG values. Estimates of the lindane concentration which inhibited consumption, respiration and absorption by 50 % (i.e. IC₅₀) were calculated using the ICPIN update of the “Bootstrap” computer software written by US Environment Agency (1993). Significance was at p = 0.05 unless otherwise stated.

5.3. RESULTS.

5.3.1 Short -term lindane exposure.

Animals were exposed to nominal concentrations of 0, 0.2, 1.0, 3.0 and 10 $\mu\text{g/l}$ lindane. After one week exposure, average measured lindane concentrations were between 20 and 30 % of nominal values (Table 5.1).

Table 5.1. Lindane actual and nominal concentrations from short term exposure experiment, as analysed by GC/ECD. Actual concentrations are presented as the mean of six samples.

Nominal $\mu\text{g/l}$ lindane	Actual $\mu\text{g/l}$ lindane	standard deviation
0	<0.001	-
0.2	0.066	0.002
1.0	0.31	0.005
3.0	0.62	0.01
10.0	2.03	0.1

Short term (i.e. 96 hour) exposure to an actual concentration of 0.62 $\mu\text{g/l}$ lindane resulted in a significant reduction in food consumption of *G. pulex* compared with control ($F_{4,59} = 6.15$; Dunnett's, $q > 2.51$) (Figure 5.4).

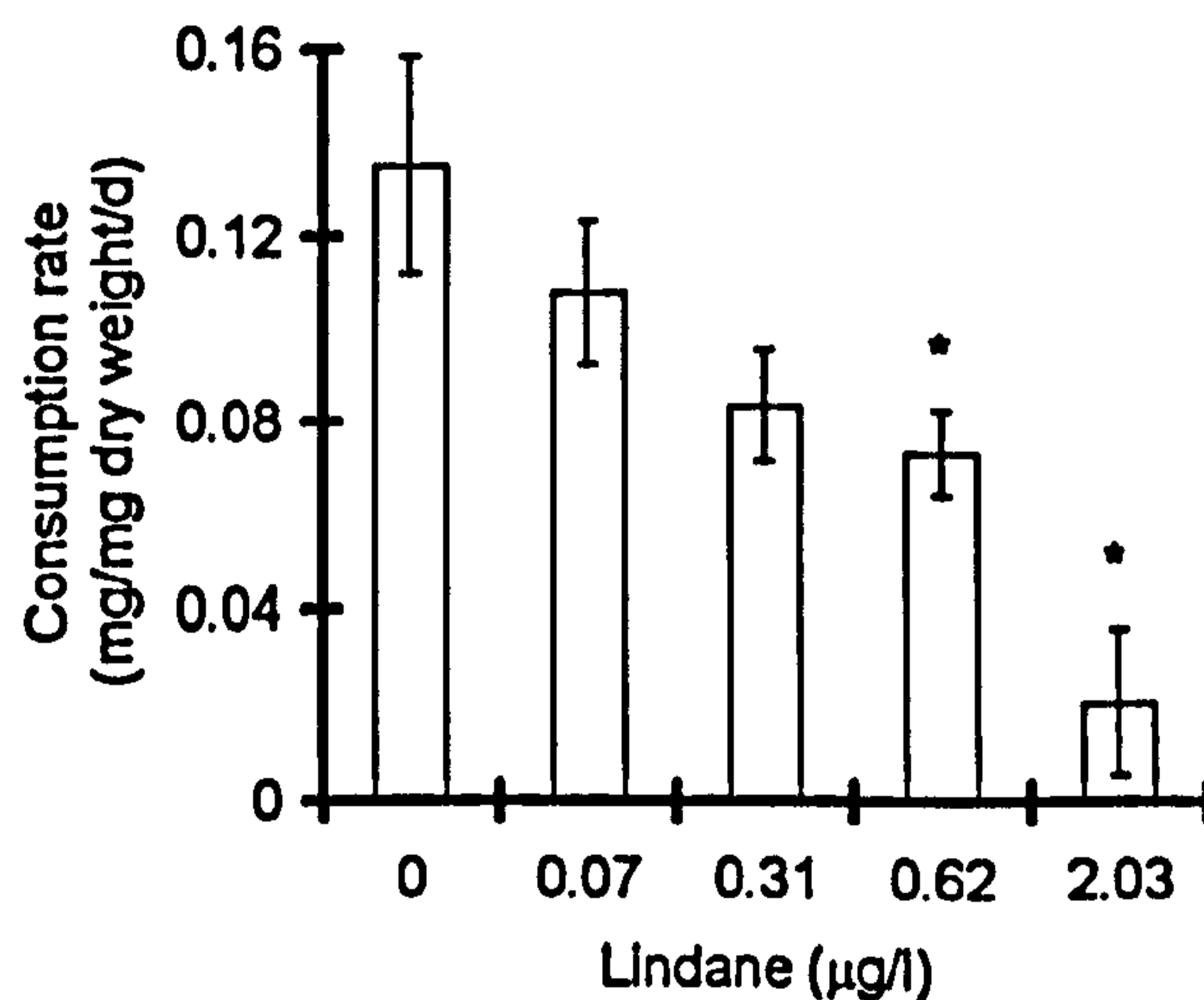


Figure 5.4. Effect of 96-hour lindane exposure on consumption rate. Actual concentration of lindane are shown. Error shown is standard error of the mean. Significant difference from control is indicated with asterisks.

The effect of lindane on feeding rate (consumption) in terms of the median inhibition concentration at 96, 168 and 336 hours exposure is shown in Table 5.2.

Table 5.2. The concentration of lindane resulting in a 50 % inhibition of food consumption by *G. pulex* exposed for 96, 168 or 336 hours.

Exposure time hours	IC ₅₀ µg/l lindane	95 % confidence limits	
		lower	upper
96	1.11	0.28	1.59
168	1.24	0.74	1.64
336	0.20	0.06	0.39

A 50 % reduction in feeding (IC₅₀) was caused by 96-hour exposure to 1.11 µg/l lindane or 168-hour exposure to 1.24 µg/l. The IC₅₀ at 336 hours was six times lower than the 168-hour value. This reduction in energy consumption was reflected in a significant decrease in *A* with increasing lindane concentration (Figure 5.3). Energy loss due to respiration (*R*) measured on animals exposed for 168 hours is also shown in Figure 5.5.

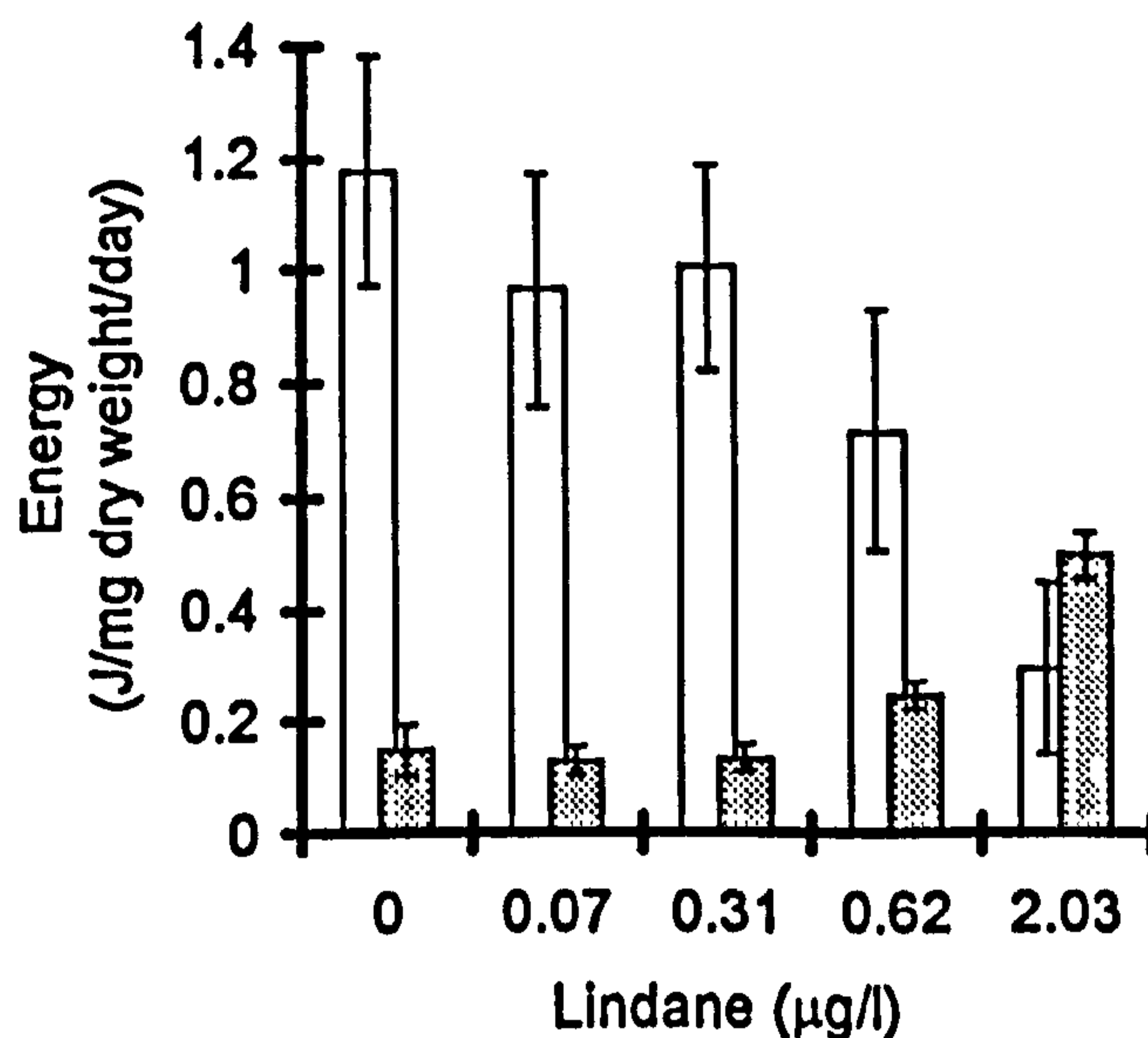


Figure 5.5. Absorbed and respired energy in Joules of *G. pulex* exposed to lindane for 168 hours. Absorbed energy values (open bars) and metabolised energy calculated from respiration (solid bars) are shown with standard error of the mean.

Table 5.3. Analysis of respirometry solutions. Lindane actual and nominal concentrations as analysed by GC/EC. Actual concentrations are the mean of three samples.

Nominal µg/l lindane	Actual µg/l lindane	standard deviation
0	<0.001	-
0.2	0.066	0.005
1.0	0.96	0.03
3.0	1.68	0.03
10.0	3.73	0.05

The effect of short-term lindane exposure on oxygen consumption was measured using a flow-through respirometer. Oxygen consumption was measured whilst animals were exposed to nominal concentrations of 0, 0.2, 1.0, 3.0 and 10 µg/l. The actual concentrations of lindane which the animals were exposed to is given in Table 5.3.

Absorbance of energy was significantly lower than the control at a concentration of 2.03 µg/l lindane ($F_{4,59} = 6.31$; Dunnett's $q > 2.51$). Loss of energy, via respiration was significantly higher than the control at the same concentration of lindane ($F_{4,59} = 21.86$; Dunnett's $q > 3.99$). The concentration at which absorption was inhibited by 50 % from control was at 0.8 µg/l lindane (95 % confidence limits of 0.34 and 1.29). Respiration was increased by 50 % from control at 0.6 µg/l lindane (95 % confidence limits; 0.52 and 0.65).

The increase in respiratory loss and decrease in energy absorbed with increasing lindane concentration resulted in a significant reduction in SfG (Figure 5.6, $F_{4,59} = 3.7$). High within treatment variability meant that SfG was only significantly different from control at 2.03 µg/l lindane (Dunnett's, $p < 0.05$, $q > 3.99$). Scope for growth was decreased by 50% relative to controls (IC_{50}) at a concentration of 0.63 µg/l lindane (95 % confidence limits 0.07 and 1.28).

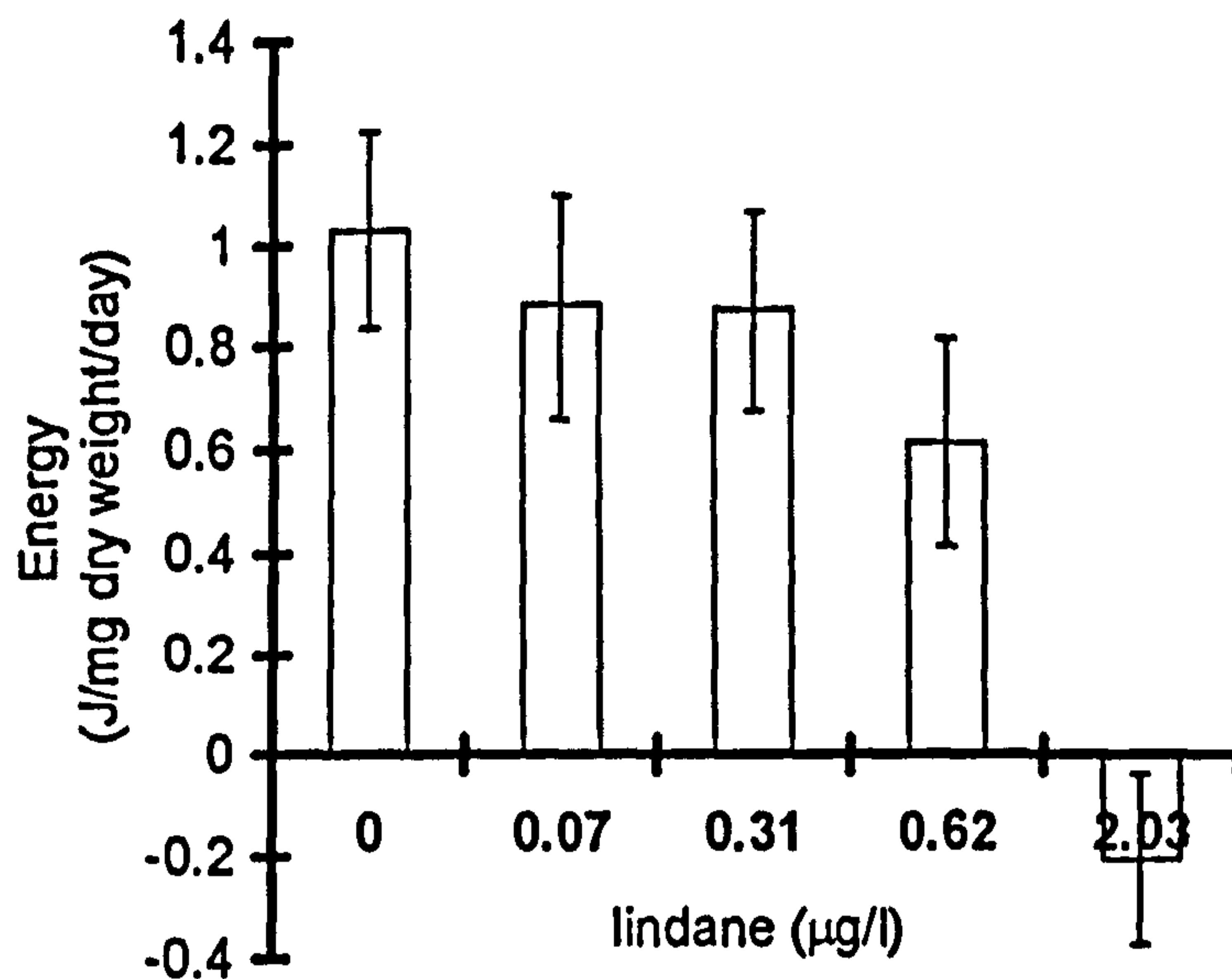


Figure 5.6. Scope for growth of *G. pulex* exposed to lindane for 168 hours. Error is the standard error of the mean.

Preliminary experimental results showed that the recovery of glycogen was high (Table 5.4). Furthermore, there was no significant difference between the glycogen content of dried and fresh animals ($t_{15}=1.25$). The significant reduction in SfG was not reflected in a reduction in glycogen concentration ($F_{4,24} = 0.23$). The glycogen body concentration for animals exposed for 168 hours to lindane concentrations is shown in Figure 5.7.

Table 5.4. Recovery of glycogen from glycogen standards. Glycogen calculated from the mean of three samples from insoluble and soluble enzyme treated standards.

Sample	glycogen added (µg)	glycogen measured (µg)	standard deviation	recovery %
50µl 1:32 standard	7.8	8.1	0.68	104
10µl 1:64 standard	0.78	0.56	0.006	72

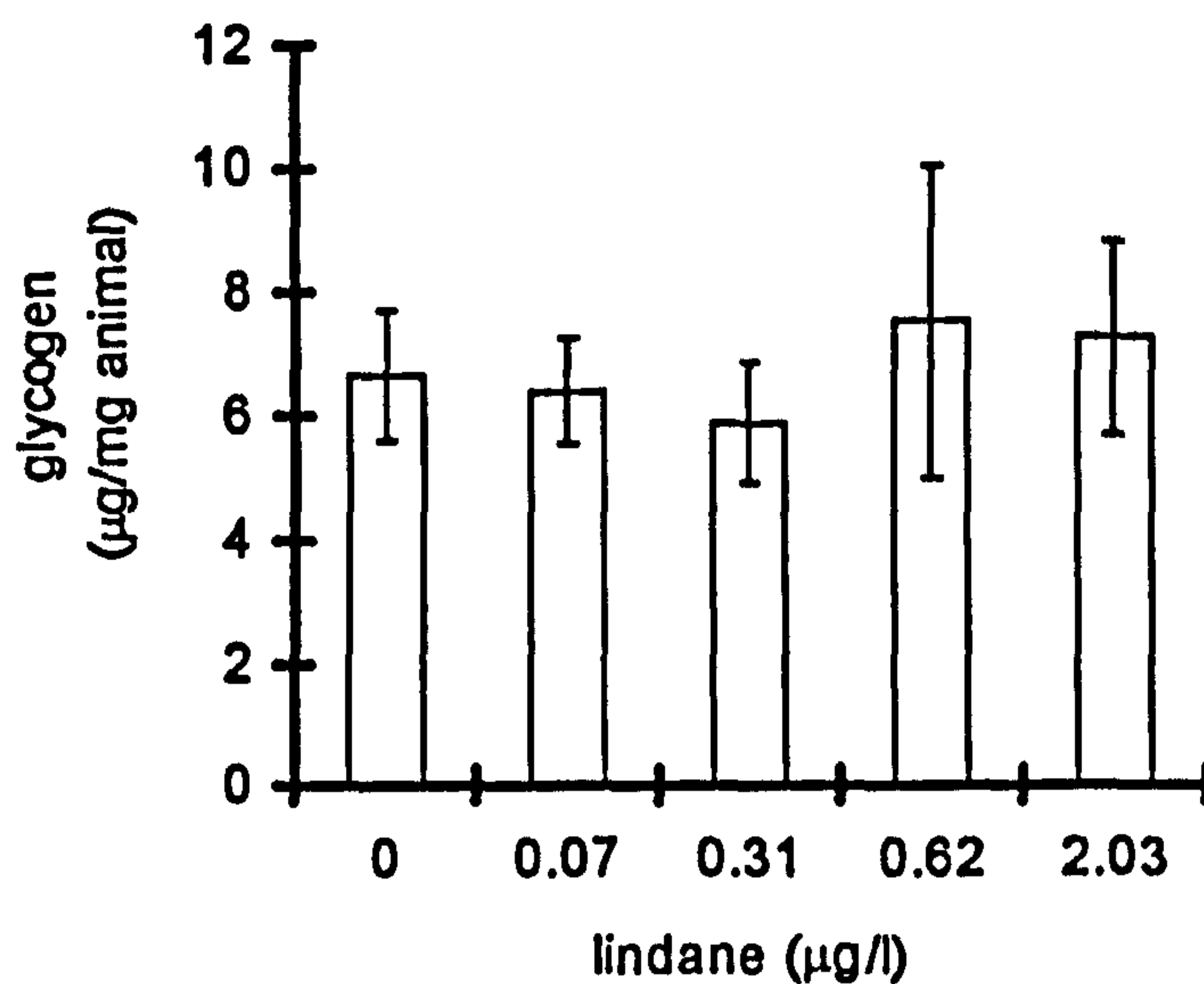


Figure 5.7. Glycogen content of *G. pulex* exposed to concentrations of lindane for 168 hours. Values are the mean of five animals per mg fresh weight, shown with standard error of the mean.

5.3.2. Long-term exposure to lindane.

The effect of long term exposure on energy budget components was investigated by maintaining animals for 35 days in either APW or nominally 0.02 µg/l lindane before exposing them to 1 µg/l for 7 days. Test solutions were changed weekly and measured lindane concentrations are given in Table 5.6.

Table 5.6. Results of GC/ECD analysis of lindane samples taken after one week exposure. Data is shown for control, pre-exposure and exposure and respirometry test solutions with one standard deviation.

	lindane concentration (µg/l)		
Sample	0	0.02	0.1
7d	<0.001	0.019 (±0.0004)	0.92 (±0.050)
21	<0.001	0.019 (±0.0003)	0.92 (±0.014)
35	<0.001	0.019 (±0.0006)	0.96 (±0.033)
respirometry	<0.001	0.019 (±0.0008)	0.97 (±0.022)

Animals maintained in APW under laboratory conditions for 35 days and then exposed to 1.0 µg/l lindane for 7 days (control/exposed) exhibited a significantly lower energy absorption ($t_{12} = 5.84$) and a significantly higher respiratory energy loss ($t_{17} = 3.52$) than animals maintained in APW for the full 42 days (Figure

5.8a). Pre-exposure to 0.02 $\mu\text{g/l}$ lindane for 35 days prior to exposure to 1.0 $\mu\text{g/l}$ lindane for 7 days (pre-exposed/exposed) did not remove the effect of elevated lindane exposure on energy absorption ($t_{11} = 10.75$) but did mitigate the subsequent effect on respiratory losses ($t_{14} = 1.71$, Figure 5.8b).

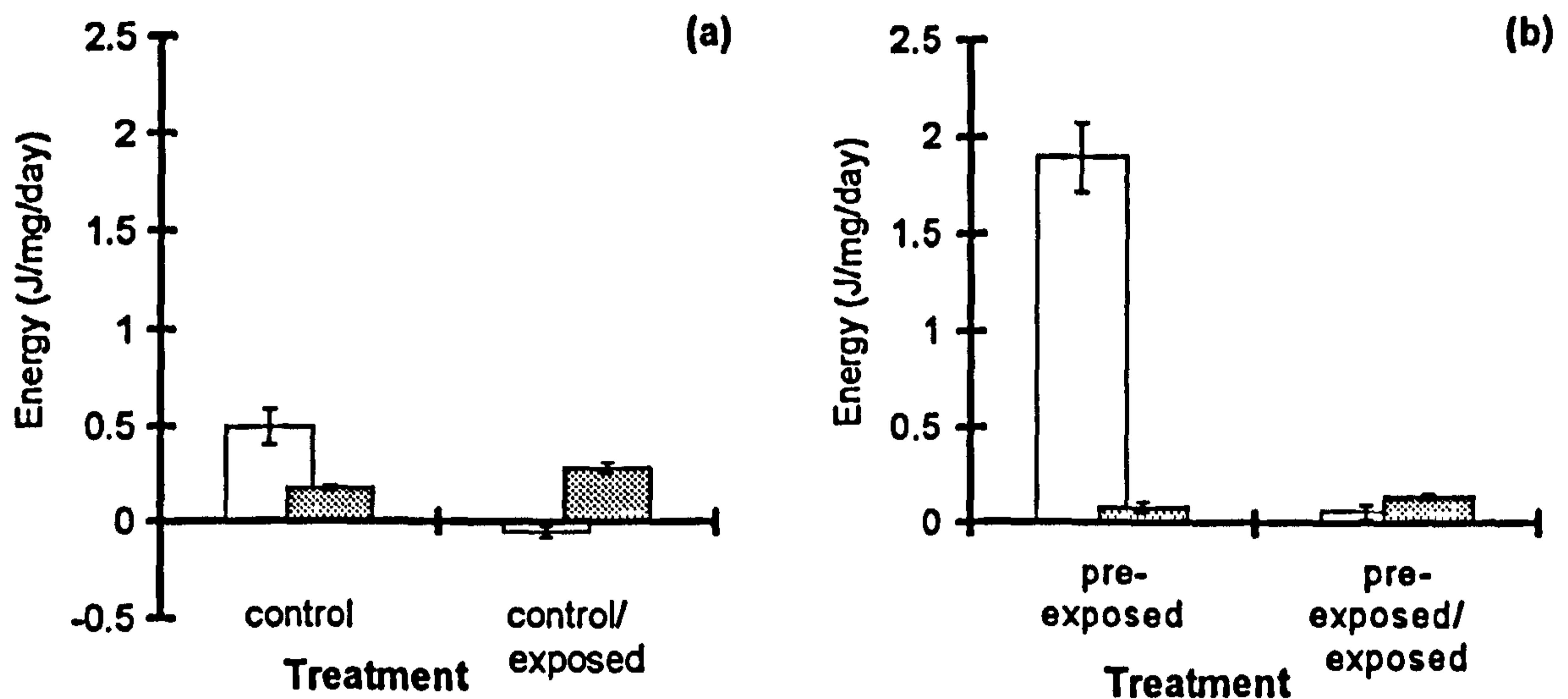


Figure 5.8. Absorbed energy (open bars) and respiratory loss (hatched bars) for control held animals and animals held for 35 days in APW and 7 days in 1.0 $\mu\text{g/l}$ (control/exposed) (a) and animals pre-exposed to 0.02 $\mu\text{g/l}$ lindane for 42 days and animals pre-exposed to 0.02 $\mu\text{g/l}$ for 35 days and further exposed to 1.0 $\mu\text{g/l}$ for 7 days (pre-exposed/exposed) (b). Error shown is the standard error of the mean.

For both groups of animals, those pre-exposed for 35 days to 0.02 $\mu\text{g/l}$ lindane and those pre-exposed for 35 days to APW, 7 days exposure to 1 $\mu\text{g/l}$ resulted in a significant decrease in SfG ($t_{14} > 6.04$). Furthermore, in both cases, the SfG of exposed animals was negative (Figure 5.9).

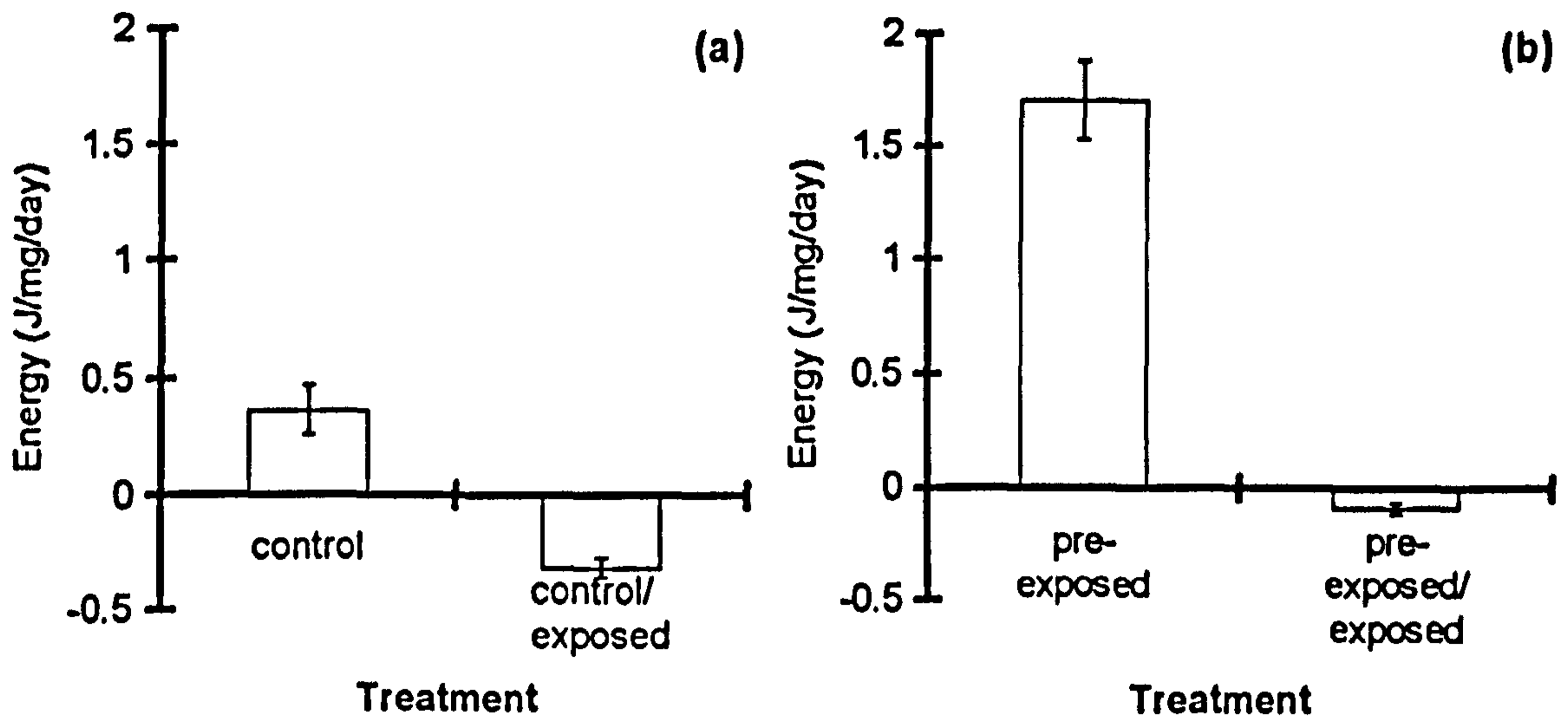


Figure 5.9. Scope for growth in animals held in clean conditions and exposed to 1 $\mu\text{g/l}$ lindane (a) and animals pre-exposed to 0.02 $\mu\text{g/l}$ and further exposed to 1 $\mu\text{g/l}$ lindane (b). Values are the mean of twelve animals with standard error of the mean.

There was no significant difference between the glycogen concentration of animals held in APW for 42 days (control) and APW for 35 days followed by exposure to 1 $\mu\text{g/l}$ lindane for a further 7 days (control/exposed) ($t_{20} = 0.75$, Figure 5.10a). In contrast *G. pulex* pre-exposed to 0.02 $\mu\text{g/l}$ lindane for 35 days prior to 7 days exposure to 1 $\mu\text{g/l}$ lindane had a significantly lower glycogen concentration than animals exposed to 0.02 $\mu\text{g/l}$ for the full 42 days ($t_{15} = 24.6$, Figure 5.10b). High coefficients of variation were associated with these data: 42 % for control, 67 % for control/exposed, 58 % for pre-exposed and 66 % for pre-exposed/exposed.

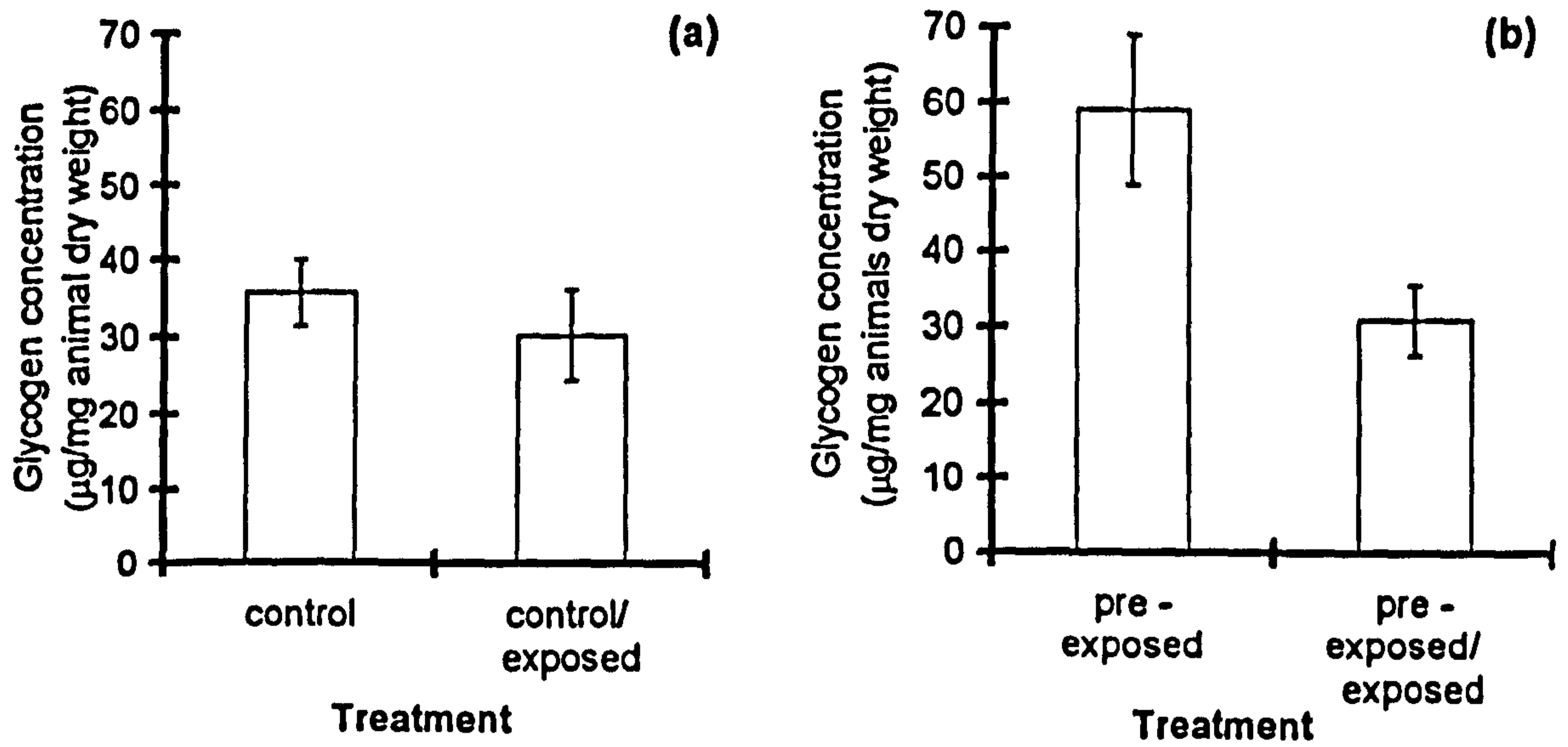


Figure 5.10. Glycogen concentration of *G. pulex* held in (a), control (APW for 42 days) and control /exposed (APW for 35 days then 1 µg/l lindane for 7 days) and (b) pre-exposed (0.02 µg/l lindane for 42 days) and pre-exposed /exposed (0.02 µg/l lindane for 35 days then 1 µg/l lindane for 7 days). Values are the mean of twelve animals for each treatment with standard error of the mean.

5.4. DISCUSSION.

The major objective of the work described in this chapter was to assess the effect of lindane exposure on the uptake and loss of energy by *G. pulex*. It was hypothesised that lindane exposure would decrease energy absorption by a narcotic effect on feeding rate and increase respiratory energy loss by an increase in metabolic energy demand due to increased cell damage and protein synthesis. It was predicted that lindane exposure would cause a decrease in feeding rate, a decrease in the amount of absorbed energy, an increase in respiration rate and a decrease in the concentration of stored energy as glycogen. Furthermore, it was predicted that on long-term, low concentration exposure that there would also be a decrease in absorption and an increase in respiration rate, but, the magnitude of these changes would be lower than on short-term exposure.

Energy budgets of *G. pulex* were affected by both short-term (i.e. 168 hour) and long-term (i.e. 42 day) lindane exposure. In the short term, decreased absorption of energy coupled with an increase in respiratory energy loss led to a decrease in scope for growth. In fact, animals exposed to 2.03 µg/l lindane for 168 hours had a negative scope for growth. Despite this, glycogen concentration appeared to be unaffected by short term exposure, suggesting that stored energy was still potentially available for growth and repair. Thirty-five day exposure to 0.02 µg/l lindane led to an increase in the amount of energy absorbed and consequent increase in SfG. However, after subsequent seven day exposure to 1 µg/l, SfG was negative. This was primarily due to a large decrease in absorbed energy. Glycogen concentrations were also decreased after 7-day exposure to 1 µg/l lindane suggesting that glycogen was used as a source of energy for maintenance and repair.

5.4.1 Effect of short-term lindane exposure on energy absorption and loss.

The rate of energy consumption (i.e. feeding rate) over 96 hours was demonstrated to be inhibited by 50 % at a lindane concentration of 1.11 µg/l (95 % CL = 0.28, 1.59). This was seven times lower than the 96-hour median lethal concentration for *G. pulex* (Table 3.3, Chapter Three). Indeed, Maltby (1994)

reported that stress-induced changes in feeding rate are strongly correlated with lethal effects. On average feeding rate is approximately eight times more sensitive than the LC₅₀ value for a range of substances including organics, metals, dissolved gases and pH. Using the relationship reported by Maltby (1994), the 96-hour LC₅₀ of 7.8 µg/l would give a predicted value of 1.0 µg/l lindane for inhibition of feeding rate, this compared well with the value determined in this study of 2.03 µg/l, as the next lowest concentration tested was 0.62 µg/l. An increase in the period of exposure led to an increase in the median inhibition concentration for feeding. At 168 hours lindane exposure, the IC₅₀ for absorption was 0.8 µg/l (95 % CL = 0.34, 1.29) compared with IC₅₀ for consumption rate of 1.24 µg/l (95 % CL, 0.74, 1.64).

Respiration rate was significantly increased by 168 hour exposure to 2.03 µg/l lindane. This was the same concentration that significantly reduced absorption. Increases in respiratory oxygen consumption have been reported previously for lindane exposure. Galindo *et al.* (1996) demonstrated a 5 % increase in oxygen consumption rate in shrimp larvae (*Penaeus vannamei*) exposed to 0.19 µg/l lindane for 1.5 hours. Furthermore, oxygen consumption of the freshwater isopod, *Asellus aquaticus*, was shown to be significantly higher than controls after 48-hour exposure to 4 µg/l lindane (Le Bras, 1987). However, Le Bras (1987) reported that at concentrations higher than 8 µg/l oxygen consumption was reduced to a rate below that of the control animals. The majority of previous studies investigating the effects of xenobiotics on the energy budget parameters of *G. pulex* have concluded that respiration rate is generally less sensitive and only occasionally as sensitive as feeding rate (Naylor *et al.*, 1989; Maltby and Naylor, 1990; Maltby *et al.*, 1990; Maltby, 1992).

The decrease in feeding and consequent energy absorption may suggest a narcotic effect of lindane on *G. pulex*. Willows (1994) reported that relationships between measures of contaminant concentration and effect of respiratory and feeding physiology could reveal the potential mechanism of toxicity. Three mechanisms of toxicity were suggested by Willows (1994);

narcosis, which reduces energy intake by a reduction in feeding rate with little effect on respiration, metabolic inhibition, and metabolic uncoupling that effect respiration. The data in the present study show a common no effect concentration of lindane on respiration and feeding. It is likely that is this a result of a combination of a neurotoxic effect, affecting feeding rate and an increase in metabolic energy demand due to increased cell damage and a impairment of respiratory function.

Increased respiration rate coupled with decreased energy acquisition will result in a decrease in SfG. Scope for growth measured at 168 hours was negative and significantly different from control at 2.03 $\mu\text{g/l}$ lindane. The 168-hour IC_{50} of 0.63 $\mu\text{g/l}$ lindane (95 % CL = 0.07, 1.28) was similar to the 168-hour IC_{50} for respiration energy loss (i.e. 0.6 $\mu\text{g/l}$, 95 % CL = 0.52, 0.65) and to the 168-hour IC_{50} for energy absorption of (i.e. 0.8 $\mu\text{g/l}$, 95 % CL = 0.34, 1.29). The negative SfG meant that at a concentration of 2.03 $\mu\text{g/l}$ lindane animals were in energy deficit and consequently the only energy available for growth would be stored energy. However, glycogen content was not reduced by 168-hour exposure to 2.03 $\mu\text{g/l}$ lindane. There are several possible explanations for this apparent discrepancy. Firstly, it is possible that glycogen is not the main short-term energy reserve or the use of glycogen is masked by differential use in different tissues. For example, exposure of the freshwater mollusc, *Lymnaea palustris*, to sublethal concentrations of hexachlorobenzene (sampled over a 21 day period) were reported to have no effect on glycogen concentration (Baturu *et al.*, 1995). This was explained by the possible differences in glycogen mobilisation in different tissues. Secondly, error in the measurement of energy budget components may have indicated an erroneous negative SfG. However, energy values derived for SfG components compared well with that of other workers. For example, values of 1.2 (SEM \pm 0.2) J/mg/day for control absorbance and 0.15 (SEM \pm 0.05) J/mg/day for control respiratory energy loss compared well with 2.25 and 0.2 J/mg/day for control absorbance and respiration values respectively reported by Naylor *et al.* (1989). Finally, there may have been error

in the measurement of glycogen due to the presence of leaf material in the gut of animals analysed for glycogen content.

Fed animals were used for glycogen analysis (section 5.2.3.). The method used does not discriminate between the polysaccharides starch and glycogen. Thus, starch in gut leaf material may have contributed to the estimation of the glycogen content in these animals. This was remedied in animals estimated for glycogen in the long-term exposure experiment as these animals were observed to have empty guts due to being without food for 15 hours. Monk (1977) reports a gut through-put time of five to seven hours on *G. pulex* fed on algal material and detritus. However, gut passage times of *G. pulex* fed on conditioned alder leaves were between 84 and 114 minutes and unaffected by exposure to cadmium and zinc ions (Maltby, pers. comm.). Animals separated from food supply (as they were in respirometry in this study), have been reported to have emptied the gut of contents in 16 to 20 hours at 15 °C (Moore, 1975). Thus, in short-term exposure experiments, fed animals were taken directly for glycogen analysis (section 5.2.3) and may have contained leaf material in their guts, which may have interfered with glycogen estimation. However, animals from respirometry experiments (i.e. unfed for 15 hours, section 5.2.2) were used for the glycogen assay from long-term exposure experiments. Thus, these animals had empty guts and there would not be possible interference of gut leaf material in the glycogen assay.

5.4.2 Long-term lindane exposure.

The amount of energy absorbed by animals pre-exposed to 0.02 µg/l lindane was highest of all the treatments (although a high coefficient of variation of 58 % should be noted). This increase in the amount of absorbed energy accounted for the highest positive SfG observed in all the treatment groups. Thus, at low 'environmental' (see section 4.2.1, Chapter Four) concentrations of 0.02 µg/l a negative effect of lindane on feeding was not apparent. This increase in absorbed energy was opposite to the predicted decrease in energy absorption on exposure to lindane. Animals pre-exposed to 0.02 µg/l lindane had an elevated glycogen

concentration that reflected the amount of absorbed energy. Increased absorption through increased consumption rate may be the result of stimulation of feeding at a low level of exposure and therefore more energy is available for storage. It has been reported that increase energy intake by the isopod, *Porcellio scaber*, from metal contaminated sites allowed increased energy allocation to reproduction with no decrease in the energy allocated to growth (Donker, 1992). This author also reports that glycogen concentrations in isopods from a metal contaminated site were not significantly different from glycogen concentrations of animals from a clean reference site. Baturu *et al.* (1995) reported that exposure of the freshwater gastropod, *Lymnaea palustris*, to the fungicide hexachlorobenzene and the herbicide atrazine did not affect glycogen concentrations, although these animals had a decreased growth rate but an increased reproductive output.

The increased energy intake on exposure to 0.02 µg/l lindane, may be due to a stimulation of feeding rate. This stimulation or apparent benefit at low levels of exposure is referred to as hormesis (Calabrese *et al.*, 1987). Hormesis has been described for many classes of chemicals and implies a higher fitness in the presence of a chemical agent at low concentrations as compared to that in the absence of that chemical (Parsons, 1989). However, this raises the question; if animals are able to increase feeding and/or absorption to increase fitness, then why do they not operate at this increased level in control conditions? With reference to section 1.4.1 and Figure 5.2, resource acquisition is assumed to be finite because the resource is finite and/or an organisms mechanism for energy uptake will limit its capacity for uptake and thus limit the amount of resource it can use. From the results in the present study, animals exposed for 42 days to 0.02 µg/l lindane increased their uptake and storage of energy, which suggests that control animals are operating a sub-maximal and perhaps sub-optimal resource acquisition. This, however, is difficult to reconcile with the assumption outlined above. It may be that there is some stimulation of feeding at low concentrations of lindane in the laboratory, resulting in increase energy available that can be allocated to storage reserves (glycogen). Although it is not clear why

control animals do not operate at this apparently beneficial increased energy absorption and consequent increased fitness.

Pre-exposure to 0.02 $\mu\text{g/l}$ may confer some tolerance on further exposure to 1 $\mu\text{g/l}$ lindane. Pre-exposed/exposed animals had a large decrease in absorbed energy after 7 day exposure to 1 $\mu\text{g/l}$ lindane, although no significant increase in respiration energy loss was observed (cf. significant increase in respiratory energy loss of exposure on control animals). The resulting SfG was negative in animals that had been pre-exposed to 0.02 $\mu\text{g/l}$ lindane and further exposed to 1 $\mu\text{g/l}$ lindane. However, negative SfG in these animals, was not as negative as that observed in exposed control animals (i.e. animals in clean conditions for 35 days and exposed to 1 $\mu\text{g/l}$ lindane for a further 7 days). Although not significant, this may be suggestive of some resistance of exposure effects as a result of pre-exposure.

Animals exposed to 1.0 $\mu\text{g/l}$ lindane after a six week period in clean APW in the laboratory had a significant decrease in absorption of energy coupled with a significant increase in respiratory energy loss. Indeed, the absorption of energy was observed to be negative in animals exposed to 1.0 $\mu\text{g/l}$ lindane for 7 days, due to a larger production of faecal material than of that food material consumed. This may be explained by the production of mucus in the gut as a response to exposure, leading a greater amount of faecal material (Bermingham, 1993). Nevertheless, there was significant reduction in SfG and a consequent negative SfG was measured as a result of exposure of control animals. Thus, no energy was available for growth and it is likely that energy available for normal functioning was limited. However, for animals held in control conditions for 35 days, the effect of 7-day exposure to 1 $\mu\text{g/l}$ lindane was not reflected in the concentration of glycogen reserves. Thus, although these animals are in apparent energy deficit (as measured by SfG) there was stored energy in the form of glycogen.

As with the short-term exposure experiment, although animals exposed to concentrations of 1 µg/l lindane and above had increased respiration and decreased absorption of energy there was no apparent affect on glycogen concentration. A negative SfG was not indicative of a decrease in the amount of energy stored as glycogen. However, the increase in absorbed energy on long-term exposure to 0.02 µg/l lindane was reflected in an increased glycogen content that was depleted by 50 % on 7-day exposure to 1 µg/l lindane. This suggests that increased glycogen energy stores were used as a source of energy on exposure, but only after an increased glycogen concentration was produced from increased energy acquisition. Again this raised the point made above that if this strategy is optimal then it would be expected that control animals would follow such a strategy. Energy may be allocated to storage rather than growth or reproduction in order to provide energy for maintenance (including the production of protective enzymes such as GST) in order to survive the exposure to lindane at low levels. Whereas, at higher exposure concentrations more energy is needed for both maintenance and repair (to damaged tissue), this is coupled with a narcotic effect on feeding that leads to a negative SfG.

5.4.3 Conclusions.

- Exposure to a sublethal concentration (i.e. 2.03 µg/l) of lindane in the short-term (168 hours) caused a decrease in energy absorption coupled with an increase in respiration.
- The hypothesis that the narcotic effect of lindane would lead to a decrease in energy intake (by a decrease in feeding rate) and an increase energy expenditure (via respiration due to increased maintenance and repair) could be accepted only for a concentration of 0.94 µg/l lindane (actual concentration) and above.
- Similar IC₅₀ values for absorption, respiration and SfG showed that energy loss and energy gain were similarly sensitive to short-term lindane exposure.
- It was predicted that the deficit in energy available for growth would lead to the use and consequent depletion of glycogen energy reserves. This prediction was not supported.

- Long term (42 day) pre-exposure to lindane at an environmentally realistic concentration (0.02 µg/l) resulted in an increased SfG; largely due to a increase in the absorbed energy.
- The hypothesis that long-term exposure to 0.02 µg/l lindane would decrease absorbed energy and increase respiration was rejected.
- It was suggested that feeding was stimulated at low lindane concentration to provide an increased energy intake. This increased energy intake resulted in increased SfG and glycogen concentration.
- It was suggested that pre-exposure may confer some tolerance on further exposure as energy expenditure via respiration was decreased in animals previously exposed to 0.02 µg/l lindane.

CHAPTER SIX

Glutathione s-transferase as a biomarker of organic xenobiotic exposure and effects.

It was the main aim of the present study to assess the use of GST as a biomarker of organic xenobiotic exposure and relate toxicant-induced changes in GST activity to changes in energy status and physiological energetics. In this way GST can be assessed as a biomarker of exposure and related to effects such that it can be used as an 'early warning' of detrimental effects at higher levels of biological organisation.

It was hypothesised that GST would be one of the first systems to respond to organic xenobiotic exposure because it is induced by, and involved in, the metabolism of such chemicals. The response of GST is part of a continuum of responses to xenobiotic exposure that signify the onset of impairment and this can be related to increasing disability of the organism eventually ending in disease and death. As GST is involved in the detoxification of organic xenobiotics then it is one of the mechanisms that enable maintenance of fitness in contaminated environments. Prolonged exposure over a number of generations may promote the selection of animals with the ability to increase activity or animals may acclimate to contaminated conditions.

Exposure to xenobiotics may have an energetic cost that is due initially to increased protein synthesis and with extended exposure, repair of damaged tissue. Finite energy resources are therefore allocated to compensate the extra energy used in the maintenance of normal functioning and repair resulting in reduced allocation of energy to storage. Thus, a mechanistic link is made between exposure and energy balance and a tentative link is made between increase GST activity and increased energy use.

The objectives each of the chapters in this study relate to the testing of the hypotheses stated above. Chapter Two established a method for the detection of GST and assessed the affect of environmental variables on GST activity. Chapter Three described the GST response on exposure to organic xenobiotics. Chapter Four assessed the use of GST as a biomarker of exposure in the field and assessed the affect of long-term exposure to pesticides on GST activity. Finally, Chapter Five considered the measurement of the energetic cost of short-term and long-term pesticide exposure.

6.1 Measurement and change of GST activity in *G. pulex*.

The measurement of exposure depended on the detection of changes in GST activity that were due to xenobiotic exposure, rather than other extrinsic or intrinsic factors. GST activity in *G. pulex* was affected by temperature, feeding and body size (Chapter Two). Activity increased at a lower holding temperature (4 °C), on feeding, and was proportionally greater in smaller size classes. In order to minimise variation due to these intrinsic and extrinsic factors, subsequent experiments used a standard sized animal (males of 10-15 mm in length) and laboratory experiments were conducted using animals that had been held in laboratory conditions for a week at 15 °C, using unfed animals (for short-term experiments).

It is obviously not possible to control extrinsic factors in the field and temporal variation in GST activity of field collected animals was observed. Variation in field *G. pulex* GST activity could not be explained by variation due to environmental factors tested in the laboratory. For example the highest activity in animals from the Craggs Stream site (Chapter Two), was measured in animals collected in December, a time when temperature is low, but although this is consistent with the laboratory result that GST activity was highest at 4 °C, animals collected in January had the lowest activity of animals collected throughout the year. Feeding may have an effect on field animals GST activity over the year. Fed animals were shown to have higher GST activity in the laboratory and the effect of feeding and different plant food materials on GST

activity in other invertebrates has been documented (e.g. Almar *et al.*, 1987; Egaas *et al.*, 1993) and is discussed in section 2.1.4. Again, this does not explain the difference between the GST activity of *G. pulex* collected in January and December. It may, however, be possible that extrinsic factors fluctuating in combination will produce different and more complex effects on GST activity than varying one factor in isolation. Fluctuations in baseline GST activity in field populations of *G. pulex* were also demonstrated in the uncontaminated site Wabe that may have been due to extrinsic factors (Chapter Four). Furthermore, laboratory and mesocosm experiment controls were observed to fluctuate over time (Chapter Three).

GST activity is affected by a variety of factors, therefore the measurement of a baseline of GST activity is to be approached with caution. This is particularly true for field studies where the 'baseline' will vary spatially and temporally and thus a 'snapshot' of activity obtained by taking a single sample, may give an erroneous indication of exposure. Furthermore, data reported in Chapter Two demonstrated that the lowest GST activity measured in experiments was variable. Nevertheless, it was tentatively suggested that a baseline of between 0.02 and 0.04 $\mu\text{mol}/\text{min}/\text{mg}$ protein was indicative of animals that did not show increased activity due to extrinsic effects. However, this should not be considered as an absolute baseline, deviations from which are indicative of chemical exposure. Rather, GST response in treated animals should be compared to that in animals under the same conditions minus chemical challenge (i.e. controls) or to reference site animals.

Laboratory exposure to lindane demonstrated a concentration dependent increase in GST activity (Chapter Three). This response was rapid (occurring within 24 hours) and sensitive; the 24-hour EC_{50} for GST activity being 15 times less than the 96-hour LC_{50} . The effect, however, was transitory, significant differences were lost at 96 hours exposure but were exhibited again at 336 hours exposure. It is perhaps noteworthy that the concentration of significant effect was the same at 24 hours and 336 hours exposure, and thus suggested a threshold of effect. A

threshold effect was also reported in animals exposed to alcohol ethoxylate in artificial streams. Again the response was rapid (at 24 hours exposure) and sensitive (34 times less than the 96-hour LC₅₀) but this effect was lost at later measurement periods.

It is recognised that concentration dependent response (dose-response) is often lost in animals exposed in fluctuating environmental conditions (*c.f.* artificial streams experiment) due to the effect of other environmental stressors (Depledge *et al.*, 1992). However, the rapidity and sensitivity of the initial response was not lost in the artificial streams. The transitory nature of detoxification enzyme response is also recognised, an initial induced response may be seen to decrease in magnitude with time in many detoxification enzymes (Timbrell *et al.*, 1994). Moreover, the rapidity of induction and subsequent fading of response has been reported for GST after an initial dose (Lagadic *et al.*, 1993), and in continuous exposure (Beverley, 1994). Furthermore, Baturo and Lagadic (1996) demonstrated an increase in GST activity in freshwater snails at 24 hours exposure to hexachlorobenzene in mesocosm ponds that decreased to control levels for the rest of the exposure period sampled at regular intervals up to 504 hours exposure.

The reason for the transience in biomarker response is unclear. However, it has been suggested that with detoxification enzymes systems, the detoxification of specific xenobiotics (e.g. particular insecticides) is due to the selective induction of isoenzymes of GST (Clark, 1990). Furthermore, it has been suggested that resistance to insecticides in insects and mites is due to several biotransforming enzymes involved in a polyvalent xenobiotic detoxifying system (Lagadic *et al.*, 1993). Therefore, a change in the enzymic response over time may explain the apparent transitory nature of GST activity over time with continued exposure. Initial exposure may induce a number of enzymes including a number of GST isoenzymes, the rapidity of this response enables challenged animals to survive exposure to various toxic compounds. Further exposure induces specific isoenzymes that are involved in the detoxification of the specific xenobiotic.

Hence, an initial GST response is measured that consequently decreases with time. This hypothesis could be tested by the measurement of specific isoenzymes of GST in *G. pulex* on exposure to specific xenobiotics.

The magnitude of the GST response in laboratory exposure to lindane was observed to decrease over time from an initial activity increase of 80 % over controls (at 24 hours exposure) to 17 % at 336. The initial GST activity increase at 24 hours alcohol ethoxylate exposure in the artificial streams of 43 % from control was similar to that in the laboratory at 96 hours of 55 %. In agreement with the lower percent increases in GST activity in exposed animals over controls at longer exposure periods, animals from the pesticide contaminated site (Ohebach, Chapter Four) had activities that varied from 39 to 13 % higher than animals from the non-contaminated site (Wabe).

The possible reasons for the decrease in GST response over time have been discussed above. The non-monotonic relationship between the magnitude of the GST response and time observed in the present study has been reported by other workers. Hans *et al.* (1993) reported an initial 75 % increase in GST activity of the earthworm, *Pheretima pothuma*, exposed to lindane (1 µg/g soil) after seven days reaching a maximum of 105.3 % of control activity, after 14 days, which then decreased to 16.5 % after 28 days exposure. However, GST induction in the freshwater mollusc, *Sphaerium corneum* showed a more monotonic increase with time albeit over a shorter time scale. For instance, Boryslawskyj *et al.* (1988) reported GST activity for the freshwater mussel *Sphaerium corneum*, which increased by 263 % for mussels exposed to 2 µg/l lindane for 24 hours and increased to 1060 % after 44 hours decreasing to 1000 % after 198 hours exposure.

Long-term pesticide exposure.

GST activity in *G. pulex* populations from contaminated sites exhibited a more sustained response to contamination. The data presented in Chapter Four demonstrated that GST activities in *G. pulex* from the contaminated sites at

Ohebach and Brunsbuttelerbach were higher than those at the clean sites Wabe and Lutterquelle. Furthermore, GST activity in *G. pulex* from the Ohebach site were consistently higher than those of the Wabe site animals, even though GST activity in animals from both sites was reported to fluctuate during the year. This again stressed the need to compare measured GST activities in contaminated site animals with those of animals in similar but uncontaminated sites. GST values were thus relative rather than absolute.

Fluctuations in GST activity in pesticide-contaminated sites could be related to pesticide-containing run-off events. Similar high flow events and seasonal variation could have accounted for a similar fluctuation pattern in clean site animals GST activity. Animals in the contaminated site (Ohebach) were subject to low concentrations of pesticide exposure for long periods interrupted by increased concentrations due to run-off events (Chapter Four). This is a common phenomenon in pesticide contamination of streams in agricultural catchments (Chapter Four). Pesticides other than lindane detected at the Ohebach site, namely parathion and fenvalerate are a organophosphate and pyrethroid insecticide respectively. Both groups are known to induce GST activity (e.g. Punzo, 1993; Rumpf *et al.*, 1994). Furthermore GST is known to be involved in resistance to (and therefore detoxification of) both organochlorine and pyrethroid insecticides (Georghiou, 1990).

The differences between animals from the contaminated and non-contaminated sites were shown to be due to increased pesticide exposure in the contaminated sites. The interpopulation differences between sites were removed on long-term laboratory exposure to a low 'environmental' concentrations of lindane. GST activity in animals from the Wabe (non-contaminated site) was not significantly increased on long-term exposure to 0.02 µg/l lindane (compared with animals kept for the same time period in clean conditions). However, a significant increase in GST activity was exhibited by these pre-exposed animals on further exposure to a short-term higher concentration of lindane (Chapter Four). It is not clear why animals from the clean site should exhibit significantly higher GST

activity compared to animals from the contaminated site after pre-exposure on further exposure of animals from both sites. It was expected that after pre-exposure then further exposure animals from both site would show similar GST activity. It is possible that the period of pre-exposure was not long enough to allow acclimation of clean site animals. Although, pre-exposure removed the differences in GST activity between sites it did not significantly increase GST activity from control in animals from either site. Induction of GST may be due to a differential response of GST isoenzymes depending on the inducing xenobiotics and the magnitude and duration of exposure. Thus, it was proposed that the higher GST activity in contaminated site animals was induced by a number of different insecticides in the field depending on their respective concentrations (Chapter Four).

Initial exposure to pesticide could induce a number of isoenzymes of GST and also a number of different protective proteins (as discussed in the previous section). However, continued exposure to a specific inducer (i.e. lindane) could be responsible for the induction of specific isoenzyme(s) of GST. These isoenzymes are involved directly in the conjugation (and consequent detoxification) of lindane. However, because the assay used in this study detected activity of most isoenzymic forms of GST, but not individual specific isoenzymes (Chapter Two), the induction of specific isoenzyme(s) was not detected. This would account for the removal of the differences between sites on pre-exposure. However, if this pre-exposure did not induce GST activity in animals from the clean site there would have been no induction of specific isoenzymes in these animals. Thus, some induction of specific isoenzymes in the contaminated site animals and no induction in the non-contaminated site animals would show an apparent removal of the differences in GST activity between sites. Further exposure to a higher concentration of lindane in the short-term was then responsible for the induction of a number of non-specific isoenzymes of GST in pre-exposed non-contaminated site animals, that could be detected as an increase in GST activity, but pre-exposed contaminated site animals maintained a continued and/or increased induction of specific isoenzymes of GST. Thus, an

increase in GST activity was detected in pre-exposed/exposed clean site animals but not in pre-exposed/exposed contaminated site animals.

6.2 The use of GST as a marker of organic xenobiotic exposure.

In the light of the above discussion the use of GST activity in *G. pulex* can be considered in terms of its usefulness as a biomarker of exposure in the field. The rapid but transitory response of GST activity to organic xenobiotic exposure both in the laboratory studies and in the artificial streams suggest that GST activity could be used to detect exposure in the short-term. The use of an *in situ* toxicity assessment approach using caged animals (section 1.1.2) may allow the detection of exposure effects on GST activity, rapidly and sensitively but the response may be very short-lived. Thus, the timing of deployment and collection would be crucial to the assessment of GST activity. Certainly, as was reported for the experiment in the artificial streams, where caged animals were used, a rapid and sensitive GST response was detected, but only at 24 hours exposure, after which GST activity showed a non-monotonic relationship with both alcohol ethoxylate concentration and time (Figure 3.6). Furthermore, this rapid, sensitive but transitory response was also found in laboratory experiments with lindane. The possible reasons for this transitory response have been discussed above.

The use of *G. pulex* from contaminated sites for analysis of GST activity as a marker of exposure to organic xenobiotics in the field is to be approached with extreme caution. It is clear that the timing of the response is important in the interpretation of the effect of exposure on GST activity in *G. pulex*. It is concluded from the work in this study that GST activities measured in exposed animals should always be compared to GST activities of animals not subject to chemical exposure. This is necessary because of the variability in 'baseline' GST activity. Consequently, assessments of GST activity as a marker of organic xenobiotic exposure are of a qualitative rather than quantitative nature. Thus, GST activity in *G. pulex* is not an absolute measure of xenobiotic exposure, but a comparative one.

On long-term exposure to environmental concentrations of lindane, animals from the clean Wabe site did not exhibit significantly increased GST activity (Chapter Four). However, on further exposure to a short-term higher concentration, pre-exposed/exposed animals showed significantly higher GST activity than contaminated site animals under the same treatment regime. Thus, long term exposure to low concentrations of pesticide of the magnitude likely to be found in the field, did not significantly increase GST activity. However, this pre-exposure had the effect of 'priming' higher GST activity on further exposure in animals from the clean site. Thus, it would appear from the results of laboratory studies in this work that even long-term exposure of *G. pulex* to contamination of the level found at field sites would not induce increased GST activity if concentrations in the field were similar to those in the laboratory (0.02 µg/l lindane). However GST was found to be a consistent marker of contamination using animals resident in contaminated sites.

6.3 Population differences.

It was suggested in Chapter Four that increases in GST activity in *G. pulex* from the Ohebach site were related to increased pesticide input from runoff. It is suggested that animals in contaminated sites are capable of increasing GST activity in order to cope with increased exposure, and indeed this was apparent in the laboratory (Figure 4.5). Interpopulation differences in GST activity between *G. pulex* from contaminated and non-contaminated sites were maintained after holding in clean conditions in the laboratory for 42 days. Furthermore, this difference was maintained on exposure to 1.0 µg/l lindane (Figure 4.6). However, after long-term exposure to a low concentration of lindane, GST activity in animals from both sites was similar. The differences in GST activity may be complicated by the induction of different isoenzymes of GST in the field compared to laboratory exposure as there was evidence of pesticides other than lindane (parathion and fenvalerate) in the Ohebach field site (Chapter Four).

Tolerance of xenobiotic exposure may be the consequence of the induction of a number protective proteins, with exposure to pesticides this may involve the

induction of a number of pesticide metabolising enzymes. It has been proposed that there is an underlying mechanism present which affects the expression of a spectrum of detoxification enzymes and proteins (Hayes and Wolf, 1988). Such a mechanism would be compatible with a chemical-induced stress response, as it is known that for general stress such as in heat shock as well as in oxidative stress many proteins are induced (Christman *et al.*, 1985). Furthermore, pesticide exposure has been reported to induce a number of detoxification enzymes in insect species (Oppenoorth, 1984). In view of the number of enzymes that are affected in these systems, it is difficult to assess which is more important in conferring the resistance phenotype. Indeed, they may all contribute and may be determined by the type of stress applied.

As discussed above, differences between the GST activity between sites were observed and maintained after holding in clean conditions. Furthermore, long-term pre-exposure to low concentrations of lindane had a differential affect on the GST activity in animals from clean and contaminated sites. It was suggested that this may be due to the differential induction of isoenzymes. Thus, there may have been a genetic basis in the differences in GST activity between animals from the contaminated and uncontaminated sites, i.e. the increased GST activity in contaminated site animals had been selected for. Insecticide resistance in insects has been reported to have a genetic basis (Oppenoorth, 1985).

The resistance of pesticide toxicity is a phenomenon that is well documented in insect pest species and has been reported for a number of classes of insecticide including organochlorines, organophosphates, pyrethroids and carbamates (Oppenoorth, 1985; Lagadic *et al.*, 1994). Increased induction and activity of GSTs has been implicated as one of the mechanisms of resistance to these substances (Fukami, 1980; Motoyama and Dauterman, 1980; Hemingway *et al.*, 1993a; Hemingway *et al.*, 1993b). For example, a resistant strain of insect larvae (*Spodoptera littoralis*) has been reported to show differences in the induction of isoforms of GST rather than the amount of total GST on treatment with lindane (Lagadic *et al.*, 1993). A similar response has been shown for the house fly

(Lagadic *et al.*, 1993). A similar response has been shown for the house fly (*Musca domestica*) (Ottea and Plapp, 1984). Lagadic *et al.* (1993) reported the basal level of CDNB conjugating GST activity in resistant *Spodoptera littoralis* was over two-fold higher than in non-induced larvae. In insects, some resistance is thought to be the consequence of selection of gene(s) coding for increased levels of GST and other detoxification enzymes (Clark, 1990). Moreover, the importance of GSTs in the resistance to organophosphate and organochlorine pesticides varies from species to species from strain to strain and from insecticide to insecticide. These variations maybe explained by resistance being part of an integrated response to insecticide challenge involving increase in the production of a number of detoxification enzymes generally (Hayes and Wolf, 1988; Clark, 1990).

Resistance to insecticides has been reported in mites, (Motoyama and Dauterman, 1972; Founier *et al.*, 1987), but it is not known whether other arthropod species show resistance in the same way as insects and mites. The selection of resistant strains detected by increased activity of GST, led to the possibility that the higher GST activity in *G. pulex* from the pesticide contaminated at Ohebach site may be the results of resistance to exposure through increased GST expression. In support of this hypothesis, it was reported by Werner (1993) that *G. pulex* from the Ohebach site showed significantly greater median survival times on exposure to the pesticide fenvalerate than *G. pulex* from a clean site.

Increased GST activity in contaminated site animals may, however, be the result of 'phenotypic plasticity' (Hoffman and Parsons 1991), this is supported by the observation that long-term exposure to a low concentration of lindane removed the difference in GST activity between contaminated and non-contaminated sites. Indeed, on further exposure 'pesticide acclimated' clean site animals exhibited significantly higher GST activity than animals from the contaminated site under the same conditions (Figure 4.7).

6.4 GST as a marker of effect.

The use of GST as a marker of the effect of lindane exposure is dependent on its linkage with effects on the physiological functioning of the animals (Chapter One). As discussed in Chapter One, a mechanistic link between the energetic cost of GST production and the cost of exposure as measured by SfG and estimated glycogen concentration in reserve is impossible, unless one is able to directly estimate the energetic cost of GST synthesis.

There was an energetic cost of exposure, this was due to concentration dependent increases in energy loss via respiration and decreases in energy absorbed. Although unchanged glycogen reserves suggested that stored energy was unaffected by exposure, at least in the short-term. It was hypothesised that lindane exposure would decrease energy absorption by narcotic effect but that respiration would be increased due to the increased use of energy to maintain normal metabolic function under lindane exposure. Thus, scope for growth would be decreased and consequently less energy would be available for growth and reproduction and less energy would be allocated to storage reserves.

Under short-term exposure conditions most of the above hypothesis could be accepted, except that glycogen reserves were unaffected by lindane exposure in the short-term. On long-term exposure to a lower concentration of lindane (a level that was expected in the field), the hypothesis was rejected as energy absorption was increased and respiration rate was decreased compared with controls. On further exposure to a short-term higher concentration of lindane 'pre-exposed' animals exhibited a decrease in SfG and a decrease in glycogen concentration. This was expected, but the apparent increase in fitness at low concentrations of lindane was not expected and is difficult to explain, as resource acquisition is assumed to be finite (section 1.4.1). The apparent increase in fitness at low chemical exposure concentrations has been described by the phenomenon of hormesis (e.g. Parsons, 1989). This does not explain why animals in control conditions do not maximise energy intake if this does in fact increase fitness.

If it is to be accepted that GST can be used as an 'early warning' of effects that mark a departure from normal functioning, the increases in GST activity should precede these changes. As demonstrated in Chapters Three and Five, the effect of exposure on GST activity increase could be detected at 0.17 µg/l lindane at 24 and 36 hours whereas, SfG was significantly reduced at 2.03 µg/l lindane at 168 hours exposure. A 24-hour EC₅₀ for GST of 0.53 (95% CL , 0.27, 1.08) µg/l lindane compared with 168-hour IC₅₀s for respiration, energy absorbance and SfG of 0.6 (95 % CL, 0.52, 0.65) µg/l lindane, 0.8 (95% CL, 0.34, 1.29) and 0.63 (95% CI, 0.07, 1.28) µg/l lindane respectively.

With reference to the hypothesis of Hatch (1962), (Figure 1.3), it can be seen that increases in GST precede the onset of departures from 'homoeostasis' i.e. the increased energy expenditure measured by energy loss via respiration and decrease in the energy intake via energy absorption. Furthermore, the threshold of GST activity increase on exposure to alcohol ethoxylate in the artificial streams at 24 hours was indicative of the threshold effect concentration on feeding rate at one weeks exposure (Chapter Three and Appendix 2.2).

Long-term pre-exposure caused an increase in absorbed energy that was reflected in an increased SfG and glycogen concentration. Pre-exposure did confer some tolerance to further exposure in that SfG was not as negative as exposed control animals. Glycogen reserves were depleted, but only to a level that was found in control animals (Chapter Five). An increase in energy demand possibly due to an increased energetic cost of protein synthesis including the production of detoxification enzymes could therefore be countered by an increase in energy absorbance and energy reserve. Thus, according to the model of Calow and Townsend (1981) (see Figure 5.2), increased resource acquisition, through increased absorption had been allocated to glycogen reserves and may be available for growth. This has been reported in other crustacean species, for example, it was demonstrated by Donker (1992) that isopods from heavy metal contaminated sites had an increased food intake allowing an increase in the allocation of energy for reproduction. On exposure of pre-exposed, clean site

animals increased GST activity (Chapter Three). The same treatment caused a negative SfG and a decrease in glycogen concentration (Chapter Five). The increase in SfG observed on pre-exposure was lost on exposure due to increased respiratory costs possibly due to increased protein synthesis, and also a large decrease in absorbed energy.

In terms of the use of GST as a marker of effects (i.e. an early warning of effects that will be detrimental to the functioning of the animal), GST activity is more sensitive than the components of SfG. Furthermore, because mechanisms of detoxification may incur an energetic cost to the organism then there is a tentative link between induction of detoxification systems, one of which is GST, and the increase in energy expenditure. However, as with the use of GST as a biomarker of exposure, induction of increased activity of GST is of limited use if it is only apparent initially and early increases in GST activity are not detectable under longer exposure times. Thus, although short-term exposure studies demonstrated that GST activity was indicative of effects at the physiological level, long-term exposure to low concentrations of lindane did not increase GST, but did affect subsequent GST activity on further exposure. It is not surprising that an energetic cost of exposure to low levels of lindane was not detected using SfG, as this concentration did not significantly increase GST activity in the laboratory.

6.5 Conclusions.

Ecotoxicology is concerned with the assessment of the toxic effect of pollutants on ecosystems (Truhaut, 1977). Thus, the role of a biomarker in ecotoxicological studies is to assess the effect of pollutants at the level of the ecosystem (Weeks, 1995). By the use of a biochemical biomarker such as GST, an indication of xenobiotic exposure is given. Furthermore, because GST is a detoxification enzyme, the marker is directly involved in the process of resistance to the chemical to which the animal is exposed (depending on the xenobiotic) and thus is likely to be one of the first systems to respond to exposure. However, whilst signalling that an exposure has taken place, biomarkers such as GST may contribute little to the prediction of the direct consequences of exposure for the

organism or population in question. In order to link GST as a marker of exposure to effect, a particular biomarker response was related to a measure of impairment which directly affects functioning at the level of the organism in terms of energy available for growth (SfG) and energy in reserve (glycogen) which can be attributed to xenobiotic exposure.

It was concluded that;

- Extrinsic factors have an affect on GST activity thus 'baseline' level of activity in the field may be subject to fluctuation.
- A tentative baseline in the laboratory of GST activity of 0.02 to 0.04 $\mu\text{mol}/\text{min}/\text{mg}$ protein was suggested.
- The detection of exposure to the organic xenobiotics alcohol ethoxylate and lindane was rapid and sensitive.
- The use of GST as a marker of pesticide exposure in the field was consistent, but GST activities of contaminated-site animals should always be compared to GST activities of reference site animals.
- The use of GST as a biomarker of exposure *in situ* is to be approached with caution as the GST response is transitory in the short-term.
- GST could be used as an 'early warning' of the on-set of energetic changes that would affect growth and reproduction in *G. pulex* in the short-term.

However, long-term exposure failed to increase GST activity, possibly because the exposure concentration was too low. Long-term exposure had no detrimental effect on SfG, and the same low concentration increased SfG. This concentration may have been too low to induce GST and therefore there was no energetic cost of exposure. Furthermore, the selective increase of specific isoenzymes to low concentrations of lindane may have no detectable energetic cost.

Interpopulation differences were removed by long-term exposure to low concentrations of lindane. This may have been due to acclimation. However, interpopulation differences were maintained on holding in clean conditions and on further exposure. This observation may suggest increased GST activity in animals from the contaminated site may have been selected for by pesticide contamination in the field.

The work presented in the present study has demonstrated that GST is a consistent marker of exposure to pesticides in the field. Furthermore, GST activity was a rapid and sensitive response to lindane and alcohol ethoxylate exposure. However, the use of GST as a biomarker of pesticide exposure with *in situ* deployment of *Gammarus pulex* must be measured over a short time scale because of the transitory nature of the GST response in the laboratory and in the artificial streams. The energetic cost of exposure to lindane was demonstrated in the short-term and as GST activity increase was more sensitive, it was suggested that GST activity could be used as an 'early warning' of effects at the physiological level. Decreased scope for growth leads to the decreased availability of energy for growth and reproduction, such that a mechanistic link is made between changes in SfG and changes at the population level. The lack of a detrimental effect of long-term low concentration exposure on SfG was such that there was no estimated energetic cost of exposure to 'environmental' concentrations. However, exposure to a number of pesticides in the field and fluctuating levels of these chemicals coupled with environmental factor may have a different effect on SfG. The linking of the effects of field exposure to pesticides on SfG to population-level effect would necessitate the measurement of SfG *in situ* in clean and pesticide contaminated sites. This could be related to population level effects by measuring population growth and reproductive output of clean and contaminated populations. This then would enable the linking of SfG to population level effect and by linking effects on GST in contaminated populations to changes in the energy available for growth and reproduction and to changes at the population level the role of GST as a biomarker of ecotoxicological effects could be assessed.

In conclusion therefore, increase in GST activity may provide a rapid and sensitive biomarker of xenobiotic exposure which may be indicative of effects at higher levels of biological organisation. However, GST activity is affected by a variety of intrinsic and extrinsic factors and should therefore only be used as part of a comparative study. Because of the transient nature of the GST response, *in situ* assessments should be based on 24-hour exposure periods.

REFERENCES

Abel, P.D. (1980).

Toxicity of γ -hexachlorocyclohexane (Lindane) to *Gammarus pulex*: mortality in relation to concentration and duration of exposure.

Freshwater Biology, 10: 251-259.

Aceto, A., Di Ilio, C., Bucciavelli, T., Pantani, C., Del'Agata, M., Pannunzio, G. and Federici, G. (1991)

Characterisation of glutathione transferase from *Gammarus italicus*.

Comparative Biochemistry and Physiology, 99B: 523-527 (1991).

Adams, J. and Greenwood, P.J. (1983).

Why are males bigger than females in pre-copula pairs of *Gammarus pulex*?

Behavioural Ecology and Sociobiology, 13: 239-241.

Adams, S.M., Shepard, K.L., Greely, M.S., Ryon, M.G., Jiminez, B.D., Shugart, L.R., McCarthy, J.F. and Hinton, D.E. (1989).

The use of bioindicators for assessing the effects of pollutant stress in fish.

Marine Environmental Research, 28: 459-464.

Alin, P., Jesson, H., Guthenberg, C., Danielson, U.H., Tahir, M.K. and Mannervik, B. (1985).

Purification of major basic glutathione transferase isoenzymes from rat liver by use of affinity chromatography and fast protein liquid chromatofocusing.

Analytical Biochemistry, 146:313-320.

Almar, M.M., Diaz-Magans, J. and Romero, F.J. (1987)

Glutathione content and glutathione s-transferase activity in the midgut gland of *Procambarus clarkii*. Sex differences, the effect of fasting and their implications in cadmium toxicity.

Comparative Biochemistry and Physiology 87C:443-435.

Antunes-Madeira, M.C. and Madeira, V.M.C. (1985).

Partition of lindane in synthetic and native membranes.

Biochemica et Biophysica Acta, 820:165-172.

Armitage, P.D., Gunn, R.J.M., Furse, M.T., Wright, J.F., and Moss, D. (1987).

The use of prediction to assess macroinvertebrate response to river regulation.

Hydrobiologia, 144:25-32.

Armitage, P.D., Moss, D., Wright, J.F. and Furse, M.T. (1983).

The performance of a new biological water quality score system based on macroinvertebrates over a wide range of unpolluted running-water sites.

Water Research, 17:333-347.

Atkinson, D.E. (1987). *Cellular Energy Metabolism and its Regulation*.

Academic Press Inc. New York. p 293.

Baldwin, W.S. and LeBlanc, G.A. (1996)

Expression and induction of an immunochemically related class of glutathione s-transferase in *Daphnia magna*.

Comparative Biochemistry and Physiology, 113B: 261-267.

Balloch, D., Davis, C.E. and Jones, F.H. (1976).

Biological assessment of water quality in three British Rivers: The North Esk (Scotland), the Ivel (England) and the Taff (Wales).

Water Pollution Control, 75: 92-100.

Barnes, R.D. (1987).

Invertebrate Zoology (5th Edition).

CBS College Publishing, USA. pp. 554-665.

Baturo, W. and Lagadic, L. (1996).

Benzo[a]pyrene hydroxylase and glutathione s-transferase activities as biomarkers in *Lymnaea palustris* (Mollusca: Gastropoda) exposed to atrazine and hexachlorobenzene in freshwater mesocosms.

Environmental Toxicology and Chemistry, 15: 771-781.

Baturo, W., Lagadic, L. and Caquet, T. (1995).

Growth, fecundity and glycogen utilization in *Lymnaea palustris* exposed to atrazine and hexachlorobenzene in freshwater mesocosms.

Environmental Toxicology and Chemistry, 14: 503-511.

Bayne, B.L. and Widdows, J. (1978).

The physiological ecology of two populations of *Mytilus edulis* L.

Oecologia, 37: 137-162.

Bayne, B.L., Brown, D.A., Burnes, K., Dixon, D.R., Ivanovici, A., Livingstone, D.R., Lowe, D.M., Moore, M.N., Stebbing, A.R.D. and Widdows, J. (1985). *The Effects of Stress and Pollution on Marine Animals*. Praeger, New York. p 384.

Bayne, B.L., Moore, M.N., Widdows, J., Livingstone, D. and Salkeld, P. (1979). Measurement of the responses of individuals to environmental stress and pollution: Studies with bivalve molluscs.

Philosophical Transactions of the Royal Society of London B, 286 :563-581.

Beenackers, A (1969).

Carbohydrate and fat as a fuel for insect flight. A comparative study.

Journal of Insect Physiology, 15: 353-361.

Bend, J.R. and James, M.O. (1978).

Xenobiotic metabolism in marine and freshwater species. In: *Biochemical and Biophysical Perspectives in Marine Biology*. Eds. D.C. Malins and Sargent, J.R. Academic Press, New York.

Berges, J.A. and Ballentyne, J.S. (1991)

**Size scaling of whole-body maximal enzyme activities in aquatic crustaceans
Canadian Journal of Fisheries and Aquatic Science 48: 2385-2395.**

Bermingham, S. (1993).

**The effect of drainage waters from an abandoned coal-mine on leaf litter
processing in a freshwater stream. PhD Thesis, University of Sheffield, UK.**

Beverley, M.C. (1994)

**Enzymology of response of an aquatic organism to xenobiotics.
PhD Thesis, University of Huddersfield, U.K.**

Beverley, M.C., Garrood, A., Craven, M. and Johnson I. (1991).

**Presentation to SETAC- Europe Founding conference. University of Sheffield 7-
10 April 1991.**

Bhagyalakshmi, A., Reddy, P.S. and Ramamurthi, R. (1984).

**In vivo sub-acute physiological stress induced by sumithion on some aspects of
oxidative metabolism in the freshwater crab.**

Water, Air and Soil Pollution, 23: 257-262.

Blat, A., Almar, M.M. and Romero, F.J. (1988).

**The effect of two sulphur-containing pesticides, fenitrothion and endosulphan , on
glutathione (GSH) content and GSH S-transferase and γ -glutamyltranpeptidase
activities in midgut gland of the American red crayfish *Procambarus clarkii*.**

Drug Metabolism and Drug Interaction, 6: 383-394.

Blockwell, S., Taylor, E., Phillips and Pascoe, D. (1996a).

**A scanning electron microscope investigation of the effects of pollutants on the
hepatopancreatic caeca of *Gammarus pulex* (L.).**

Ecotoxicology and Environmental Safety, 35: 209-221.

Blockwell, S.J., Pascoe D. and Taylor E.J. (1996b).
Effects of lindane on the growth of the freshwater amphipod *Gammarus pulex*.
Chemosphere, 32:1795-1803.

Bluzat, R. and Seuge, J. (1979).
Effects of three insecticides (lindane, fenthion and carbaryl) on the acute toxicity
to four aquatic invertebrates species and the chronic toxicity to the pulmonate
mollusc *Lymnaea*.
Environmental Pollution, 18: 51-70.

Booth, J., Boyland, E. and Simms, P. (1961).
An enzyme from rat liver catalysing conjugations with glutathione.
Biochemistry Journal, 79: 516-524.

Boquené, G., Bellanger, C., Cadiou, Y. and Galgani, F. (1995).
Joint action of pollutants on the acetylcholinesterase activity of several marine
species.
Ecotoxicology, 4: 266-279.

Boryslawskyj, M., Garrod, A.C., Pearson, J.T. and Woodhead, D. (1987).
Rates of accumulation of dieldrin by a freshwater filter feeder: *Sphaerium*
corneum.
Environmental Pollution, 43: 3-13.

Boryslawskyj, M., Garrod, A.C. and Pearson, J.T. (1988).
Elevation of GST as a stress response to organochlorine compounds in
Sphaerium corneum.
Marine Environmental Research, 24: 101-104.

Boyland, E. and Chasseaud, L.F. (1968).
Enzymes catalysing conjugations of glutathione with $\alpha\beta$ -unsaturated carbonyl
compounds.
Biochemistry Journal, 109: 651-661.

Boyland, E. and Chasseaud, L.F. (1969)

The role of glutathione and glutathione S-transferase in mercapturic acid biosynthesis.

Advances in Enzymology and Related Areas of Molecular Biology 32:173-219.

Brattsten L.B., Evans C.K., Bonetti S. and Zalkow L. H. (1984).

Induction by carrot (*Daucus carota*) allelochemicals of insecticide metabolizing enzymes in the Southern army worm (*Spodoptera eridania*).

Comparative Biochemistry and Physiology, 79B: 26-38.

Brogdon, W.G. (1984)

Mosquito protein microassay. I. Protein determinations from small portions of single mosquito homogenates.

Comparative Biochemistry and Physiology 79B: 457-459.

Brogdon, W.G. (1988)

Microassay of acetylcholinesterase activity in small portions of single mosquito homogenates.

Comparative Biochemistry and Physiology. 90C:145-150.

Brogdon, W.G. and Barber A.M. (1987).

Microplate assay of acetylcholinesterase inhibition kinetics in single mosquito homogenates.

Pesticide Biochemistry and Physiology, 29: 252-259.

Brogdon, W.G., Beach, R.F., Stewart, J.M. and Castanaza, L. (1988).

Microplate assay analysis of the distribution of organophosphate and carbamate resistance in Guatemalan *Anopheles albimanns*.

Bulletin of the World Health Organisation. 66: 339-346.

Buikema, A.L., Niederlehner, B.R. and Cairns, J. Jr. (1982).

Biological Monitoring; Part IV- Toxicity testing.

Water Research, 16: 239-262.

Burton, G.A.Jr (1991).

Assessing the toxicity of freshwater sediments.

Environmental Toxicology and Chemistry, 10: 1585-1627.

Burton, A.G. Jr., Hickey, C. W., DeWitt, T.H., Roper, D.S., Morrissey, D.J. and Nipper, M.G. (1996).

In situ toxicity testing: Teasing out the environmental stressors.

Society of Environmental Toxicology and Chemistry Europe News, November 1996, Volume 7 Issue 6: 6-9.

C.A.B International, British Crop Protection Council. (1997).

The UK Pesticide Guide. Ed. G.W. Ivens.

CAB International Wallingford UK.

Calabrese, E., McCarthy, M. and Kenyon, E. (1987).

The occurrence of chemically induced hormesis.

Health Physics, 52: 531-541

Calow, P. (1989).

Ecotoxicology?

Journal of Zoology, 218: 701-704.

Calow, P. and Townsend, C.R. (1981).

Energetics, Ecology and Evolution. In: *Physiological Ecology: An Evolutionary Approach to Resource Use*. Eds. C.R. Townsend and P. Calow. Blackwell

Scientific Publications, Oxford. pp. 3-19.

Cairns, J. (1986).

The myth of the most sensitive species.

Bioscience, 36: 670-672.

Capuzzo, J. (1985).

The effects of pollutants on marine zooplankton populations: sublethal physiological indices of population stress.

In *Marine Biology of Polar Regions and Effects of Stress on Marine Organisms* (Eds. Gray, J and Christiansen, M), pp. 475-491. John Wiley and Sons, New York.

Capua, S., Cohen, E. and Gerson, U. (1991).

Induction of aldrin epoxidation and glutathione s-transferase in the mite *Rhizoglyphus robini*.

Entomologia Experimentalis Applicata, 59: 43-50.

Chandler, J.R. (1970).

A biological approach to water quality management.

Water Pollution Control, 4: 415-422.

Chasseaud, L.F. (1976).

Glutathione Metabolism and Function.

Eds. I.M. Arias and W.B. Jakoby. Raven New York

Chasseaud, L.F. (1979).

The role of glutathione and glutathione s-transferases in the metabolism of chemical carcinogens and other electrophilic agents.

Advances in Cancer Research, 29: 175-274.

Chutter, F.M. (1972).

An empirical biotic index of the water quality of water in South African streams and rivers.

Water Research, 6: 19-30.

Christman, M.F., Morgan, R.W., Jacobson, F.S. and Ames, B.N. (1985).

Positive control of a regulon for defences against oxidative and heat-shock proteins in *Salmonella typhimurium*.

Cell, 41: 753-762.

Clark, A.G. (1989).

The comparative enzymology of the glutathione s-transferases from non-vertebrate organisms.

Comparative Biochemistry Physiology, 92B: 419-446.

Clark, A.G. (1990).

The glutathione S-transferases and resistance to insecticides. In: *Glutathione S-Transferases and Drug Resistance. Proceedings of the 3rd International GST conference, Edinburgh, Scotland, 28-30 August, 1989*. Eds. J.D. Hayes, C.B. Pickett and T.J. Mantle. Taylor and Francis, London. pp.369-378.

Clark A.C, Hitchcock M and Smith J.N. (1966).

Metabolism of gammahexane in flies, ticks and locusts.

Nature 209: 103.

Clark, L., Gomme, J. and Hennings, S.(1991).

Study of pesticides in water from a chalk catchment, Cambridgeshire (England, U.K.).

Pesticide Science, 32: 15-34.

Collier, T.K. and Varanasi, U. (1991).

Hepatic activities of xenobiotic metabolizing enzymes and biliary levels of xenobiotics in English sole (*Pleuronectes vetulus*) exposed to environmental contaminations.

Archives of Environmental Contamination and Toxicology, 20: 462-473.

Collier, T.K, Stein, J.E, Wallace, R.J and Varanasi, U. (1986)

Xenobiotic metabolising enzymes in spawning English sole (*Parophrys vetulus*) exposed to organic-solvent extracts of marine sediments from contaminated and reference areas.

Comparative Biochemistry and Physiology, 84C: 91-298.

Cook, S.E.K. (1976).

**Quest for an index of community structure sensitive to water pollution.
Environmental Pollution, 11: 269-288.**

Crane, M., Delaney, P., Mainstone, C. and Clarke, S. (1995).

**Measurement by in situ bioassay of water quality in an agricultural catchment.
Water Research, 29: 2441-2448.**

Crossland, N.O and Beasley, N.M.R, (1990).

**A review of the fate and toxicity of lindane in aquatic environments.
Shell Research Ltd, External report; SBER.90.010.
Shell Internationale Research Maatschappij B.V. 1990.**

Darby, F.J. (1973).

**Storage of enzyme preparations.
Forensic Science, 2: 451-457.**

Del Boccio, G., Di Ilio, C., Ålin, P., Jörnvall, H. and Mannervik, B. (1987).

**Identification of a novel glutathione s-transferase in human skin homologous with
class alpha glutathione transferase 2-2 in the rat.
Biochem. J. (1987) 277, pp 21-25.**

Depledge, M.H. (1989).

**The rational basis for detection of the early effects of marine pollutants using
physiological indicators.
Ambio, 18:301-302.**

Depledge, M.H. and Fossi, M.C. (1994).

**The role of biomarkers in environmental assessment (2).
Invertebrates.
Ecotoxicology, 3: 161-172.**

Depledge, M.H., Amaral-Mendes, J.J., Daniel, B., Halbrook, R.S., Kloepper-Sams, P., Moore, M.N. and Peakall, D.B. (1992).

The conceptual basis of the biomarker approach. In: *Biomarkers: research and application in the assessment of environmental health*. Eds. D.B. Peakall and L.R. Shugart. NATO ASI Series H: Cell Biology, Volume 68, Springer-Verlag, Heidelberg, Berlin. pp. 15-29.

Dickson, G.W. and Giesy, J.P. (1981).

The effects of starvation on muscle phosphoadenylate concentrations and adenylate energy charge of surface and cave crayfish.

Comparative Biochemistry and Physiology, 71A: 357-361.

Dickson, G.W. and Giesy, J.P. (1982).

Seasonal variation of phosphoadenylate concentrations and adenylate energy charge in dorsal tail muscle of the crayfish, *Procambarus acutus acutus* (Decapoda: Astacidae).

Comparative Biochemistry and Physiology, 72A: 195-199.

Dierickx, P.J. (1984).

Glutathione s-transferase in aquatic macroinvertebrates and its interaction with different organic micropollutants.

The Science of the Total Environment, 40: 93-102.

Di Ilio, C., Aceto, A., Piccolomini, R., Allocati, N., Faraone, A., Cellini, L., Raragaan, G. and Federici, G. (1988).

Purification and characterisation of three forms of glutathione s-transferase from *Proteus mirabilis*.

Biochemical Journal, 255: 971-975.

Donker, M. (1992).

Energy reserves and distribution of metals in populations of the isopod *Porcellio scaber* from metal contaminated sites.

Functional Ecology, 5/6: 29-36.

Donkin, P. and Widdows, J. (1986). Scope for growth as a measurement of environmental pollution and its interpretation using structure-activity relationships.

Chemistry and Industry, November 1986, 732-737.

Dorn, P.B., Salanitro, J.P., Evans, S.H. and Kravetz, L. (1993).

Assessing the aquatic hazard of some branched and linear non-ionic surfactants by biodegradation and toxicity.

Environmental Toxicology and Chemistry, 12: 1751-1762.

Dorn, P.B., Rodgers, J.H., Dubey, S.T., Gillespie, W.B. and Figueroa, A.R. (1996).

Assessing the effects of a C14-15 linear alcohol ethoxylate surfactant in stream mesocosms.

Ecotoxicology and Environmental Safety, 34: 196-204.

Douglas, K.T. (1988).

Reactivity of Glutathione in Model systems for Glutathione s-transferase and Related enzymes. In: *Glutathione Conjugation; Mechanisms and Biological Significance*. Eds. H. Sies and B. Ketterer. Academic Press, London. pp. 2-43.

Downer, R and Matthews, J (1976).

Patterns of lipid distribution and utilization in insects.

American Zoology, 16: 733-745.

Drotar, A., G.A. Burton, Jr., J.E. Tavernier and Fall, R. (1987).

Widespread occurrence of bacterial thiol methyltransferases and biogenic emission of methylated sulfur gases.

Applied and Environmental Microbiology 53:1626-1631.

Edwards, C.A. and Fisher, S.W. (1991).

The use of cholinesterase measurements in assessing the impact of pesticides on terrestrial and aquatic invertebrates. In: *Cholinesterase-inhibiting Insecticides; Their Impact on Wildlife and the Environment*. Ed. P. Mineau. Elsevier. London. pp. 255-275.

Egaas, E. (1993).

A comparative study of effects of atrazine on xenobiotic metabolizing enzymes in fish and insect and of the *in vitro* phase II atrazine metabolism in some fish insects and one plant species.

Comparative Biochemistry and Physiology, 106C:141-149.

Egass, E., Svendsen, N.O. Korbros, S., Skaare, J.U. and Jensen, E.G. (1991).

Activities and properties of xenobiotic metabolizing enzymes in the peach-potato aphid (*Myzus persicae* Sulzer) feeding on paprika (*Capsicum annuum* L.) or swedes (*Brassica napus rapifera* Metzger).

Comparative Biochemistry and Physiology, 99C:105-110.

Egass, E., Skaare, J.U., Svendsen, N.O. and Sandvik, M., Falls, J.G.,

Dauterman, W.C., Collier, T.K. and Netland, J. (1993).

A comparative study of the effects of atrazine on xenobiotic metabolising enzymes in fish and insect, and of the *in vitro* phase II atrazine metabolism in some fish, insects, mammals and one plant species.

Comparative Biochemistry and Physiology, 106C: 141-149.

Egass, E., Skaare, J.U., Svendsen, N.O. and Sandvik, M. (1994).

Pesticide induced biochemical changes in terrestrial insects, benthos and fish markers of contamination of soils and waters.

In: *Biomarkers for pesticide contamination*. (1994) pp. 89-116.

Elliot, J. and Davison, W. (1975).

Energy equivalents of oxygen consumption in animals energetics.

Oecologia, 19: 195-201.

Engel, D.W. and Brouwer, M. (1984).

Trace metal-binding proteins in marine molluscs and crustaceans.

Marine Environmental Research, 14: 139-151.

Engel, D.W. and Brouwer. M. (1987).

Metallothioneins: A monitoring tool. In: *Pollution and Physiology of Estuarine Organisms*. Eds. F.J. Vernberg, F.P. Thurnberg, A. Calabrese and W.B. Vernberg. Columbia: University of South Carolina Press. pp 421-438.

Ernst, W.H.O and Peterson, P.J. (1994).

The role of biomarkers in environmental assessment (4).

Terrestrial plants.

Ecotoxicology, 3: 180-192.

Feijtel, T. and van de Plassche, T. (1995).

Environmental risk characterisation of major surfactants used in the Netherlands. Draft report for the National Institute of Public health and environmental Protection, Bilthoven, The Netherlands.

Finney D.J. (1971).

Probit Analysis. Cambridge University Press, 3rd Edition, p. 333.

Fish, H. (1992).

Freshwaters.

In: *Understanding our Environment (An Introduction to Environmental Chemistry and Pollution)*. Ed. R.M. Harrison. Royal Society of Chemistry, Cambridge. pp 53-90.

Förlin, L. and Andersson, T. (1985).

Storage conditions of rainbow trout liver cytochrome P-450 and conjugation enzymes.

Comparative Biochemistry and Physiology 80B :569-572.

Fournier, D., Cuany, A., Pralavorio, M., Bride, J.M. and Bergé, J.B. (1987).

Analysis of methidathion resistance mechanisms in *Phytoseiulus persimilis* A.H. Pesticide Biochemistry and Physiology, 28: 271-278.

Fukami, J. (1980).

Metabolism of several insecticides by glutathione S-transferase.

Pharmacological Theory, 10: 473.

Furse, M.T., Moss, D., Wright, J.F., and Armitage, P.D. (1984).

The influence of seasonal and taxonomic factors on the ordination and classification of running-water sites in Great Britain and on the prediction of their macroinvertebrate communities.

Freshwater Biology, 14: 257-280.

Galgani, F., Boquen , G. and Cadiou, Y. (1992).

Evidence of variation in cholinesterase activity in fish along a pollution gradient in the North sea.

Marine Ecology Progress Series, 91: 77-82.

Galindo, R., Medina, J. and Villagrana, L. (1996).

Physiological and biochemical changes in shrimp larvae (*Penaeus vannamei*)

Intoxicated with organochlorine pesticides.

Marine Pollution Bulletin, 32: 872-875.

Garrood, A. Akroyd, C., Beverley, M.C., Boryslawskyj, M. and Pearson J. T. (1989)

Poster presentation at 3rd International GST Conference, Edinburgh University - August 1989.

Gee, J.R. (1988).

Population dynamics and morphometrics of *Gammarus pulex* L.: evidence of seasonal food limitation in a freshwater detritivore.

Freshwater Biology, 19: 333-343.

Giesy, J.P., Duke, C.S., Denzer, S.R. and Dickson, G.W. (1981).

Phosphoadenylate concentrations and energy charge in two freshwater crustaceans: responses to physical and chemical stressors.

Proceedings of the International Association for theoretical and Applied Limnology, 21: 205-220.

George, S.G. (1993).

Enzymology and Molecular Biology of Phase II Xenobiotic-Conjugating Enzymes in Fish.

In: *Aquatic Toxicology (Molecular Biology and Cellular Perspectives)*.

Malins, D.C and Ostrander, G.K (Eds). pp 37-61.

Lewis Publishers, CRC Press, Florida USA.

George, S.G. and Young, P., Leaver, M. and Clarke, D. (1990).

Activities of pollutant metabolising and detoxification systems in the liver of the plaice, *Pleuronectes platessa*: sex and seasonal variations in non-induced fish.

Comparative Biochemistry and Physiology 96C :185-192.

Georghiou, G.P. (1990).

Overview of insecticide resistance.

In: *Managing resistance to agrochemicals. Form fundamental research to practical strategies*. Eds. M.B. Green, H.M. Le Baron and W.K. LeBaron. ACS Symposium Series No. 421, American Chemical Society, Washington DC. pp. 18-41.

Gledhill, T., Sutcliff, D.W. and Williams, W.D. (1976).

Key to the British Freshwater Crustacea: Malacostraca.

Freshwater Biological Association Scientific Publications 32:1-71.

Westlake, D.F., Casey, JH., Dawson, F.H., Lable M., Mann R.H.K. and Marker A.F.H. (1972). The chalk stream ecosystem. In: *Productivity problems of freshwaters*.

Eds. Z. Kajak and A. Hillbricht-Ilkowska. IBP-UNESCO

Symposium on Productivity Problems of freshwaters. Kazimierz Dolny, Poland. pp 615-635.

Grant, D.F., Bender, D.M. and Hammock, B.D. (1989).

Quantitative kinetic assays for glutathione s-transferase and general esterase in individual mosquitoes using an EIA reader.

Insect Biochemistry, 19:741-751.

Green, R.H. (1979).

Sampling Design and Statistical Methods for Environmental Biologists.

John Wiley and Sons, Ltd., New York. p 257.

Green, D.W.J, Williams, K.A and Pascoe, D. (1986).

Studies on the acute toxicity of pollutants to freshwater macroinvertebrates: 4.

Lindane (gamma-hexachlorocyclohexane).

Archiv. fur. Hydrobiologie, 106: 263-273

Grieg-Smith, P.W. (1991).

Use of cholinesterase measurements in surveillance of wildlife poisoning in farmland. In: *Cholinesterase-inhibiting Insecticides; Their Impact on Wildlife and the Environment*. Ed. P. Mineau. Elsevier. London. pp 127-150.

Habig, W.H. and Jakoby, W. (1981a).

Assays for differentiation of glutathione s-transferase.

Methods in Enzymology, 77: (Article 51), 398-405.

Habig, W.H. and Jakoby, W. (1981b).

Glutathione s-transferases (Rat and Human).

Methods in Enzymology, 77: (Article 27), 219-230.

Habig, W.H., Pabst, M.J and Jakoby, W. (1974).

Glutathione s-transferases (the first enzymatic step in mercapturic acid formation).

Journal of Biological Chemistry, 249:7130-7139.

Hamelink, J.L. and Waybrant, R.C. (1976).

DEE and lindane in a large-scale model lentic ecosystem.

Transactions of the American Fish Society, 105: 124-134.

Hamer, D.H. (1986).

Metallothionein.

Annual Review of Biochemistry, 55: 913-951.

- Hans, R.K., Khan, M.A., Farooq, M. and Beg, M.U. (1993).
Glutathione s-transferase activity in an earthworm (*Pheretima posthuma*) exposed to three insecticides.
Soil Biology and Biochemistry, 25: 509-511.
- Hansen, P.D. (1980).
Uptake and transfer of the chlorinated hydrocarbon lindane (gamma-BHC) in a laboratory freshwater food chain.
Environmental Pollution (Series A), 21: 97-108.
- Harwell, M.A., Cropper, W.P. and Ragsdale, H.L. (1978).
Nutrient cycling and stability: a reevaluation.
Ecology, 58:600-606.
- Hatch, T. (1962).
Changing objectives in occupational health.
Industrial Hygiene Journal, Jan/Feb: 1-7.
- Hawkins, A.J.S., Bayne, B.L. and Day, A.J. (1986).
Protein turnover, physiological energetics and heterozygosity in the blue mussel, *Mytilus edulis*: the basis of variable age specific growth.
Proceedings of the Royal Society of London, B, 229: 161-176.
- Haya, K., Waiwood, B.A. and Van Eckhaute, A. (1983).
Adenylate energy charge and ATPase activity: Potential Biochemical Indicators of Sublethal Effects caused by Pollutants in Aquatic Organisms. In *Aquatic Toxicology*. Ed J.O. Nriagu. John Wiley and Sons, Inc New York. pp 307-333.
- Hayakawa, T., Lemahieu, R.A. and Udenfriend, S. (1974).
Studies on glutathione-s-arene oxidase transferase; a sensitive assay and partial purification of the enzyme from sheep liver.
Archives of Biochemistry and Biophysics 162: 223-230.

- Hayaoka, T. and Dauterman, W.C. (1982).
Induction of GST by phenobarbital and pesticides in various house fly strains and its effect on toxicity.
Pesticide Biochemistry and Physiology, 17:113-119.
- Hayes, J.D. and Wolf, C.R. (1988).
Role of Glutathione transferase in Drug Resistance. In: *Glutathione Conjugation; Mechanisms and Biological Significance*. Eds. H. Sies and B. Ketterer.
Academic Press, London. pp. 316-350.
- Hazleton, G.A. and Lang, C.A. (1983).
Glutathione s-transferase in the yellow fever mosquito [*Aedes aegypti* (Louisville)] during growth and ageing.
Biochemistry Journal, 210: 281-287.
- Hellawell, J. (1977).
Biological surveillance and water quality monitoring.
In: *Biological Monitoring of Inland Fisheries*, Ed. J.S. Alabaster.
Applied Science, London. pp. 69-88.
- Hemingway, J., Small, G.J. and Monro, A.G. (1993a).
Possible mechanisms of organophosphorus and carbamate insecticide resistance in german cockroaches. (Dictyoptera: Blattellidae) from different geographical areas.
Journal of Economic Entomology, 86:1623-1630.
- Hemingway, J., Dunbar, S.J., Monro, A.G. and Small, G.J. (1993b).
Pyrethroid resistance in german cockroaches (Dictyoptera:Blattellidae):
Resistance levels and underlying mechanisms.
Journal of Economic Entomology, 86:1631-1638.
- Hendricks, J.D., Meyers, T.R. and Shelton, D.W. (1984).
Histological progression of hepatic neoplasia in rainbow trout (*Salmo gairdneri*).
National Cancer Institute Monographs, 65: 321-336.

Hilbish, T.J. and Koehn, R.K. (1985).

Dominance in physiological phenotypes and fitness at an enzyme locus.
Science, 229: 52-54.

Hill, I.R., Mattiesen, P. and Heimbach, F. (1993).

Guidance Document on Sediment Toxicity Tests and Bioassays for Freshwater and Marine Environments.

From the 'Workshop on Sediment Toxicity Assessment' held at Slot Moermond Conrescentrum Renesse, The Netherlands, 8-10 November 1993.

Society of Environmental Toxicology and Chemistry- Europe.

Hinton, D.E., Baumann, P.C., Gardner, G.R., Hawkins, W.E., Hendricks, J.D., Murchelano, R. A. and Okihiro, M.S. (1992).

Histopathologic Biomarkers. In: *Biomarkers; Biochemical, Physiological and Histological Markers of Anthropogenic Stress*. Eds. Huggett, R.J., Kimerle, R.A., Mehrle, P.M., Bergman, H.L. Society of Environmental Toxicology and Chemistry Special Publication Series. Lewis Publishers. London. pp 155-209.

Hinson, J.A. and Kadlubar, F.F. (1988).

Glutathione and Glutathione Transferases in the Detoxification of Drug and Carcinogen Metabolites. In: *Glutathione Conjugation; Mechanisms and Biological Significance*. Eds. H.Sies and B. Ketterer. Academic Press, London. pp. 236-280.

Hodgson, E. (1985).

Microsomal mono-oxygenases.

In: *Comprehensive insect physiology, biochemistry and pharmacology*, Volume 11. Eds. G.A. Kerkut and L.I. Gilbert. Pergamon Press, Oxford. pp. 225-321.

Hoffmann, A. and Parsons, P. (1991).

Evolutionary genetics and environmental stress.

Oxford University Press Inc., New York.

Holland, D.L. (1978).

Lipid reserves and energy metabolism in the larvae of benthic marine invertebrates. In: *Biochemical and Biophysical Perspectives in Marine Biology*, Volume 4. Eds. D.C. Malins and J.R. Sargents. Academic Press, Inc., New York. pp 85-123.

Hollingworth, R.M., Alstott, R.L., and Litzenberg, R.D. (1973).

Glutathione s-aryl transferases in the metabolism of parathion and its analogues. *Life Sciences*, 13: 191-199.

Holme, D.J. and Peck, H. (1993).

Analytical Biochemistry.

Longman Scientific and technical Publications, England.

Huggenberger, F. (1972).

Lindane in soil columns as influenced by water movement.

Oil science society American Proceedings, 36: 544-555.

Huggett, R.J., Kimerle, R.A., Mehrle, P.M., Bergman, H.L., Dickson, K.L., Fava, J.A., McCarthy, J.F., Parrish, R., Dorn, P.B., McFarland, V. and Lahvis, G. (1992). Introduction, In: *Biomarkers; Biochemical, Physiological and Histological Markers of Anthropogenic Stress*. Eds. Huggett, R.J., Kimerle, R.A., Mehrle, P.M., Bergman, H.L. Society of Environmental Toxicology and Chemistry Special Publication Series. Lewis Publishers. London. pp 1-3.

International Organisation for Standardization (ISO) (1979).

Assessment of the biological quality of rivers by a macroinvertebrate 'score'.

ISO/TC 147/SC 5/WG 6 N 5, p 18.

Ireland, D.S., Burton, G. A. and Hess, G.G. (1996).

In situ toxicity evaluations of turbidity and photoinduction of polycyclic aromatic hydrocarbons.

Environmental Toxicology and Chemistry, 15: 574-581.

Ivanovici, A.M. (1980).

Application of adenylate energy charge to problems of environmental impact assessment in aquatic organisms.

Helgoland Meeresunters, 33: 556-565.

James, M.O. (1989a).

Cytochrome P450 monooxygenase in crustaceans.

Xenobiotica, 19: 1063-1076.

James, M.O. (1989b).

Biotransformation and disposition of PAH in aquatic invertebrates. In:

Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment.

Ed. V. Varanasi. CRC Press, Florida.

James, M.O and K.M. Kleinow (1993).

Trophic Transfer of Chemicals in the Aquatic Environment.

In: *Aquatic Toxicology; Molecular Biochemistry and Cellular Perspectives.* Eds

D.C. Malins and G.K. Ostrander. Lewis Publishers, CRC Press, Florida USA. pp

1-23.

Jones, J.R., Tracy, B.H., Sebaugh, J.L., Hazelwood, D.H. and Smart, M.M. (1981).

Biotic index testing for ability to assess water quality of Missouri Ozark streams.

Transactions of the American Fish Society, 110: 627-37.

Ketterer, B., Meyer, D.J. and Clark, A.G. (1988).

Soluble Glutathione Transferase Isoenzymes. In: *Glutathione Conjugation;*

Mechanisms and Biological Significance. Eds. H. Sies and B. Ketterer.

Academic Press, London. pp 74-137.

Kleinow, K., Melancon, M.J. and Lech, J.J. (1987).

Biotransformation and induction: implications for toxicity, bioaccumulation and monitoring of environmental xenobiotics in fish.

Environmental Health Perspectives, 71: 105-119.

Koehn, R.K. (1978).

Physiology and biochemistry of enzyme variation: the interface of ecology and population genetics. In: *Ecological genetics: the interface*. Ed. P.F. Brussard. Springer-Verlag, New York. pp. 51-72.

Kolkwitz, R. and Marsson, M. (1908).

Okologie der pflanzlichen Saprobein.
Ber. dtsh. bot. Ges. 26: 505-519.

Kolkwitz, R. and Marsson, M. (1909).

Okologie der tierischen Saprobein.
Int. Rev. ges Hydrobiol. 2: 126-152.

Kurihara, N., Tanak, K. and Nakajima, M. (1979).

Mercapturic acid formation from lindane in rats.
Pesticide Biochemistry and Physiology, 10: 137-150

Lagadic, L., Cuany, A., Berge, J. and Echaubard, M. (1993).

Purification and partial characterisation of glutathione s-transferases from insecticide-resistant and lindane-induced susceptible *Spodoptera littoralis* (Boisd.) larvae.

Insect Biochemistry and Molecular Biology, 23: 467-474.

Lagadic, L, Caquet, T. and Ramade, F. (1994).

The role of biomarkers in environmental assessment (5).
Invertebrate populations and communities.
Ecotoxicology, 3: 193-208.

Laskowski, R. (1995).

Some good reasons to ban the use of NOEC, LOEC and related concepts in ecotoxicology.
Oikos, 73: 140-144.

Le Blanc, G.A. and Cochrane, B.J. (1985).

Modulation of substrate-specific glutathione s-transferase activity in *Daphnia magna* with concomitant effects on toxicity tolerance.

Comparative Biochemistry and Physiology, **82C**: 37-42.

LeBlanc, G.A. Hilgenberg, B. and Cochrane, B.J. (1988).

Relationships between the structures of chlorinated phenols, their toxicity and their ability to induce glutathione s-transferase activity in *Daphnia magna*.

Aquatic Toxicology, **12**: 147-156.

Le Bras, S. (1987).

Influence du lindane sur le métabolisme respiratoire d'*Asellus aquaticus* L.

Relation concentration -perturbation.

Hydrobiologia, **148**: 115-122.

LeBras, S. (1990).

Sensitivity of *Asellus aquaticus* to lindane in relation to biotic (size and metabolism) and abiotic factors (concentration of insecticide and temperature).

Revue des Sciences de L'eau, **3**: 183-193.

Lech, J.J., Vodcnik, M.J. and Elcombe, C.R. (1982).

Induction of monooxygenase activity in fish. In *Aquatic Toxicology*. Ed. L.J.

Weber. Raven Press. New York.

Lee, R., Polhemus, J., Cheng, L. (1975).

Lipids of the water-strider *Gerris remigis* Say (Heteroptera: Gerridae). Seasonal and developmental variations.

Comparative Biochemistry and Physiology, **51B**: 451-456.

Lee, R.F. (1982).

Mixed function oxygenase (MFO) in marine invertebrates. (1982).

Marine Biology Letters, **2**: 87-105.

Lee, R.F. (1988).

Glutathione s-transferase in marine invertebrates from Langesund fjord.
Marine Ecology Progress Series, 46: 33-36.

Liess, M. (in press).

Significance of agricultural pesticides on stream macroinvertebrate communities.
Proceedings of the International Association for Theoretical and Applied
Limnology.

Liess, M., Schulz, R. and Werner, U. (1993).

Macroinvertebrate dynamics in ditches as indicator for surface water runoff. An
ecological aspect for assessment of agricultural impact on running water
ecosystems.

Modeling Geo-Biosphere Processes, 2: 279-292.

Liess, M., R. Schulz and M. Neumann (1996).

A Method for monitoring pesticides bound to suspended particles in small
streams.

Chemosphere, 32: 1963-1969.

Livingstone, D.R. (1991).

Organic xenobiotic metabolism in marine invertebrates. In: *Advances in
Comparative and Environmental Physiology*. Ed. R. Gilles. Springer-Verlag,
Berlin. pp. 45-185.

Livingstone, D.R. (1989).

Cytochrome P-450 and oxidative metabolism in molluscs.
Xenobiotica, 19: 1041-1062.

Livingstone, D.R. (1993).

Biotechnology and pollution monitoring in the aquatic environment.
Journal of Chemical Technology and Biotechnology, 57: 195-211.

Livingstone, D.R., Förlin, L. and George, S.G. (1994).

Molecular biomarkers and toxic consequences of impact by organic pollution in aquatic organisms. In: *Water Quality and Stress Indicators in Marine and Freshwater Systems: Linking Levels of Organisation*. Ed. D.W. Sutcliffe. Freshwater Biological Association. pp 154-171.

Lowe, D.M., Moore, M.N. and Evans, B.M. (1992).

Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*.

Marine Ecology Progress Series, 91: 135-140.

Lowery, O.H., Rosebrough, N.J., Farr, F.A. and Randall, R.J. (1951).

Protein measurement with folin phenol reagent.

Biological Chemistry, 193: 265-275.

Marrs, K.A. (1996).

The functions and regulation of glutathione s-transferase in plants.

Annual Review Plant Physiology Plant Molecular Biology, 47:127-158.

Macek, K.J., Buxton, K.S., Derr, S.K., Dean, J.W. and Sauter, S. (1976).

Chronic toxicity of lindane to selected aquatic invertebrates and fishes.

National Technology Information Service PB-256, 334: 58.

Mackay, D.W. Soulsby, P.G. and Poodle, T (1973).

The biological assessment of pollution in streams.

Association of River Authorities Year Book and Directory, 189-197.

Magnum, C.P. and Towle, D.W. (1977).

Physiological adaptation to unstable environments.

American Science, 65:67-75.

Maitland, P.S. (1966).

Notes on the biology of *Gammarus pulex* in the River Endrick.

Hydrobiologia, 28: 142-152.

Malbouisson, J.F.C, Young, T.W.K. and Bark A.W. (1995).

Use of feeding rate and re-pairing of precopulatory *Gammarus pulex* to assess toxicity of Gamma-hexachloro-cyclohexane (lindane).

Chemosphere, 30:1573-1583.

Maltby, L. (1994).

Stress, shredders and streams: using *Gammarus* energetics to assess water quality. In: *Water Quality and Stress in Marine and Freshwater Systems: Linking Levels of Organisation*. Ed. D.W. Sutcliffe. Freshwater Biological Association. pp 98-110.

Maltby, L. (1992).

The use of physiological energetics of *Gammarus pulex* to assess toxicity: A study using artificial streams.

Environmental Toxicology and Chemistry, 11: 79-85.

Maltby, L. and Calow, P. (1989).

The application of bioassays in the resolution of environmental problems; past, present and future.

Hydrobiologia, 188/189: 65-76.

Maltby, L and Naylor, C (1990).

Preliminary observations on the ecological relevance of the *Gammarus* 'scope for growth' assay: effect of zinc on reproduction.

Functional Ecology, 4: 393-397.

Maltby, L., Naylor, C. and Calow, P. (1990).

Effect of stress on a freshwater benthic detritivor : scope for growth in *Gammarus pulex*.

Ecotoxicology and Environmental Safety, 19: 285-291.

Maltby, L., Forrow, D.M., Boxall, A.B.A., Calow, P. and Betton, C.I. (1995).

The effects of motorway runoff on freshwater ecosystems: 1. Field study.

Environmental Toxicology and Chemistry, 14: 1079-1092.

Mannervik, B. (1985).

The isoenzymes of glutathione s-transferase.

Advances in Enzymology, 57: 357-417.

Mannervik, B., Ålin, P., Guthenberg, C., Jensson, H., Tahir, M., Warholm, M. and Jörnvall, H. (1985).

Identification of three classes of cytosolic glutathione transferase common to several mammalian species: Correlation between structural data and enzymic properties.

Proceedings of the National Academy of Sciences USA, 82: 7202-7206.

Margalef, R. (1956).

Diversidad de especies en las comunidades naturales.

Publ. Inst. de Biol. Apl., 9: 5-28.

Matthiessen, P., Sheahan, D., Harrison, R., Kirby, M., Rycroft, R., Turnbull, A., Volkner, C. and Williams, R. (1995).

Use of a *Gammarus pulex* bioassay to measure the effect of transient carbofuran runoff from farmland.

Ecotoxicology and Environmental Safety, 30: 111-119.

Mayer, F.L., Versteeg, D.J., McKee, M.J., Folmar, L.C., Graney, R.L., McCume, D.C. and Rattner, B.A. (1992).

Physiological and Nonspecific Biomarkers. In: *Biomarkers; Biochemical, Physiological and Histological Markers of Anthropogenic Stress*. Eds. Huggett, R.J., Kimerle, R.A., Mehrle, P.M., Bergman, H.L. Society of Environmental Toxicology and Chemistry Special Publication Series. Lewis Publishers. London. pp 5-85.

Metcalf, R.L. (1989).

Insect resistance to insecticides.

Pesticide Science, 26: 333-358.

**PAGE
MISSING
IN
ORIGINAL**

Morgenstern, R. and Depierre, J.W. (1988).

Membrane-bound Glutathione Transferases. In: *Glutathione Conjugation; Mechanisms and Biological Significance*. Eds. H. Sies and B. Ketterer.

Academic Press, London. pp 157-175.

Morgenstern, R., Lundqvist, G., Mosialou, E. and Andersson, C. (1990).

Membrane-bound glutathione transferase: function and properties. In:

Glutathione S-Transferases and Drug Resistance. Proceedings of the 3rd International GST conference, Edinburgh, Scotland, 28-30 August, 1989. Eds.

J.D. Hayes, C.B. Pickett and T.J. Mantle. Taylor and Francis, London. pp 57-64.

Moriarty, F.(1991).

Ecotoxicology; The Study of Pollutants in Ecosystems.

Academic Press, London.

Moss, D., Furse, M.T., Wright, J.F., and Armitage, P.D. (1987).

The prediction of the macroinvertebrate fauna of polluted running-water sites in Great Britain using environmental data.

Freshwater Biology, 17: 41-52.

Motoyama, N. and Dauterman, W.C. (1980).

Glutathione S-transferases: their role in the metabolism of organophosphorus insecticides. In: *Reviews in Biochemical Toxicology*. Volume 2. Eds. E.

Hodgson, J.R. Bend and R.M. Philpot. Elsevier/North Holland,

NewYork/Amsterdam. pp. 49-69.

Motoyama, N. and Dauterman, W.C. (1972).

In vitro metabolism of azinphosmethyl in susceptible and resistant house-flies.

Pesticide Biochemistry and Physiology, 2: 113-122.

Nagel, R (1993).

Ecotoxicology - an emerging science.

In: Fish Ecotoxicology and Ecophysiology.

Bruanbeck T, Hanke W, Senger H (Eds). pp 7-11.

Verlagsgesellschaft Weinheim.

Naylor, C., Maltby, L. and Calow, P. (1989).

Scope for growth in *Gammarus pulex* a freshwater detritivor.

Hydrobiologia, 188/189: 517-523.

Nimmo, I.A. (1985).

The glutathione s-transferase activity in the gills of the Rainbow trout (*Salmo gairdneri*).

Comparative Biochemistry and Physiology, 80B: 365-369.

Nimmo, I.A. (1986).

The glutathione s-transferase activity in the pyloric caeca of *Salmo gairdneri*.

Comparative Biochemistry and Physiology, 83B: 831-835.

Nimmo, I.A. and Spalding, C.M. (1985).

The glutathione s-transferase activity in the kidney of *Salmo gairdneri*.

Comparative Biochemistry and Physiology, 82B: 91-94.

Oppenoorth, F.J. (1984).

Biochemistry of insecticide resistance.

Pesticide Biochemistry and Physiology, 22: 187-193.

Oppenoorth, F.J. (1985).

Biochemistry and genetics of insecticide resistance.

In: *Comprehensive insect physiology, biochemistry and pharmacology*, Volume

12. Eds. G.A. Kerkut and L.I. Gilbert. Perganom Press, Oxford. pp. 713-773.

Ortiz de Montellano, P.R. (1986).

Cytochrome P-450: Structure, Mechanism and Biochemistry.

Ed. P.R. Ortiz de Montellano. Plenum Press. New York.

Ottea, J.A. and Plapp, F.W. (1984).

Glutathione s-transferase in the housefly: biochemical and genetic changes associated with induction and insecticide resistance.

Pesticide Biochemistry and Physiology, **22**: 203-208.

Pabst, M.J., Habig, W.H. and Jakoby, W.B. (1974).

Glutathione s-transferase A: a novel kinetic mechanism in which the major reaction pathway depends on substrate concentrations.

Journal of Biological Chemistry, **249**: 7140-7148.

Parsons, P. (1989).

Acetaldehyde utilization in *Drosophila*: an example of hormesis.

Biological Journal of the Linnean Society, **37**: 183-189.

Pascual, P., Martinez-Lara, E., Toribio, F., Lopez-Barea, J. and Barcena, A.J. (1991).

HPLC isoenzyme patterns of glutathione transferase from marine fishes with different levels of pollution.

Biochemical Society Transactions, **19**: 302S.

Peakall, D.B. (1994).

The role of biomarkers in environmental assessment (1). Introduction.

Ecotoxicology, **3**: 157-160.

Peakall, D.B. and Walker C.H. (1994).

The role of biomarkers in environmental assessment (3). Vertebrates.

Ecotoxicology, **3**: 173-179.

Peters, R.H. (1983).

The ecological implications of body size.

Cambridge University Press. Cambridge U.K.

Phillips, D.J.H (1980).

Quantitative Aquatic Biological Indicators. Their use to monitor trace metal and organochlorine pollution. Applied Science, London.

Pratt, J.M. and Coler, R.A. (1976).

A procedure for the routine biological evaluation of urban runoff in small rivers. *Water Research*, 10: 1019-1025.

Preston, A. (1979).

Standards and Environmental criteria: the practical application of the results of laboratory experiments and field trials to pollution control.

Philosophical Transactions of the Royal Society of London, B 286: 611-624.

Punzo, F. (1993).

Detoxification enzymes and the effects of temperature on toxicity of pyrethroids to the fall armyworm *Spodoptera fugiperda* (Lepidoptera: Noctuidae).

Comparative Biochemistry and Physiology, 10C: 155-158.

Repetto, G., Sanz, P. and Repetto, M. (1988).

In vivo and *in vitro* effect of triclofon on esterases of the red crayfish *Procambarus clarkii*.

Bulletin of Environmental Contamination and Toxicology, 41: 597-603.

Ramage, P.I.N. and Nimmo, I.A. (1984).

The substrate specificities and substrate compositions of the hepatic glutathione s-transferases of *Salmo gairdneri*.

Comparative Biochemistry and Physiology, 78B: 189-194.

Rodriguez-Ariza, A., Abril, N., Navas, J.I., Dorado, G., Lopez-Barea, J. and Pueyo, C. (1992).

Metal, mutagenicity and biochemical studies on bivalve molluscs from Spanish coasts.

Environmental and Molecular Mutagenesis, 19:112-124.

Roddie, B., Kedwards, T. and Crane, M. (1992).

Potential impact of watercress farm discharges on the freshwater amphipod, *Gammarus pulex* L.

Bulletin of Environmental Contamination Toxicology, 48: 63-69.

Rumpf, S., Eason, C.T. and Penman, D.R. (1994).

Biomarkers of insecticide contamination in lacewings.

Poster presentation at SECOTOX annual meeting, Zurich, September 1994.

Saunders, H.O. (1969).

U.S. Bureau of sport fisheries and wildlife. Technical paper No. 25. U.S. Government printing office, Washington D.C.

Saleh, M.A., Motoyama, N. and Dauterman, W.C. (1978).

Reduced glutathione in the housefly concentration during development and variation in strains.

Insect Biochemistry, 8: 311-316.

Schulz, R. (in press).

Macroinvertebrate dynamics in a stream receiving insecticide-contaminated runoff. Proceedings of the International Association for Theoretical and Applied Limnology.

Sheehan, D., Crimmins, K.M. and Burnell, G.M. (1991).

Evidence for glutathione s-transferase activity in *Mytilus edulis* as an index of chemical pollution in marine estuaries. In: *Bioindicators and Environmental Management*. Eds. D.W. Jeffrey and B. Madden. Academic Press, London. pp 420-425.

Sheehan, D. and Casey, J.P. (1993).

Microbial glutathione s-transferases.

Comparative Biochemistry and Physiology, 104B: 1-6.

Shirley, T. and Stickle, W (1982).

Responses of *Leptasterias hexactis* (Echinodermata: Asteroidea) to low salinity II.

Nitrogen metabolism, respiration and energy budget.

Marine Biology, 69: 155-163.

Shugart, L.R. (1988).

Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay.

Aquatic Toxicology, 13: 32-52.

Shugart, L., Bickham, J., Jackim, G., McMahon, G., Ridley, W., Stein, J. and Steinert, S. (1992).

DNA Alterations. In: *Biomarkers; Biochemical, Physiological and Histological Markers of Anthropogenic Stress*. Eds. Huggett, R.J., Kimerle, R.A., Mehrle, P.M., Bergman, H.L. Society of Environmental Toxicology and Chemistry Special Publication Series. Lewis Publishers. London. pp 125-153.

Sibly, R. and Calow, P. (1986).

Physiological ecology of animals: an evolutionary approach.

Blackwell Scientific Publications, Oxford.

Sladeczek, V. (1973).

Systems of water quality from the biological point of view.

Archiv. Hydrobiol. Beih. Erg. Limnol., 7: 1-218.

Slooff, W. (1983).

Benthic macroinvertebrates and water quality assessment, some toxicological considerations.

Aquatic Toxicology, 4: 73-82.

Slooff, W. and De Zwart, D. (1983).

Bioindicators and chemical pollution of surface waters.

Environmental Monitoring and Assessment, 3: 237-245.

Smyly, W.J.P. (1957).

The life history of the bullhead or miller's thumb (*Cottus gobio* L.).

Proceedings of the Zoological Society of London, 128: 431-453.

Spalding, R.F. and D.D. Snow (1989).

Stream levels of agrochemicals during a spring discharge event.

Chemosphere, 19: 1129-1140.

Sparkes, T., Keogh, D. and Pary, R. (1996).

Energetic costs of mate guarding behaviour in male stream isopods.

Oecologia, 106: 166-171.

Stegeman, J.J., Brouwer, M., Di Giulio, R.T., Förlin, L., Fowler, B.A.,

Sanders, B.M. and Van Veld, P.A. (1992).

Molecular responses to environmental contamination: Enzymes and protein systems as indicators of chemical exposure and effect. In: *Biomarkers;*

Biochemical, Physiological and Histological Markers of Anthropogenic Stress.

Eds. Huggett, R.J., Kimerle, R.A., Mehrle, P.M., Bergman, H.L. Society of Environmental Toxicology and Chemistry Special Publication Series. Lewis Publishers. London. pp 235-335.

Stegeman, J. (1981).

Polycyclic hydrocarbons and their metabolism in the marine environment. In:

Polycyclic Aromatic Hydrocarbons and Cancer. Volume 3. Eds. H. Gelboin and P.Ts'o. Academic Press. New York. pp 1-60.

Stenersen, J., Kobro, S., Bjerke, M. and Arend, U. (1987).

Glutathione transferase in aquatic and terrestrial animals from nine phyla.

Comparative Biochemistry and Physiology, 86C: 73-82.

Stephenson R.R (1983).

Effects of water hardness, water temperature, and size of the test organism on the susceptibility of the freshwater shrimp, *Gammarus pulex* (L.), to toxicants.

Bulletin of Environmental Contamination and Toxicology, 31: 459-466.

Sternburg, J. and Kerns, C.W. (1956).

Pentachlorocyclohexene, an intermediate in the metabolism of lindane by house flies.

Journal of Economic Entomology, 49: 548.

Stickle, W., Rice, S. and Moles, A. (1984).

Bioenergetics and survival of the marine snail *Thais lima* during long-term oil exposure.

Marine Biology, 80: 281-289.

Stitt, M., Bulpin, P. and Rees, T. (1978).

Pathway of starch breakdown in photosynthetic tissues of *Pisum sativum*.

Biochimica et Biophysica Acta, 544: 200-214.

Stuhlbacher, A. and Maltby, L. (1992).

Cadmium resistance in *Gammarus pulex* (L.).

Archives of Environmental Contamination and Toxicology, 22: 319-324.

Sutcliffe, D.W. and Carrick, T.R. (1985).

Freshwater shrimps (*Gammarus* and *Crangonyx*).

The Freshwater Biological Association, Windermere, England.

Sutcliffe, D.W., Carrick, T.R. and Willoughby, L.G. (1981).

Effects of diet, body size, age and temperature on growth rates in the amphipod *Gammarus pulex*.

Freshwater Biology, 11: 183-214.

Sugiyama, Y. Yamada, T. and Kaplowitz, N. (1981).

Glutathione s-transferases in elasmobranch liver.

Biochemistry Journal, 199: 749-756.

Talmage, S.S. (1994).

Environmental safety assessment of major surfactants: alcohol ethoxylates and alkylphenol ethoxylates.

The Soap and Detergent Association. Lewis Publishers Ann Arbor, MI.

Tanaka, K., Nakajima, M. and Kurihara, N. (1981).

The mechanism of resistance to lindane and hexadeuterated lindane in the third Yumenoshima strain of housefly.

Pesticide Biochemistry and Physiology, 16:149-157.

Tate, L.G. and Herf, D.A. (1978).

Characterization of glutathione s-transferase activity in tissues of the blue crab *Callinectes sapidus*.

Comparative Biochemistry and Physiology, 61C: 165-169.

Tattersfield, L.J., Young, L.J., Pearson, N. and Davies, E.H. (1996).

Neodol 25-9: Fate and effects in outdoor artificial streams and pools.

Shell Research Report, SBER.95.013, Shell Internationale Research Maatschappij BV. The Hague.

Taylor, E., Maund, S. and Pascoe, D. (1991).

Toxicity of four common pollutants to the freshwater macroinvertebrates *Chironomus riparius* and *Gammarus pulex*.

Archives of Environmental Contamination and Toxicology, 21: 371-376.

Thaker, A.A and Haritos, A.A. (1989).

Cadmium bioaccumulation and effects on soluble peptides, protein and the enzymes in the hepatopancreas of the shrimp *Callinassa tyrrhena*.

Comparative Biochemistry and Physiology, 94C: 63-70.

- Thomas, P., Wofford, H.W. and Neff, J.M. (1981).
Biochemical stress responses of striped mullet (*Mugil cepalus* L.) to Fluorone
Analog.
Aquatic Toxicology, 1: 329-342.
- Thyband, E. and LeBras, S. (1988).
Absorption and elimination of lindane by *Asellus aquaticus*.
Bulletin of Environmental Contamination and Toxicology, 40: 731-735.
- Timbrell, J.A., Draper, R. and Waterfield, C.J. (1994).
Biomarkers in toxicology: New use of some old molecules?
Toxicology and Ecotoxicology News, 1: 4-14.
- Thyband, E., LeBras, S. and Cosson, R.P. (1987).
Study of the comparative sensitivity of *Asellus aquaticus* with reference to
pesticides.
Oecologia Applicata, 8 :355-361.
- Timbrell, J.A. (1991).
Principles of Biochemical Toxicology. Second Edition.
Taylor and Francis. London.
- Truhaut, R. (1977).
Ecotoxicology: Objectives, principles and perspectives.
Ecotoxicology and Environmental Safety, 1: 151-173.
- Ulmann, E. (1972).
Lindane - Monograph of an insecticide.
Verlag K Schillinger, Freiburg.
- Van Gestel, C.A.M. and Van Brummelen, T.C. (1996).
Incorporation of the biomarker concept in ecotoxicology calls for a redefinition
of terms.
Ecotoxicology, 5: 217-225.

Van Handel, E. (1974).

Lipid utilization during sustained flight of moths.

Journal of Insect Physiology, 20: 2329-2332.

Veerasingham, M. and Crane, M. (1992).

Impact of farm waste on freshwater invertebrate abundance and the feeding rate of *Gammarus pulex* L.

Chemosphere, 25: 869-874.

Voogt, P.A. (1983) Lipids: Their distribution and metabolism. *In The Mollusca, Volume 1. Metabolic Biochemistry and Molecular Biomechanics*. Ed. P.W.

Hochachka. Academic Press London. pp. 510-521.

Wang, I.Y., Tung, E., Wang, A., Argenbright, L., Wang, R., Pickett, C.B. and Lu, A.Y.H., (1986).

Multiple Ya subunits of glutathione s-transferase detected by monoclonal antibodies.

Archives of Biochemistry and Biophysics, 245: 543-547.

Wardlaw (1985).

Practical statistics for experimental biologists.

John Wiley and Sons, Chichester UK. p. 104 .

Warren, C.E. and Davis, G.E. (1967).

Laboratory studies on the feeding, bioenergetics and growth of fish.

In: *The Biological Basis of Freshwater Fish Production*. Ed S.D. Gerking.

Blackwell Scientific Publications Oxford. pp 175-214.

Watson, D.G. (1987).

Post-radiolabeling for detecting DNA damage.

Mutagenesis, 2: 319-331.

Weast, R.C, Selby, S.M. and Hodgman, C.D. (1964).

Handbook of Chemistry and Physics, (45th Edition).

Chemical Rubber Company, Ohio.

Webster, J.R., Waide, J.B and Pattern, B.C. (1975).

Nutrient cycling and the stability of ecosystems. In: *Mineral cycling in southeastern ecosystems*. Eds. F.G. Howell, J.B. Gentry and B.C. Pattern. Springfield, VA:ERDA.

Weeks, J.M., (1995).

The value of biomarkers for ecological risk assessment: academic toys or legislative tools?

Applied Soil Ecology, 2: 215-216.

Weeks, J.M. and Svendsen, C. (1996).

Neutral red retention by lysosomes from earthworm (*Lumbricus rubellus*) coelomocytes: a simple biomarker of exposure to soil copper.

Environmental Toxicology and Chemistry, 15: 1801-1805.

Welch, W.J. (1990).

The mammalian stress response: Cell physiology and biochemistry of stress proteins. In: *The Role of the Stress Response in Biology and Disease*. Eds. R. Morimoto, A. Tissieres and C. Georgopoulos. Cold Spring Harbour, N.Y. Cold Spring Harbour Laboratory.

Welton, J.S. (1979).

Life-history and production of the amphipod *Gammarus pulex* in a Dorset chalk stream.

Freshwater Biology, 9: 263-275.

Welton, J.S., Ladle, M., Bass, J.A.B. and John, I.R. (1983).

Estimation of gut throughput time in *Gammarus pulex* under laboratory and field conditions with a note on the feeding of young in the brood pouch.

Oikos, 41:133-138.

Werner, U. (1993).

Ökotoxikologische Untersuchungen zur Insektizidempfindlichkeit von *Gammarus pulex* (L.). Diploma Dissertation, Technical University of Braunschweig, Germany.

Werner, U. (1994).

Population dynamics of *Gammarus pulex* (L.) in two lowland brooks of Lower Saxony in relation to temperature regime.

Lauterbornia, 15: 125-135.

Westlake, D.F., Casey, H., Dawson, F.H., Ladle, M., Mann, R.H.K. and Marker, A.F.H. (1972).

The chalk-stream ecosystem. In: *Productivity Problems of Freshwaters*. (Eds. Z. Kajak and A. Hillbricht-Ilkowska), Proceedings of the IBP-UNESCO Symposium on Productivity of Freshwaters, Kazimierz Dolny, Poland. pp. 615-635.

Widdows, J. and Bayne, B.L. (1971). Temperature acclimation of *Mytilus edulis* with reference to its energy budget.

Journal of the Marine Biological Association, U.K, 51: 827-843.

Widdows, J., Donkin, P. and Evans, S.V. (1987).

Physiological responses of *Mytilus edulis* during chronic oil exposure and recovery.

Marine Environmental Research, 23: 15-32.

Widdows, J. and Page, D.S. (1993).

Effects of tributyltin and dibutyltin on the physiological energetics of the mussel, *Mytilus edulis*.

Marine Environmental Research, 35:233-249.

Wilhm, J.I. and Dorris, T.C. (1968).

Biological parameters for the water quality criteria.

Bioscience, 18: 477- 481.

Williams, K., Green, D. and Pascoe, D. (1984).

Toxicity testing with freshwater macroinvertebrates: Methods and application in environmental management.

In: *Freshwater Biological Monitoring*. Ed D. Pascoe and R.W., Edwards.

Pergamon Press, Oxford. pp 81-91.

Willoughby, L.G. and Sutcliffe, D.W. (1976).

Experiments on feeding and growth of the amphipod *Gammarus pulex* (L.) related to its distribution in the River Duddon.

Freshwater Biology, 6; 577-586.

Willoughby, L.G. and Earnshaw, R. (1982).

Gut passage times in *Gammarus pulex* (Crustacea: Amphipoda) and aspects of summer feeding in a stony stream.

Hydrobiologia, 97: 105-117.

Willows, R.I. (1994).

The ecological impact of different mechanisms of chronic sub-lethal toxicity on feeding and respiratory physiology. In: *Water Quality and Stress in Marine and Freshwater Systems: Linking Levels of Organisation*. Ed. D.W. Sutcliffe.

Freshwater Biological Association. pp 88-97.

Willows, J. and Page, D.S. (1993).

Effects of tributyltin and dibutyltin on the physiological energetics of the mussel, *Mytilus edulis*.

Marine Environmental Research, 35: 233-249.

Wood, J.M.(1987).

Biological processes involved in the cycling of elements between soil or sediments and the aqueous environment.

Hydrobiologia 149: 31-42.

Woodiwiss, F.S. (1964).

The Trent Biotic Index- Macroinvertebrates in biological surveillance.

Elaboration of the Scientific Bases for Monitoring of Surface Water by

Hydrobiological Indicators. Report of the First U.K./U.S.S.R. Seminar held at Valdai, U.S.S.R., 12-14 July, 1976, pp 58-81.

Wright, J.F., Moss, D., Armitage, P. D. and Furse, M.T. (1984).

A preliminary classification of running-water sites in Great Britain based on macroinvertebrate species and the prediction of community type using environmental data.

Freshwater Biology, 14: 221-256.

Wright, J.F., Armitage, P.D., Furse, M.T. and Moss, D. (1989).

Prediction of invertebrate communities using stream measurements.

Regulated Rivers: Research and Management, 4: 147-155.

Wrona, F. and Davies, R. (1984).

An improved flow-through respirometer for aquatic macroinvertebrate bioenergetic research.

Canadian Journal of Fisheries and Aquatic Science, 41: 380-385.

Yu, S.J. (1987).

Biochemical defence capacity in the spined soldier bug (*Podisus maculiventris*) and its lepidopterous prey.

Pesticide Biochemistry and Physiology 28: 16-223.

Yu, S.J. (1982).

Host plant induction of glutathione s-transferase in the fall armyworm.

Pesticide. Biochem. Physiol. 18: 101-106.

Zamer, W and Shick, J.M. (1987).

Physiological energetics of the intertidal sea anemone *Anthopleura elegantissima*.

Marine Biology, 93: 481-491.

Zar, J.H., (1996).

Biostatistical Analysis. 3rd Edition.

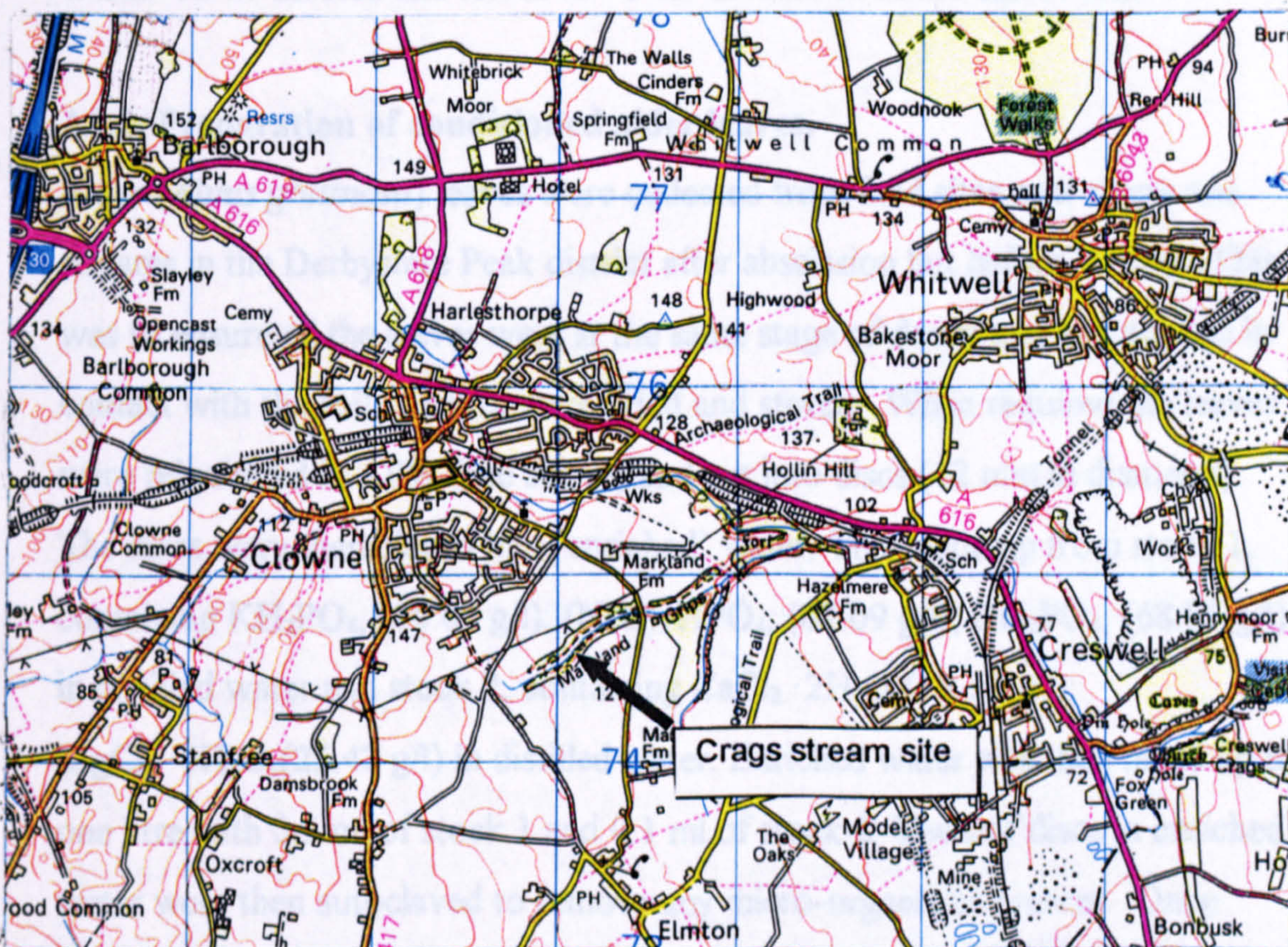
Prentice-Hall international (U.K.) London.

APPENDIX 1

A1.1. The field site at Craggs Stream.

The section of map below indicates the location of the field site at Craggs stream, near Clowne, Derbyshire (NGR: SK497745). *Gammarus pulex* used in the present study were collected from this site for the work detailed in Chapters Two, Three and Five.

Figure A1.1. Section of map of the field site at Craggs stream. The position of the site is indicated. Map reproduced from: Ordnance Survey, 'Landranger' 120 (Mansfield, Worksop and surrounding area), scale: 2 cm to 1 km (1: 50,000).



A1.2. Preparation of artificial pond water (APW).

Four stock solutions were prepared as shown in Table A1.1.

Table A1.1. Stock solution for APW. Stock solution are numbered according to the order added to distilled water.

Stock solution	Concentration (g/l)
1. $\text{CaCl}_2 \cdot \text{H}_2\text{O}$	58.80
2. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	24.65
3. NaHCO_3	12.95
4. KCl	1.15

To make up APW, 5 ml of each of the stock solutions were added in the order indicated in Table A1.1 (to avoid precipitation) to make up one litre of distilled water. APW aerated and left at 15 °C for at least 24 hours before use.

A1.3. Preparation of conditioned alder leaves.

Alder (*Alnus glutinosa*) leaves were collected from field sites near rivers and streams in the Derbyshire Peak district after abscission but before leaf fall. This was to ensure all the leaves were at the same stage of decay and had not been in contact with the soil. Leaves were dried and stored. When required the leaves were rehydrated in APW (see above) and cut into discs (12 mm in diameter). The discs were then placed into 'enriched' water. This made up from stock 1, containing KH_2PO_4 , (66.04 g/l); $(\text{NH}_4)_2\text{HPO}_4$, (87.09 g/l); KH_2PO_4 , (68.05 g/l) in distilled water and stock 2, containing $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, (18.38 g/l); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, (25.42 g/l) in distilled water. Enriched water was then made up to one litre with 0.5 ml of stock 1 and 0.1 ml of stock 2. The leaf discs in enriched water were then autoclaved to remove any micro-organisms present. Once cooled the discs were inoculated with the fungus *Cladosporium* sp. Using aseptic technique in a laminar-flow cabinet, 12 mm discs were cut from *Cladosporium* sp. that had been incubated for four weeks on malt extract both (30g/l) with mycological peptone (5 g/l) at 15 °C. Fungal discs were then added to leaf discs (fungi : leaves, 1:20) and incubated at 15 °C for 10 days. After this time leaf discs were carefully removed and dried at 60 °C in for 48 hours after which they could be stored in a desiccator. Leaves used for experiment were rehydrated for 48 hour in the appropriate test solution prior to experiments.

A1.4. Extinction coefficient in the microplate reader.

The extinction coefficient was determined for the microplate reader with a well volume of 200 μl as stated in section 2.2.8. The extinction coefficient was determined by a linear regression of absorbance (OD) against the assumed concentration of conjugated product, S-(2,4-dinitrophenyl)-glutathione (DNP-GSH). A plot of OD determined for a microplate with assay well filled to 200 μl against concentration of DNP-GSH is shown in Figure A1.2.

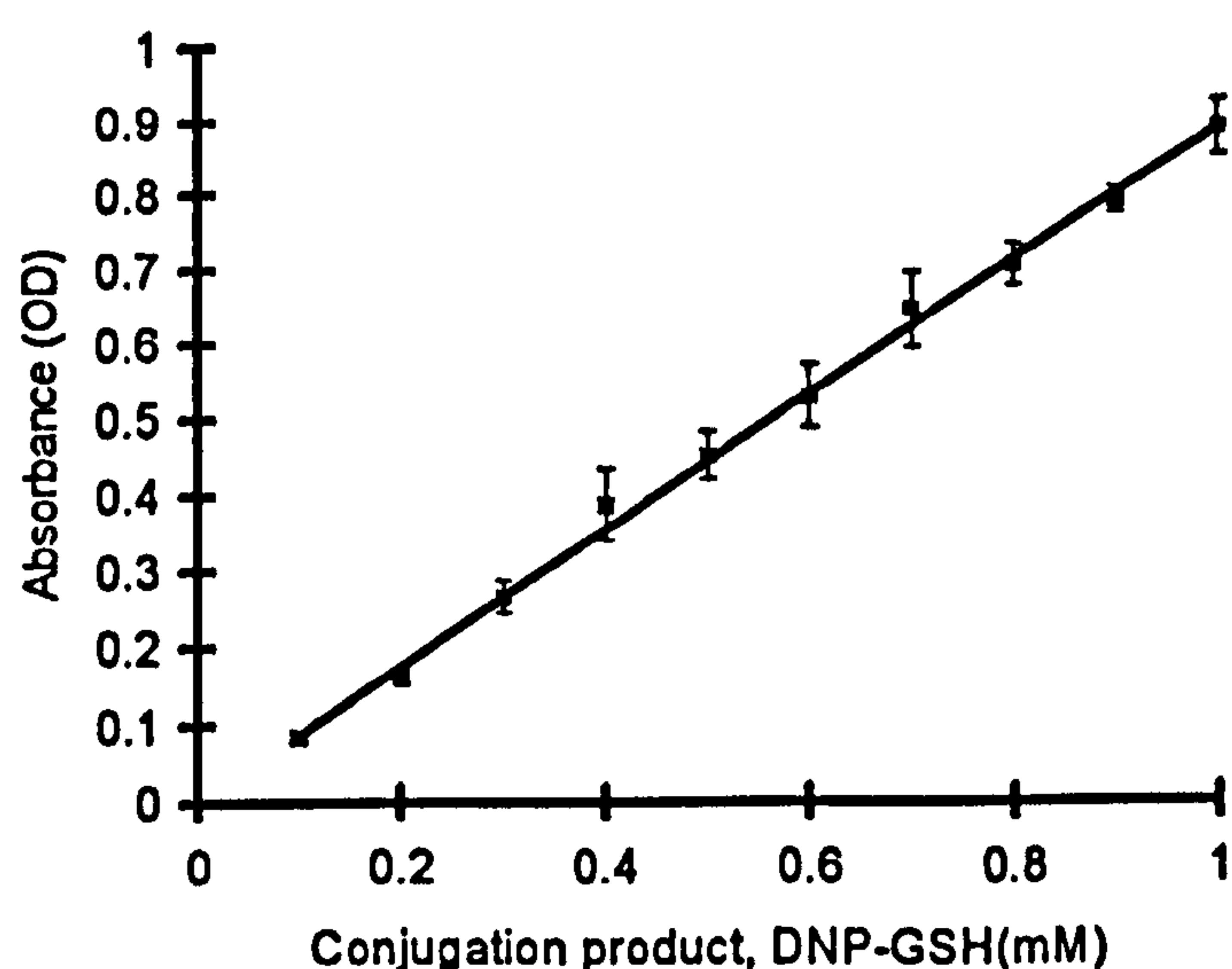


Figure A.1.2. Determination of the 'extinction coefficient' of the conjugation product DNP-GSH for reading taken using the microplate reader with 200 μl well volumes. Data is the mean of three well readings, error shown is one standard deviation.

A linear regression of the data and gave an equation of $y = 0.891x$ with a correlation coefficient of 0.99. Thus an 'extinction coefficient' derived for the conjugation product was 8.9 OD mM^{-1} . There are no units of cm because the path length of the wells is determined by volume and this was constant at 200 μl .

APPENDIX 2

A2.1 Analysis of alcohol ethoxylate test solutions from the artificial streams experiment.

Solutions were analysed at Shell Research Ltd. Chemical analysis was by HBr fission and gas chromatography with mass selective detection. Data obtained from Shell Research Ltd. (Tattersfield *et al.*, 1996) for alcohol ethoxylate concentrations in each of the eight streams over the test period that animals were exposed is presented in Table A2.1.

Table A2.1. Nominal and actual concentrations of alcohol ethoxylate as analysed at Shell Research Ltd. Mean values for reading over the 14 day experimental period were used as actuals in the present study.

Nominal ($\mu\text{g/l}$)	Day number							Mean actual concentration ($\mu\text{g/l}$)
	1	3	5 actual	7 conc. ($\mu\text{g/l}$)	9	11	13	
Control	0	0	0	0	0	0	3	0.4
25	14	19	12	16	28	19	22	18.6
50	30	41	27	40	47	41	42	38.3
100	59	88	56	46	84	63	84	61.3
200	167	213	112	140	150	165	139	115.1
300	298	287	228	240	353	310	262	282.6
400	455	241	272	380	383	332	278	334.4
800	652	774	559	600	679	556	671	641.6

The mean concentration was that used as actual concentration in the present study.

A2.2. Leaf material consumption for animals caged in the artificial streams.

Animals were caged in the artificial streams with leaf material as food (section 3.2.4, Figure 3.2). After seven days leaf material was removed, dried and weighed (section 3.2.4.). Consumption at seven days is shown in Figure A2.1.

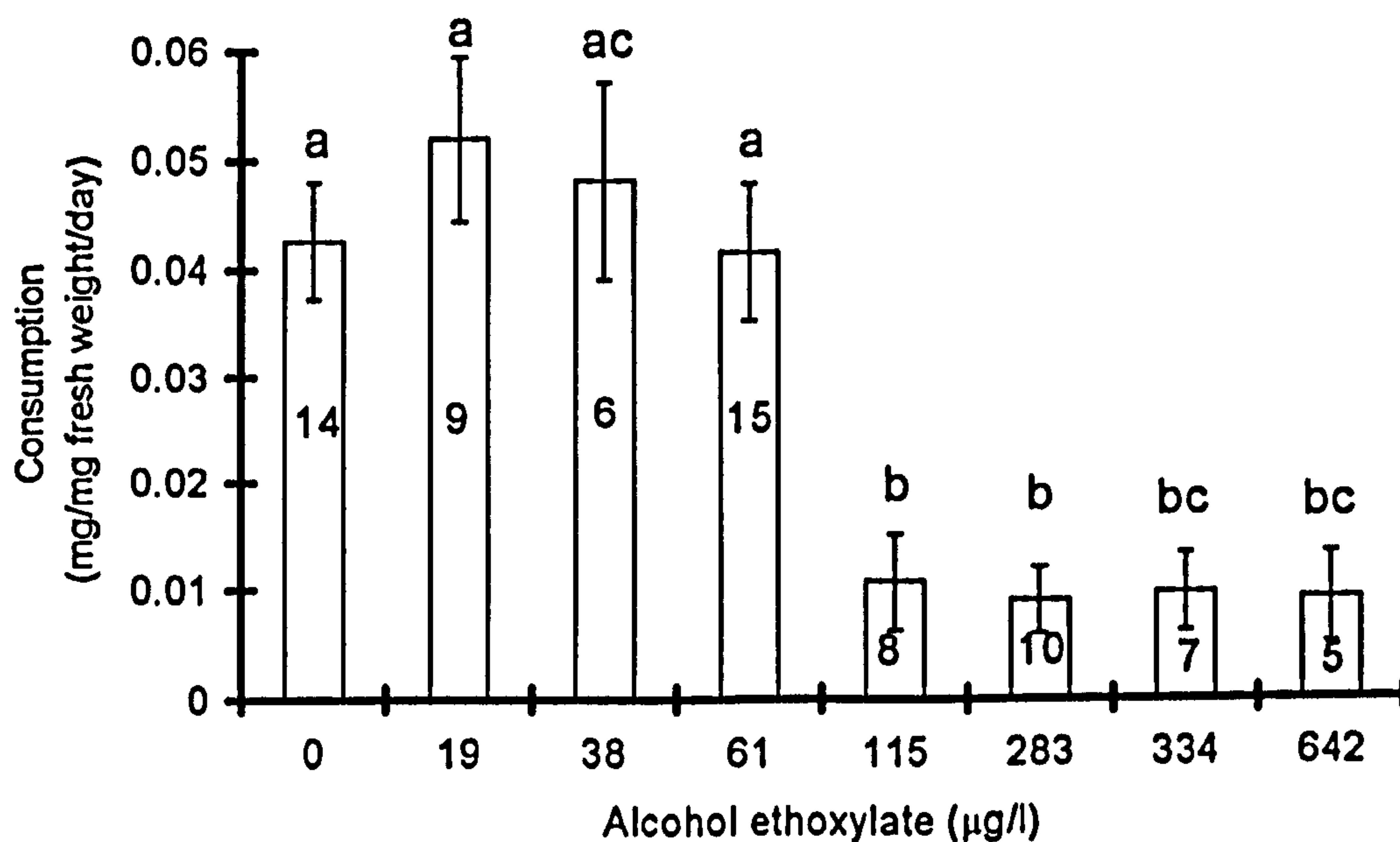


Figure A2.1. Consumption of leaf material at 7 days exposure to alcohol ethoxylate in the artificial streams. Data is presented as the mean values, the number of animals analysed is indicated in each bar. The error bars represent the standard error of the mean. Data were analysed with oneway ANOVA and found to be significantly different ($F_7 = 88.8$), individual differences were analysed using Tukey's multicomparison test. Values not significantly different are indicated with the same letter ($q > 99.9$). Significance was $p = 0.05$.

APPENDIX 3

A3.1. Calibration of oxygen electrodes.

The use of the Radiometer oxygen electrode and Strathkelvin Instruments (Model 781) oxygen meter is detailed in section 5.2.1. The oxygen electrode and meter were operated in a controlled temperature room at 15 °C. The electrode chamber was filled with distilled water and the electrode connected to the oxygen meter which was switched on and left for 15 minutes. A saturated solution of sodium sulphite (BDH) was prepared in distilled water at 15 °C. The oxygen meter was set to 'read'. Sodium sulphite solution was injected into the electrode chamber ensuring that the chamber was filled with solution. The reading was adjusted to zero using the 'zero' control. The meter was then left for two minutes and the display adjusted once more to using the 'fine' control. The electrode chamber was rinsed four times with distilled water by injecting distilled water into the chamber. Oxygen saturated APW at 15 °C was then injected into the electrode chamber using a Hamilton 50 microlitre syringe. This was repeated to ensure the electrode chamber was filled with APW. The meter was left for two minutes and adjusted to 100 per cent oxygen saturated with the 'fine' control. The meter and electrode were then ready for samples to be measured. All of the above procedure was repeated for each session using the electrode and meter.