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FACULTY OF ENGINEERING, SCIENCE AND MATHEMATICS

School of Civil Engineering and the Environment

The Sustainable Use of Water to Mitigate the Impact of Watercress Farms on Chalk Streams in Southern England

By

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Thesis for the degree of Doctor of Philosophy

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF ENGINEERING, SCIENCE & MATHEMATICS
SCHOOL OF CIVIL ENGINEERING & THE ENVIRONMENT

Doctor of Philosophy

THE SUSTAINABLE USE OF WATER TO MITIGATE THE IMPACT OF WATERCRESS FARMS ON CHALK STREAMS IN SOUTHERN ENGLAND

By Melanie Joanne Dixon

Cruciferous plants release isothiocyanates when their tissues are wounded. Release of phenethyl isothiocyante (PEITC), from watercress (*Nasturtium officianale* (R.Br)) is thought to affect invertebrates in chalk receiving waters downstream of watercress farms and is potentially exacerbated by discharge from crop washing on site. There is currently no standard method for measuring PEITC in aqueous samples and little is known about its behaviour in the aquatic environment.

Water in which frozen watercress leaf/stem tissue had been washed was analysed using solid phase extraction and gas chromatography-mass spectrometry techniques. PEITC could be consistently identified from samples prepared with as little as 1g watercress and was measured at concentrations of 397 – 696 µg/g watercress washed. Ecotoxicological testing showed disruption of *Gammarus pulex* (L.) reproductive behaviour in watercress wash water and PEITC solution. Two-hour exposure to wash water prepared at 1g watercress per litre water resulted in a mean precopular separation ET_{50} of 89 ± 6 minutes (four tests). This may account for the unsustainable population in the Bourne Rivulet (downstream of Lower Link Farm, Hampshire) where repeated exposure to an elevated level of PEITC occurs. *In situ* acute 7-day caged *G. pulex* tests at the watercress farm showed that untreated factory wash water resulted in significantly higher mortality (18 ± 5 % of test organisms) compared to control levels (3 ± 1 %) and that after treatment by recirculation of wash water through watercress beds mortality analogous to control levels was found (5 ± 1 %).

Temporal and spatial changes in macroinvertebrate populations of the Bourne Rivulet over the last 20 years corresponded with changes in farm management practice to improve the watercress farm discharge quality. In particular, the abundance of *G. pulex* had dramatically increased from 205 individuals in Spring 2007 to 2405 individuals in Autumn 2008 after factory wash water discharge was 'treated' by recirculation through watercress beds. *In situ* testing may be used at watercress farms to identify where PEITC has the potential to cause an unsustainable population. Recirculation of wash water through watercress beds, as a surrogate wetland treatment system, is a straight forward and practical mitigation measure to implement.

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Declaration of Authorship

I, Melanie Joanne Dixon, declare that the thesis entitled

The Sustainable Use of Water to Mitigate the Impact of Watercress Farms on Chalk Streams in Southern England

and the work presented in the thesis are both my own and have been generated by me as the result of my original research. I confirm that:

this work was done wholly or mainly while in candidature for a research degree at this university;

where any part of this thesis has previously been submitted for a degree or any qualification at this university or any other institution, this has been clearly stated;

where I have consulted the published work of others this is always clearly attributed;

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I have acknowledged all main sources of help;

where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

none of this work has been published before submission.

Signed:

Dated:

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I would like to especially thank some of the long list of people who have helped me complete this work.

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Abbreviations

ACR	Acute to Chronic Ratio
AMU	Atomic Mass Units
ANOVA	Analysis of Variance
ASPT	Average Score Per Taxon
BMWP	Biological Monitoring Working Party
CAMS	Catchment Management Strategy
CoGAP	Code of Good Agricultural Practice
Defra	Department of Food and Rural Affairs
EC_x	Concentration at which x% of test organisms show effect, e.g. EC_{50}
ET_{50}	Time at which 50% of the test organisms show effect
GC-MS	Gas Chromatography – Mass Spectrometry
HPLC	High Performance Liquid Chromatography
LOEC	Lowest Observed Effect Concentration
MDS	Multidimensional Scaling
NGR	National Grid Reference
NMR	Nuclear Magnetic Resonance technique
NOEC	No Observed Effect Concentration
NPK	Ratio of Nitrogen, Phosphate and Potassium in fertiliser
Ntaxa	Number of Taxa
OS	Ordnance Survey
PEITC	Phenethyl isothiocyanate
PITC	Phenyl isothiocyanate
RE	River Ecosystem value
SAC	Special Area of Conservation
SE	Standard Error
SPE	Solid Phase Extraction
SRP	Soluble Reactive Phosphate
SSSI	Site of Special Scientific Interest
UKAS	United Kingdom Accreditation Service
WOE	Weight of Evidence approach

1 WATERCRESS CULTIVATION AND ITS IMPACT ON CHALK STREAMS

1.1 Introduction

Producing and processing watercress (*Rorippa nasturtium-aquaticum* (L.) Hayek, also known as *Nasturtium officianale* (R.Br), illustrated in Plate 1.1-a) could not take place without reliable and plentiful supplies of high quality water. Chalk headwaters provide an ideal location for cultivation of watercress as the nutrient content is naturally high and the constant temperature provides protection from winter frosts and promotes vegetation growth during the colder months (Berrie, 1992).

Plate 1.1-a Watercress (*Nasturtium officianale* (R.Br))

England has the principal resource of chalk streams and rivers in Europe, many of which are designated conservation sites (e.g. Rivers Test, Itchen and Avon Sites of Special Scientific Interest; Rivers Avon and Itchen Special Areas of Conservation) (Environment Agency, 2004b). The watercress industry has flourished on the chalk streams of England over the last 150 years and crops can be found where the surface geology of a band of chalk runs from the south west to north east of the country.

There are 161 chalk rivers and streams in England (Environment Agency, 2004b) and a traditional image of their pristine habitat with clear flowing waters, healthy plant growth and abundant trout fisheries exists as an important part of the country's heritage. However, although there are stretches which remain in this condition,

many of England's chalk rivers have been subjected to anthropogenic impacts, for example siltation of the river bed gravels due to bank damage by cattle (Environment Agency, 2004b).

The watercress industry exerts its own particular pressures on the chalk stream environment. For example, by contributing to low flows due to abstraction of aquifer water to supply the water flow to the cropping beds. Watercress farms may also contribute a pollution load to the river in the form of discharge of nutrients, which are applied to the growing crop, or increase the sediment load during times when the watercress beds are cleaned. In the case of the watercress farm owned by Vitacress Salads Limited at Lower Link Farm, St. Mary Bourne, Hampshire, the chalk stream headwater is maintained by water used in watercress and baby leaf salad production and processing. However, the aquatic macroinvertebrate community in this stream differed from others in southern English chalk rivers in that although freshwater shrimp (*Gammarus pulex* (L.) illustrated in Plate 1.1-b) were present, their numbers were relatively low.

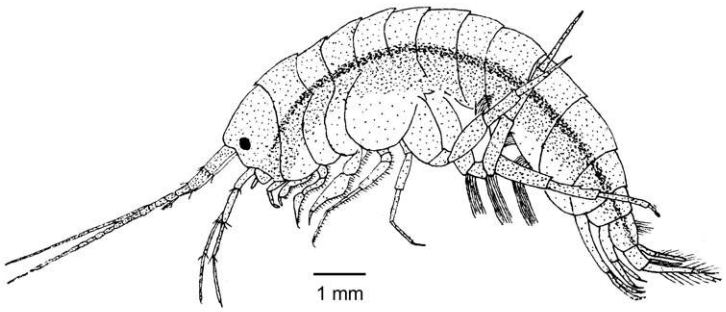

Plate 1.1-b Freshwater Shrimp (*Gammarus pulex* (L.))

The impact observed on the *G. pulex* in the stream may have been due to the release of isothiocyanates by the harvested and processed watercress and other salad crops. Many crop plants, particularly Cruciferae, produce natural pesticides such as this as a defence against herbivores. This thesis examines aspects of the nature of the impact on *G. pulex* caused by exposure to water used in growing, harvesting and processing watercress and other baby leaf salads. It assesses whether mitigation measures in

place at Lower Link watercress farm are effective in addressing this situation. It also considers the historical and current macroinvertebrate biology of the Bourne Rivulet, as an indicator of its environmental quality and as a measure of biological responses to process and practice changes that have taken place on site.

The second part of this Chapter provides a more detailed overview of watercress cultivation in England. Traditional, small scale farming methods are described, along with those carried out by larger commercial operations, such as at Lower Link Farm. Further background information is also provided on chalk stream ecology typically found where watercress cultivation takes place. The nature of the impact on chalk stream ecology and the influence of watercress cultivation is also discussed, in particular in relation to The Bourne Rivulet downstream of the large scale watercress cultivation and processing operation at Lower Link Farm.

1.2 The Watercress Industry in Southern England

1.2.1 Historical

Watercress is native to Europe and Asia and has naturalised in other countries. Its culinary and medicinal use can be traced back to the ancient Greeks (Keenleyside *et al.*, 2006) and it has been cultivated commercially for approximately 200 years at locations on chalk streams in England. It is also cultivated on a large scale in the United States of America and to a lesser extent in many other places, for example, Australia and New Zealand.

By the late 1800s and early 1900s, when watercress featured as a staple part of the working class diet, the watercress industry in England was flourishing and a family business run in the southern counties of England by Eliza James had a near monopoly on watercress supplied to the London trade (The Watercress Alliance, 2009). Many of the farms in Hampshire were founded by Ms James and it was her Trademark 'Vitacress' that is used today by the largest watercress grower in the UK.

1.2.2 Distribution of Watercress Farms

Watercress cultivation is inherently connected to the chalk geology of England; the aquifer fed springs, arising from chalk provide an ideal environment for watercress cultivation with a constant flow of relatively warm winter and cool summer water. Figure 1.2-a shows the location of chalk streams and rivers in England. Although watercress is cultivated throughout England (there are growers located in the north of England in Lincolnshire and North Yorkshire), the majority of watercress has historically been and is currently cultivated in southern England and this region is the focus of this thesis. There are over 60 hectares (148 acres) of watercress beds on the chalk winterbournes, streams and rivers in Hampshire, Dorset and Wiltshire. These are listed in Appendix A.

Figure 1.2-a Chalk Rivers in England (Natural England, 2009)

1.2.3 Development of Cultivation Methods

Traditionally, production methods were small-scale; watercress was propagated vegetatively in running water channelled to flow through levelled cropping beds. It was harvested throughout the winter. 'Traditional' watercress growers are defined by Environment Agency licensing requirements as those who replant their beds no more than once a year between the beginning of June and the end of September (Natural England, 2009). Farms that operate in this manner harvest throughout the autumn, most of the winter and spring but not during the summer when the crop runs to seed. They typically carry out much less bed cleaning and thus wash out less silt to the receiving water. There are a few growers who still operate using this method and

some now additionally incorporate some of the intensive year-round harvesting methods used by the majority of growers. Intensive cultivation methods use sown crops throughout spring and early summer, in addition to allowing the cut crop to re-grow. The cut tops of watercress plants may also be used to restock beds in autumn. The majority of growers now operate a year round production system which has peak production in the summer months. The UK market is also supplemented by crops grown abroad (for example, in Portugal) in the winter months.

In the UK there are a variety of cultivation types, the smallest being operated by small scale traditional growers with just one or two hectares of watercress beds. There are some formerly traditional growers who now operate with some intensive methods but who also maintain the traditional methods for a proportion of their crop. The largest scale commercial growers operate year round and may additionally supplement their winter crop harvests with produce grown overseas.

1.2.4 Legislative Requirements

The majority of UK watercress production is by watercress growers who are members of the National Farmers Union Watercress Association. They operate within the standards and guidance of their voluntary code of practice (Assured Produce, 2006). This code seeks to ensure high standards of hygiene for the product and the protection of the environment with respect to products used for pest, weed and disease control and techniques for harvest and storage. The code includes lists of approved insecticides and fungicides and specific off label approvals for watercress (i.e. the use of a named product for situations other than those included on the product label). A series of control points are also provided which the code strongly recommends and their compliance forms part of the Assured Produce assessment certification/approval decision. Examples of the control points given include: the protection of the beds from intrusion by livestock; the use of water channels and cropping beds with impervious sides and which are constructed and maintained to eliminate the risk of pollution by contaminated water.

Growers are additionally subject to legislative requirements of the Water Framework Directive (2000) and must adhere to Environment Agency consent conditions for

abstraction and discharge as determined by the Water Resources Act (1991). A section dedicated to watercress farming is included in the Code of Good Agricultural Practice (CoGAP) (Department for Environment Food and Rural Affairs, 2009b). This document provides practical guidance to help farmers, growers and land managers protect the environment in which they work. Parts of the CoGAP form a Statutory Code under Section 97 of the Water Resources Act (1991) and give advice on avoiding water pollution. Reference is also made to the Wildlife and Countryside Act (1981), where watercress cultivation may affect protected habitats such as Sites of Special Scientific Interest. The disposal of settled solids cleaned out from lagoons and watercress beds are also subject to control by The Environmental Permitting (England and Wales) Regulations (2007).

A survey of phosphate fertiliser use throughout the watercress cultivation industry (Agriculture and Horticulture Development Board, 2009) also provides action points for growers to use phosphate fertilisers more efficiently to meet commercial requirements for optimum yield, shelf life and establish acceptable levels of phosphate in discharge waters.

Pesticide use in watercress cultivation is subject to statutory regulation under Section III of the Food and Environmental Protection Act (1985). This is administered by the Department of Food and Rural Affairs (Defra). The Control of Pesticides Regulations (1986) provide detailed conditions for the consent of pesticide use. Watercress growers are additionally required to adhere to associated discharge consents set by the Environment Agency. Very few pesticides are approved for use in watercress cultivation due to the high risk to the aquatic environment within which farms are located.

1.2.5 Small Scale Cultivation using Traditional Methods

Watercress production using the traditional method is now relatively uncommon. Natural England (2009) reports that there is only one traditional grower in Wiltshire and none in Dorset. In Hampshire there are still traditional growers on the

rivers Test, Itchen, Blackwater at Sherfield English and Loddon and Lyde near Basingstoke. In West Sussex there are traditional watercress growers on Ham Brook near Chichester.

Mapleleaf Watercress, based in Mapledurwell, Hampshire for example, supplies traditionally farmed watercress to local retailers and direct to customers. The watercress beds are farmed using natural artesian flow from springs at the source of the River Loddon, near Basingstoke, Hampshire (see Figure 1.2-b). The watercress beds were thought to have been planted originally by monks at the nearby Andwell Priory (Fort, 2008). They have been farmed traditionally by the owners for the last one hundred years.

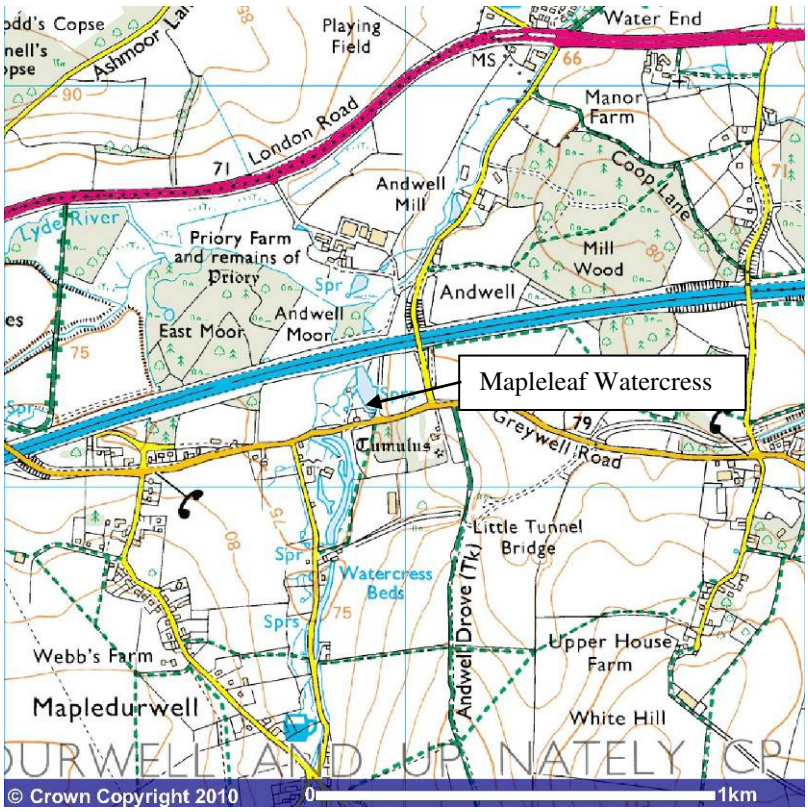

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Figure 1.2-b Location of Watercress Beds - Mapledurwell, Hampshire

Watercress farmed traditionally has two growing seasons. The relative warmth of the spring water sustains winter growth and cutting begins, for example at Mapledurwell, in February with peak production in April and May when beds may

be cut every six weeks depending on the growing conditions. The watercress beds are cut in rotation, section by section and the cut stalks are then left to re-grow by vegetative propagation for the next harvest. Once harvested, the watercress is bunched and the stalks trimmed by hand and packed into polystyrene boxes for delivery. Harvested watercress is briefly washed (dunked) in cold water containing a weak chlorine solution prior to shipping to retailers and wholesalers.

At the end of the peak growing season, the watercress plants are left until June to flower and seed. The watercress is dried *in situ* and seed collected. Traditional growers are restricted to cleaning each bed once a year and at Mapledurwell this is carried out in June following seed collection. One bed is cleared and cleaned each week. Where possible, the water flow through the beds is almost cut off and the silt prevented from being flushed into the receiving water by blocking the bed water outlet channels (in accordance with the watercress growers Code of Practice (Assured Produce, 2006). The beds are then cleared of plant matter and the gravel substrate is cleaned by raking.

The cleaned beds are then restocked with watercress seedlings grown in a propagation unit from seed kept from the previous season. Later in the summer and in autumn, the cut tops of watercress plants are used to re-stock the beds if required. These are simply strewn across the surface of the cleaned bed and allowed to root into the substrate. The second cutting season runs from July to September after which the plants are left in the beds to overwinter.

1.2.6 Intensive Cultivation by a Large Commercial Operation

Lower Link Farm at St. Mary Bourne, Hampshire (Plate 1.2-a) is the largest watercress farm in Europe (18 ha) and is operated by Vitacress Salads Limited. The farm was established in the early 20th Century and is located on the headwaters of the Bourne Rivulet, a tributary of the River Test. The watercress beds are fed with aquifer water pumped from boreholes on site.

Watercress seedlings are propagated off-site under plastic (polytunnels) in peat-based compost at a density of 10-20 seeds per cm^2. The sown plugs are sprayed with

fungicide and the seedlings are transplanted at 14 days old to the gravel beds at St. Mary Bourne where slow release fertiliser is applied in pellet form. They take root within 2-3 days and after a week fertiliser is applied in liquid form (NPK 10, 0, 5) on a daily basis (between 9am and 3pm) via a drip-pipe to the top of the beds. *Ad hoc* applications of calcium nitrate are made to the borehole supply carriers above the beds as necessary (i.e. when the leaves are showing signs of chlorosis - nitrate deficiency). Borehole water is supplied to the bed at a very low flow rate whilst the seedlings are small, gradually increasing with crop age. The flow rate is altered mechanically, by removing/replacing wooden boards across the inflow channels to the beds, on an *ad hoc* basis by the farm foreman. Water flow is also increased during colder weather to keep the bed temperature higher.

Plate 1.2-a Watercress Cultivation at Lower Link Farm

During the peak production months of May to September the watercress is harvested approximately 25 days after the seedlings are transplanted to the beds (i.e. when the plants are 5 to 6 weeks old). During these months harvesting and bed–cleaning takes place on the farm on a daily basis. Once harvesting the crop is complete, the remaining plant material is cleared from the bed and the gravel substrate is raked (mechanically and by hand). Water flow through the bed us used to remove accumulated sediment from the raked gravel. Therefore, during the cleaning process the bed flow has a very high suspended solid load and is diverted to a settlement tank

to prevent pollution of the receiving water. At other times of the year bed-clearing is less frequent (once a week/fortnight). During the winter months (December to February), the crops are more often left to re-grow from cut stubble.

There are two discharges from the watercress farm to the Bourne Rivulet (See Figure 1.2-c). The outfall to the West Rivulet discharges borehole water from watercress beds on the west side of the site. The outfall to the East Rivulet discharges borehole water from beds on the east side of the site, as well as factory wash water and site storm discharge.

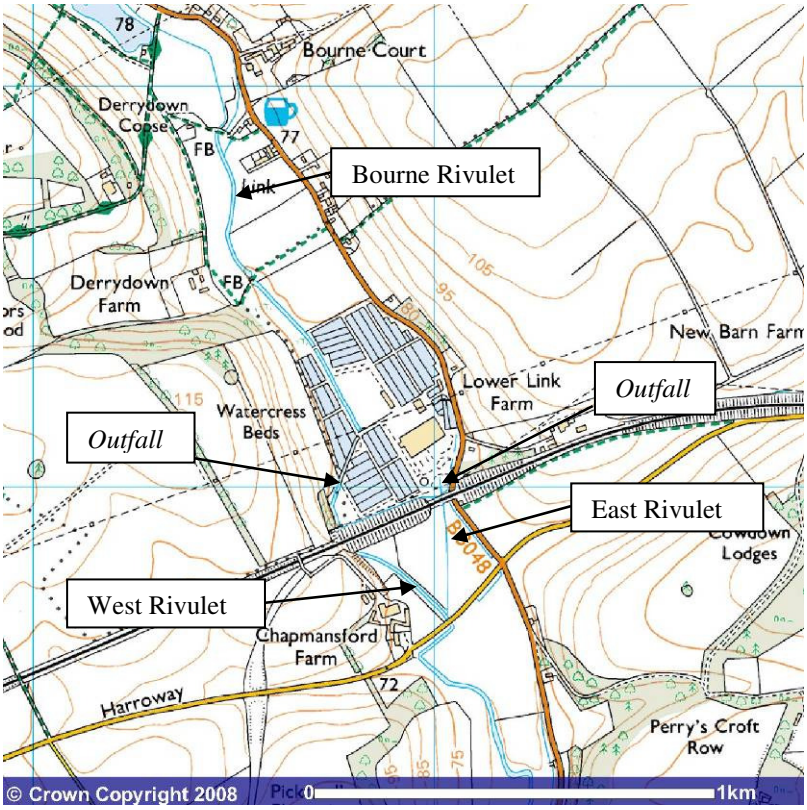

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Figure 1.2-c Watercress Farm Outfalls to the Bourne Rivulet

The Environment Agency imposes water quality consent conditions (see Table 1.2-a) and routinely monitors from 5 locations in the vicinity of the watercress farm. *Ad hoc* samples have also been taken on a number of occasions.

Table 1.2-a Environment Agency Water Quality Consent Conditions

Parameter	Bourne Side (West Rivulet)	Viaduct discharge (East Rivulet)
pH	6-9	6-9
free chlorine	absent	absent
Suspended solids	20 mg/L	20 mg/L
Solid matter from crops	≤5 x 5mm	≤5 x 5mm
Total Zinc	75 µ/L	75 µ/L
Malathion	0.5 µg/L	-
Hydrocarbons	-	5 mg/L

A number of recent changes have been made to the East Rivulet and the outfall. Work was undertaken in November 2005 to remove accumulated silt and widen the channel of a 250 m stretch of the East Rivulet at and below the outfall. The work was designed to improve flows and encourage a better habitat for fish, invertebrates and aquatic plants.

Furthermore, in January 2007, the culverts and outfall to the East Rivulet were excavated and a 200 m length of virgin stream created from the newly excavated channels. These were designed with a variety of vegetated bank types to form a sinuous channel of varying widths and flow types. Coarse flints and gravels were re-introduced to the new stream bed to create a variety of geomorphological features. The new channel was then planted with plant species from BritishFlora (Cain Bio-Engineering Ltd, 2009). In addition to the physical improvements made to the outfall and channel, a series of process modifications were also made on-site, for example, the installation of two 2 mm parabolic screens and a suspended solids settlement tank. These are detailed in full in Table 4.1-a.

In addition to watercress harvested from Lower Link Farm and other Vitacress Salads Ltd farms, there are more than 30 different types of salad leaf also processed on site. These include watercress and other leaves from Vitacress Salads Ltd farms overseas in Portugal, Spain, USA and Kenya. Isothiocyanate containing crops which are currently washed and processed on site include: watercress, black cabbage, kale, mizuna, rocket and tatsoi. Other crops are: coriander, lambs lettuce, iceberg lettuce, parsley, green batvia lettuce, lollo rosso, mottistone lettuce, red chard, green chard,

red cos, spinach, tango lettuce, pea shoots, white chard, beetroot shoots, julienne carrot, sugar snap pea shoots and radish.

All the crops are processed in the factory building on site at Lower Link Farm. They are washed in clean spring water from the boreholes on-site and packaged individually or in mixes, before being loaded for delivery. Spring water is re-used during the wash process before being discharged to the outfall on the East Rivulet. The wash house operates on a daily basis (including weekend days) typically between the hours of 0730 and 1700 on weekdays and 0630 and 1600 at the weekend, the exact times being dependent on the schedule of orders to fulfil.

1.3 Impact of Watercress Cultivation

1.3.1 Chalk Stream Ecology

The fauna and flora are diverse in chalk streams and rivers and are strongly influenced by the physical and chemical conditions, e.g. the extent of aquifer recharge and flow characteristics (Mainstone, 1999). A broad range of conditions generally exists along the length of a chalk river, from the headwaters and ephemeral winterbourne sections to the large size river reaches. Communities therefore differ along the length of the river.

Mainstone (1999) details the characteristic plant communities of the differing stretches of chalk river. The beds of water crowfoot (*Ranunculus* spp.) are an important characteristic feature, although vegetation management (e.g. weed cutting and bank management) has traditionally been used to maintain its preferred high flow rate and define the structure of the plant community and allow it to dominate. Fast growing aquatic annual plants such as pond water crowfoot (*Ranunculus peltatus*) and watercress (*Nasturtium officianale*) dominate in spring and summer in winterbourne sections, with non-aquatic grasses and herbs prevailing in more intermittent/drier reaches. Emergent and marginal reeds are more common in perennial sections, with brook water crowfoot (*Ranunculus penicillatus*) dominating in spring/summer. Classic chalk streams and larger river reaches typically support brook water crowfoot, watercress, starwort (*Callitriche platycarpa*), blue water speedwell (*Veronica anagallis-aquatica*) and lesser pond sedge (*Carex acutiformis*). Larger river reaches have higher species-richness than other lowland river communities in the UK with more than 50 species per km.

Watercress occurs naturally as a common macrophyte in most reaches of chalk streams and can dominate during the summer period. Mainstone (1999) reports it as ‘expected (>75% occurrence)’ in perennial headwaters, classic chalk streams and classic chalk rivers and as ‘very likely (50-75% occurrence) in winterbourne reaches.

Winterbourne sections of chalk streams are characterised by invertebrate species which have prolonged resting stages to withstand dry periods. Examples given by Mainstone (1999) include the pea mussel, (*Pisidium casertanum*) , which is tolerant to drying out and the mayfly, *Paraleptophlebia werneri*) , which lays eggs resistant to drying out. Similarly species belonging to the Coleoptera (beetles) and Hemiptera (bugs), which are capable of rapid colonisation once the water flow is resumed, are usually found. A large diversity of macroinvertebrate species are found along the perennial sections and community composition varies with changes in the habitat structure within the channel. For example, Ephemerellidae (mayfly) prefer shallow riffle, gravel substrate. Additionally, there is a seasonal change in species composition with changing flow conditions and vegetative growth within the channel. There are some rare species such as the riffle beetle (*Riolus cupreus*) or endangered species such as the southern mayfly (*Coenagrion mercuriale*)which are only found in chalk rivers. There are also generalists, such as *G. pulex* or *Erpobdella octoculata,* which are found along the river length.

The characteristic fish species of chalk rivers is the brown trout (*Salmo trutta*) (Environment Agency, 2004b) and the diversity of habitats gives the potential for colonisation by a large range of fish species such as grayling (*Thymallus thymallus*), bullhead (*Cottus gobio*), brook lamprey (*Lampetra planeri*) and salmon (*Salmo salar*). Many chalk river reaches of have been traditionally managed for fishery interests and some are stocked with brown and rainbow trout (*Oncorhynchus mykiss*).

1.3.2 Impact and Influence on Chalk Stream Ecology

Actual and potential impact on chalk stream ecology below watercress farms is well documented. Casey and Smith (1994) attribute a wide concentration of phosphate and potassium downstream of watercress beds to the addition of fertilisers and speculate that this could alter the structure of plant communities in the streams. Lower than normal nitrate levels are also described as nitrate is removed by the growth of watercress. Increased zinc concentrations, a potential macroinvertebrate toxicant, were related to the application of zinc to control crook root, a practice which is no longer widespread within the industry. Casey & Smith (1994) also

found that the normally low level of suspended solids was increased, although this factor has been addressed at many farms and reduced by the installation of sediment traps or holding ponds. Casey (1981) concluded that discharge from watercress bed outflow would have a beneficial effect on the Bere Stream headwater acting to maintain flow levels during periods of low flow.

The fauna downstream of Lower Link farm is considered to have been adversely affected (Medgett, 1998), probably by preparation of the produce. In a survey of operational practice on Hampshire watercress farms, watercress was often found to be washed in chlorinated water, the disposal of which presented a pollution risk (Fewings, 1999). The use of settlement ponds, treatment tanks or the disposal of chlorinated waste to land were identified as preventative measures in many instances. Some traditional farms were also described as using redundant production beds for settlement. Recommendation was also made to investigate whether different levels of PEITC are released during different harvesting operations (e.g. hand pick *vs.* mechanical) and the "evaluation of the PEITC link to the absence of *Gammarus*". More recently, further work was recommended (Natural England, 2009) to explain the effects seen in invertebrate populations in watercress beds and discharge streams, in particular in relation to phenylethyl isothiocyanate (PEITC). The biochemistry of PEITC, a watercress secondary metabolite, is discussed in Chapter 2 and its impact on *G. pulex* in Chapter 3.

1.3.3 Impact on Macroinvertebrates in the Bourne Rivulet

Approximately 90% of the watercress beds in southern England are located on or upstream of a chalk river Site of Special Scientific Interest (SSSI) (Natural England, 2009). The watercress beds on the Bourne Rivulet, for example, although not a SSSI itself, are upstream of the River Test, which is designated a SSSI along it entire length. It is described as a classic chalk stream within which are found nationally rare, as well as nationally scare macroinvertebrate species (Environment Agency, 2004b).

The situation is complex and unusual at Lower Link watercress farm as, in addition to the watercress beds, there is a large salad processing and packing plant which

discharges wash water to the Bourne Rivulet. Biological surveys of the Bourne Rivulet (Medgett, 1998, Cotter, 2005, Marsden, 2006) showed that there had been a community response to inputs to the watercourse from the watercress farm discharge. There was a notable reduction or absence of *G. pulex* in many of the samples taken, a reduction of biotic scores and taxon richness, with a gradient of improvement to approximately 2 km downstream.

However, in the cultivated watercress habitat, anecdotal reports (Vitacress Salads Ltd, 2007) and my own informal observations on site at watercress farms found numerous (although not formally quantified) Gammaridae within the watercress beds, grazing on dead and decaying plant matter. Farm workers at Vitacress Salads Ltd also report that gammarids cause damage by grazing on the very young watercress seedlings.

A survey (White and Medgett, 2006) found that Elmidae and Gammaridae were virtually excluded from samples taken downstream of Lower Link watercress farm and outfall and there were comparatively higher numbers of Asellidae, Oligochaeta and Planariidae than at other sites on the Bourne Rivulet. In these instances samples may have reflected a change in the predator-prey relationship in addition to the response to pollution insensitivity or tolerance. A continued measurable effect on macroinvertebrate communities below Lower Link watercress farm was also noted (Medgett, 2008), although an improvement in numbers of *G. pulex* and other pollution sensitive groups was found in samples taken from the East Rivulet, which they attributed to changes made to the farm process and practice at Lower Link Farm.

1.4 Aims and Rationale of Thesis

1.4.1 Research Hypotheses

There is a recorded impact on *G. pulex* in the Bourne Rivulet; the artificially maintained chalk stream receiving water below the outfall from Lower Link watercress farm and processing plant. This is thought to be due to isothiocyanates produced during the harvesting and processing of watercress and other baby leaf salads. Several unpublished studies have been carried out in relation to this issue (Medgett, 1998, Marsden, 2005, Cotter, 2005, Marsden, 2006, Murdock, 2008a) and biological monitoring carried out (White and Medgett, 2006, Medgett, 2008, Murdock, 2007, 2008a, 2009). However, evidence to show a definitive link between the production of PEITC by the watercress crop (and its processing) and the effect evident in the receiving water has not been provided.

The research hypotheses are:

- it is possible to identify and quantify levels of PEITC from water in which watercress has been washed;
- the isothiocyanates produced by watercress have a detrimental effect on *G. pulex* survival and reproductive behaviour;
- mitigation measures in place at Lower Link Farm to reduce the impact of water used in the production and processing of watercress on the receiving water are successful;
- in the receiving water, macroinvertebrates other than *G. pulex* have been affected.

1.4.2 Thesis Structure

The subsequent two Chapters give more detail on the measurement of isothiocyanates (in particular phenethyl isothiocyante, PEITC) produced by watercress and its impact on *G. pulex*. Measurement of isothiocyanates contained in watercress wash water is problematic as there is no standard methodology available. Chapter 2 gives further information on the biochemistry, identification and

measurement of PEITC and its role in relation to invertebrate behaviour and human health benefits. The Chapter then describes the development of a method to measure PEITC from freshly prepared watercress wash water, adapted from methods used to identify and measure isothiocyanates or their glucosinolate precursor from leaf preparations. Data are used to calculate levels of PEITC found in freshly prepared watercress wash water samples.

In Chapter 3 a series of ecotoxicological tests is reported which measures acute and sublethal impact of watercress wash water and PEITC solution on *G. pulex* juveniles and reproductive adults. A novel approach to sublethal testing is used which has particular relevance to the pulsed nature of the isothiocyanate containing discharge at the watercress farm. It also takes into consideration the volatile nature of PEITC. Data are used to describe the sensitivity of *G. pulex* to watercress wash water.

Chapter 4 addresses the mitigation measures in place at Lower Link watercress Farm to reduce potential impact of watercress cultivation on chalk stream invertebrates in the receiving water. The Chapter describes the series of changes to the farm management and the factory process over the last 15 years. An assessment is then made of the effectiveness of one of the most recent changes made, whereby the wash water discharge is re-circulated back through a series of watercress beds prior to discharge to the chalk stream receiving water i.e. a surrogate constructed wetland. A series of toxicity tests carried out *in situ* at Lower Link Farm is reported and their significance to the receiving water environment is described.

In Chapter 5 the chalk river distribution, diversity and conservation status in England is described, along with the influences to which they are subjected. A long term biological data set was available, as a result of monitoring of the macroinvertebrate community in the Bourne Rivulet below Lower Link Farm. The long term data are used to illustrate the changes in the Bourne Rivulet macroinvertebrate populations which have taken place over a period of two decades. Particular reference is made to the concurrent changes in farm management practice.

Finally, Chapter 6 discusses the sustainability of watercress farming in relation to the maintenance of the chalk stream environment. An overview of the source and fate of

PEITC is given along with implications for watercress cultivation with respect to the potential impact on *G. pulex* and the wider macroinvertebrate community. The potential application by the UK watercress industry of methodology used and results of this thesis are discussed, along with observations related to the evolution of chalk stream management in relation to their particular function/use. Further explanation of the limitations of the study is given along with suggestions for further work.

2 PHENETHYL ISOTHIOCYANATE FROM WATERCRESS WASH WATER

2.1 Introduction

This Chapter initially explores the identification and biochemistry of isothiocyanates from cruciferous plants, in particular phenethyl isothiocyanate (PEITC) produced by watercress. The role of isothiocyanates in relation to invertebrate behaviour and the human health benefits that have been attributed to them are also discussed.

Chapter 1 has highlighted the impact on chalk stream ecology which can occur below watercress farms and noted that this may potentially be due to isothiocyanates produced by the crop itself. If we were able to measure PEITC in samples taken from watercress bed flow or watercress wash water, or even from the receiving water below a watercress farm, it would be possible to identify where and to what level PEITC production was present, whether there were peaks in its production and whether PEITC degradation was occurring anywhere within the system. Measurement of PEITC could be used to show a definitive link to effects recorded on macroinvertebrate communities in chalk receiving waters. However, measurement of PEITC from an aqueous matrix is problematic and no standard methodology is available. Methods to measure isothiocyanates or their glucosinolate precursor from leaf preparations or blood serum have been reported and these are discussed further in Section 2.2.

Section 2.3 describes the development of a method to measure PEITC from freshly prepared watercress wash water and the results of analyses using this method. The objectives for this experimental work were:

- To identify PEITC from freshly prepared watercress wash water using gas chromatography-mass spectrometry (GC-MS) techniques;
- To attempt to quantify the levels of PEITC in samples of watercress wash water;
- To assess the variability of levels of PEITC in standardised preparations of watercress wash water with a known ratio of leaf wet weight to water.

A discussion of the outcomes of the method development experimental work is presented in Section 2.4 along with consideration of its suitability for application to industry.

2.2 Biochemistry and Role of Phenethyl Isothiocyanate

2.2.1 Glucosinolates

PEITC is derived from the catabolism of glucosinolates present in cell vacuoles within tissues of plants containing them (Bones and Rossiter, 1996). Glucosinolates occur naturally in watercress and other cruciferous plants (plants of the order Brassicales, in particular the family Brassicaceae, also known as Cruciferae), many of which are important economic food crops. There is a large body of literature detailing the biochemistry and distribution of glucosinolates (Kjñr, 1976, Gil and MacLeod, 1980, Bones and Rossiter, 1996, Fenwick *et al.*, 1982). More recently Mithen (2001) reviews the biochemistry, genetics and biological activity of glucosinolates and their degradation. Fahey *et al.* (2001) detail the chemical diversity and distribution of glucosinolates among plants in the context of their therapeutic and prophylactic properties.

Plant species may contain several different forms of glucosinolate and the distribution of these has been found to vary between the roots, leaves, stems and seeds (Fahey *et al.*, 2001). The glucosinolate levels may vary considerably within plants over a 24 hour period (Rosa, 1997) and the watercress glucosinolate concentration has been found to increase in response to long days, low night temperatures (<20ºC) and supplementary light (Engelen-Eigles *et al.*, 2006) and also to treatments with nitrogen and sulphur (Kopsell *et al.*, 2007).

2.2.2 Isothiocyanate and Myrosinase

Isothiocyanates are produced as secondary metabolites when glucosinolate (the stable water-soluble precursor) is hydrolysed by the action of a myrosinase enzyme released when the plant is wounded. A thioglucoside linkage is cleaved by the enzyme resulting in a glucose group and an unstable intermediary. This intermediary rapidly rearranges to produce sulphate and either a thiocyanate, isothiocyanate or nitrile, depending on substrate, pH or availability of ferrous ions (Bones and Rossiter, 1996). Isothiocyanates are usually produced at neutral pH

(similar to those of the aquifer fed supply of pH 7 to 8 to Lower Link Farm) while nitrile production occurs at lower pH. Wilkinson *et al.* (1984) determined the myrosinase activity of cruciferous vegetables by measuring the initial rate of glucose formulation from glucosinolate hydrolysis. They also showed that myrosinase activity was affected by variation in ascorbate concentrations and for watercress there was very little ascorbate independent myrosinase activity. Palaniswarmy (2003) reported peak levels of ascorbic acid coincided with peak production of PEITC in watercress plants sampled at intevals between 21 and 81 days of age.

2.2.3 Phenethyl Isothiocyanate (PEITC)

Isothiocyanates have been separated and identified from plant extracts by high performance liquid chromatography (HPLC) ((Zhang *et al.*, 1996, Fahey *et al.*, 2001) or gas chromatography – mass spectrometry (GC-MS) (Cole, 1976, Gil and MacLeod, 1980, Palaniswamy *et al.*, 2003). The primary hydrolysis product of the glucosinolate present in greatest quantities in watercress (i.e. 2-phenethyl glucosinolate, also known as gluconasturtiin) is 2-phenethyl isothiocyanate (PEITC). The amount of PEITC derived varies between different studies, most likely due to cultural conditions and age of plant (Palaniswamy *et al.*, 1997), but possibly also due to sensitivity of analysis. Cole (1976) reported a mean PEITC value of 74 µg/g on analysis of 8 week old watercress plants grown in the UK under glass (time of year not specified). Palaniswarmy *et al*. (2003) recorded levels of PEITC ranging from 233 µg/g leaf fresh weight for 3 week old seedlings to 688 µg/g leaf fresh weight for 11 week old plants maintained in controlled temperature (day 25 ºC / night 22 ºC) and light conditions. Concentrations increased with plant age until the plants were about 9 weeks old, with no further increase measured in plants subsequently harvested. Thus we would expect the levels of PEITC from cultivated watercress to vary depending on the time of year the crop is grown, the age of plants when they are harvested (5 weeks to several months old) and the country, i.e. local environmental conditions where they are grown.

Gil and MacLeod (1980) noted that the relative abundance of the glucosinolate breakdown products of watercress leaves altered significantly when it was mixed with another member of the Cruciferae. They concluded that; "Although the

natural degradation pathway gives mainly isothiocyanates, it appears to be particularly sensitive and can be readily subjugated in favour of nitrile formation by applying heat to the system or incorporating another member of the Cruciferae with natural nitrile-directing properties." This may be of particular relevance to the salad leaf processing plant at Lower Link Farm and it may be of interest to compare the amount of PEITC in watercress leaf *vs.* a watercress and mixed salad leaf combination that is processed there.

The stability and degradation of PEITC in the receiving water below watercress farms has not been reported. Pharmacokinetic studies to establish bioavailability of PEITC to mammals have measured the stability of PEITC in a range of pH buffers (Ji *et al.*, 2005). The half-life was found to vary from 56.1 to 68.2 hours at room temperature (25°C), being more stable at pH 3.0 than at a neutral or alkaline pH. The half-life at 4°C and pH 7.4 however, was significantly increased to 108.1 hours. The increased stability of PEITC at lower temperatures is relevant to the refrigerated process operation at Lower Link Farm and the borehole water of consistent pH (7.3) and low water temperature (~10.5°C).

2.2.4 PEITC as a Chemical Defence Mechanism

A number of studies identify the role of the degradation products in the defence of the plant against herbivorous insects (Newman *et al.*, 1992, Kerfoot *et al.*, 1998). They suggest that freshwater systems possess few specialist herbivores and chemical feeding deterrents provide the most effective means of protection against generalists. They provide evidence that watercress is chemically defended from herbivory by the glucosinolate-myrosinase system. Prusak *et al.* (2005) found that many other US native freshwater macrophytes (abundant species were used, although none commercially cultivated) also use chemical defences against the crayfish (*Procambarus acutus*), a generalist herbivore. Shelton (2005) investigated small-scale variation in glucosinolate production within *Raphanus sativus* cruciferous plants and the variation caused by induction (i.e. when a plant systematically increases its level of defence in response to herbivory) and found that variation may have significant effects on herbivores and could be an important component of plant defence. For example, unpredictable changes to toxin levels may cause insects with

inducible detoxification systems to be 'out of phase' with their food. Also, random variation of the plant defence would also result in reduced selection by herbivores to resistance to the plant defences, allowing the plant to compete with the short generation and recombination potential of insect herbivores.

Glucosinolates are attractants for a number of specialist herbivores and they have been found to act as both feeding and oviposition stimulants. Roessingh *et al.* (1992) demonstrated the role of glucosinolates in the oviposition behaviour of the cabbage root fly. The white butterfly (*Peiris rapae* (L.)) feeds almost exclusively on plants in the Brassicaceae and although they contain diverse phytochemicals which are thought to serve defensively, there is evidence to show that isothiocyanates may be deleterious to larval growth and development at high doses (Agrawal and Kurashige, 2003). Some larvae, for example, the turnip sawfly (*Athalia rosae* (L.)) and diamondback moth (*Plutella xylostella* (L.)) sequester glucosinolates within their haemolymph as a predator defence mechanism (Opitz *et al.*, 2010). The production of isothiocyanates, which would be toxic to the larvae, is inhibited by the competitive action of a sulphatase enzyme produced by the larvae. This prevents the glucosinolate-myrosinase reaction and converts glucosinolate into a desulphoglucosinolate, which cannot be degraded and is excreted with the faeces (Müller and Sieling, 2006, Ratzka *et al.*, 2002). Such specialists have evolved to cope with the host plant defences, although as induction of glucosinolates in response to herbivory increases plant defences, this may be the stimulus for the selection of more effective plant defences against specialists.

There are also a number of between-plant interactions which are reported in connection with crucifers. Vaughn and Boydston (1997) found that volatile isothiocyanates released by chopped up cruciferous plants inhibited the seed germination of several crop and weed species. Isothiocyanates have been tested for their suitability for weed control and PEITC, in particular, has been reported to show high activity against wheat germination and seedling growth (Bialy *et al.*, 1990). A comprehensive review of the use of seed meal containing glucosinolates for controlling plant pests recommends that meals with isothiocyanate-producing glucosinolate concentrations in excess of 200 μmol/g tissue will most effectively control a wide variety of plant pests (Brown and Morra, 2005).

2.2.5 Human Health Benefits of PEITC

Many papers report the identification and chemistry of PEITC in relation to the human health benefits. In particular PEITC has been found as particularly effective as an inhibitor of carcinogenesis and also has preventive properties in relation to a number of different cancer types. Ingestion of an isothiocyanate metabolite from cruciferous vegetables was found to inhibit the growth of human prostate cancer cell xenografts (Chiao *et al.*, 2004) and crude watercress extract was found to have significant chemo-protective properties (anti-genotoxic, anti-proliferative and anti-metastatic (invasion) *in vitro* in human colon cancer cell lines (Boyd *et al.*, 2006). Furthermore, broccoli and watercress were found to suppress the metabolic pathways which are associated with invasive potential and invasiveness of human breast cancer cells (Rose *et al.*, 2005). It is possible to measure PEITC in plasma and urine samples, obtained from subjects who have eaten watercress, by a liquid chromatography–tandem mass spectrometry technique (Ji and Morris, 2003). The putative benefits of a diet high in PEITC producing plants are under more thorough investigation to elucidate the specific pathways and mechanisms by which PEITC acts to prevent and reduce cancerous cell growth.

2.3 Analysis of PEITC by GCMS

2.3.1 The GCMS Process

There are several different methods reported for analysis of PEITC from plant extracts of cruciferous crops as described in Section 2.2.3, although there is no accredited or industry standardised test and none reliably measure PEITC from an aqueous matrix. This study has focused on the development of a procedure to identify and quantify PEITC from watercress wash water using GC-MS technology. Data from the literature where analyses for isothiocyanates specifically from watercress leaf/stem tissue have been carried out have used GC-MS methods (Cole, 1976, Palaniswamy *et al.*, 2003, Gil and MacLeod, 1980) and these methods provide a useful start point for method development. A US patent for extraction of PEITC for neutraceutical compositions and methods (Ribnicky *et al.*, 2002) is also reported, although this method details extraction of PEITC from land cress and from seeds rather than leaf tissue.

Gas chromatography mass spectrometry (GC-MS) is an instrumental technique which is used to separate, identify and quantify complex mixtures of chemicals. It comprises a gas chromatograph (GC) coupled to a mass spectrometer (MS). The sample solution is injected into the GC inlet where it is vaporized and taken into the chromatographic column by a carrier gas (helium). The sample flows through the column and the compounds are separated according to their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase). The latter part of the column passes through a heated transfer line and ends at the entrance to an ion source. Compounds eluting from the column are subjected to a beam of electrons which ionise the sample molecules resulting in the loss of one electron and their conversion to positive ions. When the resulting peak from this ion is seen in a mass spectrum, it gives the molecular weight of the compound. Due to the large amount of energy imparted to the molecular ion it usually fragments producing further smaller ions with characteristic relative abundances that provide a 'fingerprint' for that molecular structure. This information may be then used to identify compounds of interest. The positive ions are separated according to their

mass related properties by a mass analyser and the information recorded, displayed and analysed using a computer.

2.3.2 Equipment Set-up

A Thermo Finnegan Trace GC Ultra was used with a Thermo Finnegan Polaris Q MS. The capillary column used was a Restek Rtx 5MS (30 m length, 0.25 mm internal diameter, crossbond 5% diphenyl 95% dimethyl polysiloxane). Helium was used as the carrier gas (flow rate 1.2 ml/min) and split injection with split flow of 60 ml/min. Reference was made to chromatographic conditions used by Palaniswarmy *et al*. (2003) for the analysis of PEITC and PITC. The injection port temperature was 220 °C, the transfer line temperature was 230 °C and the ion source temperature was 205 °C. An initial temperature of 60 °C was held for 3.5 min and was increased to a final temperature of 320°C at the rate of 40 °C per minute. The analysis time was ~16 min.

Prior to each sample run a leak test was performed, along with gas calibration and an air-water test. A blank (methanol wash) was analysed at the start and end of each sequence to check for column bleed. The peaks were identified and quantified using a PEITC standard (at 163 m/z) and a phenethyl isothiocyanate (PITC) internal standard (at 135 m/z) analyzed under identical chromatographic conditions. A 5µl injection was used for all samples and standards and the relative abundance as area under the peak was measured.

2.3.3 PEITC and PITC Standards

Phenyl isothiocyanate (PITC) was used as an internal standard as it did not have the same retention time as PEITC. Reagent grade PEITC standard (Sigma Aldrich UK Product No. 253731, molecular weight 163.24 AMU, density; 1.094 g/cm^3) and PITC internal standard (Sigma Aldrich UK Product No. 139742, molecular weight 135.19 AMU, density; 1.13 g/cm^3) were used. The PEITC and PITC reagents were of 99% and 98% purity respectively and were used without further purification. PEITC was kept under nitrogen to prevent oxidation. Analytical grade methanol

(Fisher Chemicals UK Product No. M/4056/PB17, 99% purity) was used as the solvent and was used without further purification.

Stock solutions of PEITC in methanol (0.1094 µg/µl) and PITC in methanol (0.113 µg/µl) were prepared and the retention time and general level of detection of PEITC and PITC by the GC were established. Example chromatograms showing peaks for PEITC and PITC are given in Appendix B. Component identification was carried out using computer matching against the Mass Spectral Search Program v. 2.0 (National Institute of Standards and Technology, 2008). Then, serial dilutions of the PEITC and PITC stock solutions were prepared and analysed to find the limits of detection. The PEITC standard retention time was between 9.28 and 9.31 minutes and the limit of detection was 0.05 ng PEITC. The PITC internal standard retention time was 8.1 minutes and the limit of detection was 0.07 ng PITC.

An assessment of the GC column response over a range of concentrations of the standards was made. A sequence of standards were prepared by serial dilution of a PEITC stock solution (of concentration 1.094 µg/µl) with methanol which resulted in between 5 ng and 220 ng PEITC being injected onto the GC-MS. A PEITC standard curve was constructed from analyses carried out on six occasions (Figure 2.3-a).

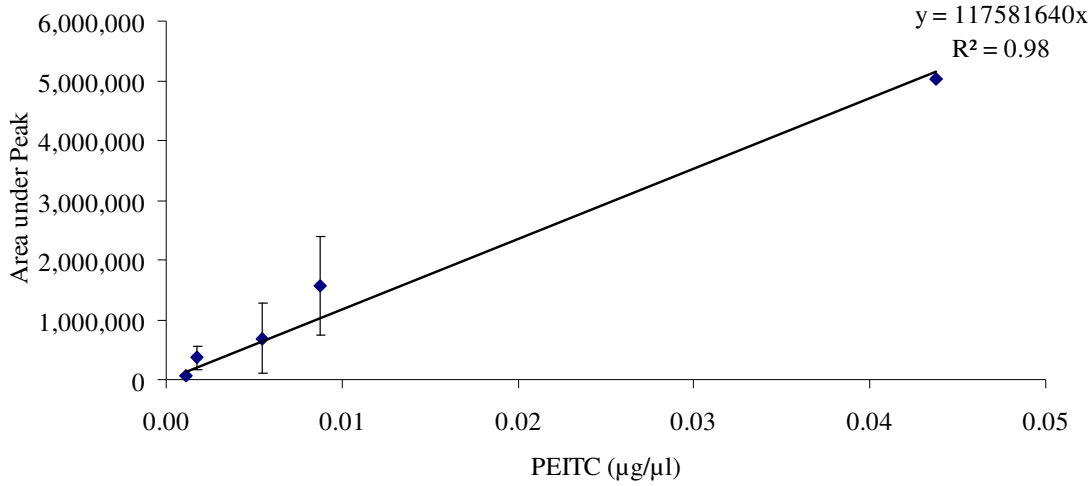

Figure 2.3-a PEITC Standard Curve

Vertical bars show standard error where concentrations were repeated on separate occasions. The linear trend line, regression equation and coefficient of determination are shown. The area under peak represents the relative abundance of PEITC in the injected sample.

A PITC standard curve was constructed from a series of dilutions of PITC stock solution (of concentration 1.13 µg/µl) carried out on two occasions (Figure 2.3-b). Error bars were not included on the PITC curve as the concentrations tested on each occasion were not the same. The coefficient of determination (R^2) was used to judge linearity and both curves showed linearity over the tested range of concentrations.

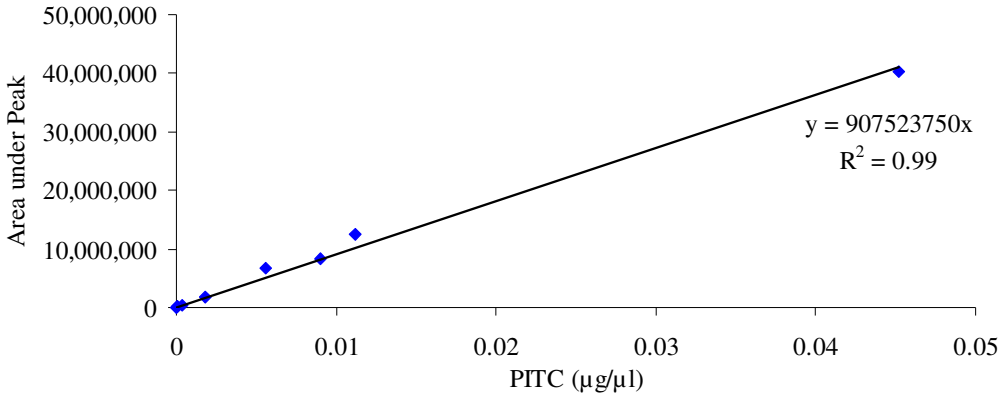

Figure 2.3-b PITC Internal Standard Curve

The linear trend line, regression equation and coefficient of determination are shown. The area under the peak represents the relative abundance of PITC in the injected sample.

2.4 Identification of PEITC from Wash Water Samples

2.4.1 Preparation of Samples

Examples of watercress sample preparation methods available in the literature include grinding in water (Palaniswamy *et al.*, 2003), grinding in liquid nitrogen (Ribnicky *et al.*, 2002), maceration in water (Cole, 1976), chopped/blended in water (Gil and MacLeod, 1980), agitation of leaves and stems with hexane (Breme *et al.*, 2007), homogenating crushed frozen plant tissue in hot methanol (Blua and Hanscom, 1986), placing freeze-dried tissue in methanol (Kopsell *et al.*, 2007) placing frozen watercress in cold water (Newman, 1990a), followed by extraction. The method of sample preparation was chosen to represent that which would minimise between-plant variability due to growth conditions and age of plant (§ 2.2.1) and was most applicable to the harvest and washing process at a watercress farm, where cut stem and leaf tissue are washed in water.

A single batch of mature watercress was harvested from the watercress farm at Warnford, Hampshire. It was freshly harvested then frozen to -20 ºC to ensure cell wall lysis and therefore a maximum PEITC release could be assumed. By using a single batch of watercress for all tests, the variability of PEITC levels produced due to differing growth conditions and plant age (Engelen-Eigles *et al.*, 2006, Palaniswamy *et al.*, 2003) would be eliminated.

Test samples were prepared by 'washing' a measured (wet) weight of frozen watercress leaf/stem in water. Laboratory cold mains water supply was used, after it had been allowed to flow for a period of at least one minute. The weighed frozen watercress leaves and stems were placed in a beaker; a measured volume of dilution water was added and stirred once. The mixture was then filtered using a 250 µm mesh to remove the course debris and the resulting wash water used as the test sample. A process of solid phase extraction was then carried out to isolate the PEITC and this is described in Section 2.4.2.

2.4.2 Overview of the Solid Phase Extraction Process

Solid phase extraction (SPE) uses a solid phase and a liquid phase to concentrate the amount of analyte in a solution. SPE was used to change the matrix of the analyte from water to an analytical grade solvent suitable for analysis by GC-MS, although it can also be used for removal of interfering substances and concentration of the analyte.

During the process of SPE, the sample is forced or drawn through a column packed with an adsorbent solid. Non-polar interactions occur between hydrocarbon residues of the functional groups of the adsorbent and the analyte. Since most organic compounds have a non-polar structure, they can be adsorbed to non-polar adsorbents by van-der-Waals forces (i.e. a temporary dipole creates weak intermolecular dispersion force between non-polar molecules). Interfering components and matrix molecules are not retained. The analyte can then be removed from the adsorbent by elution with a suitable analytical grade solvent.

Before sample addition, conditioning of the adsorbent is necessary to ensure reproducible interaction with the analyte. Conditioning (also called solvation) results in a wetting of the adsorbent and produces an environment suitable for adsorption of the analyte. Non-polar adsorbents are usually conditioned with 2 to 3 column volumes of a solvent which is miscible with water (e.g. methanol), followed by the solvent in which the analyte is dissolved. After conditioning, the adsorbent bed must not run dry otherwise solvation is destroyed. The sample can then be applied using negative or positive pressure with a flow rate of ~3 ml per minute. This is followed by drying of the adsorbent bed and then by elution of the retained analyte with a suitable eluent at a slow speed of ~1 ml per minute.

2.4.3 Experimental Set-up & Method

A vacuum manifold was used to draw the solvent through the SPE cartridge (see Plate 2.4-a). A valve and gauge on the manifold allowed control of the vacuum applied to regulate and maintain a constant flow rate through the cartridge. A collection tube was placed beneath each cartridge (inside the vacuum manifold) to collect the liquid that passed through.

The SPE cartridges were conditioned by passing 3 x 6 ml solvent (methanol) to wet the adsorbent surface and penetrate the bonded phase, followed by 3 x 6 ml MilliQ water to wet the silica surface, through under vacuum. Between conditioning and sample addition the SPE column packing was not allowed to dry by leaving approximately 1 mm of solvent above the adsorbent bed during this process. Then immediately, 9 ml sample was added and the cartridges were dried under vacuum for one hour. They were washed with 3 ml methanol and 1.5 ml of the collected sample transferred to GC-MS vials.

Plate 2.4-a Vacuum Manifold and SPE columns

2.4.4 Choice of Solid Phase Extraction Cartridge

Preliminary analysis of an aqueous dilution of the PEITC standard was carried out using a range of different SPE columns to confirm the adsorbent phase with the highest affinity for PEITC retention. Eleven different types of SPE column were used and it was anticipated that a C18 phase (endcapped, with octadecyl-modified silica) would be the most suitable to retain the complex organic PEITC. Octadecyl-modified silica is a non-polar sorbent which retains most organic analytes from aqueous matrix (Thermo Fisher Scientific Inc., 2008).

A PEITC stock solution was prepared at a concentration of 1.094 µg/µl and a 1:100 dilution in water was made (0.01094 µg/µl) which would result in approximately 10 ng of PEITC injected onto the GC-MS column. As for the wash water samples, dilution water from the laboratory cold mains supply was used, after it had been allowed to flow for a period of at least one minute.

Detection and measurement of PEITC from an aqueous dilution after solid phase extraction was possible with all of the columns tested. The level of PEITC extracted from the columns varied greatly with the highest level being over 200 times greater than the lowest (see Figure 2.4-a). The Chromabond C18ec column, which had a reservoir volume of 6 ml and 1000 mg adsorbent mass, retained the most PEITC and was chosen to use for all further solid phase extraction of PEITC from samples.

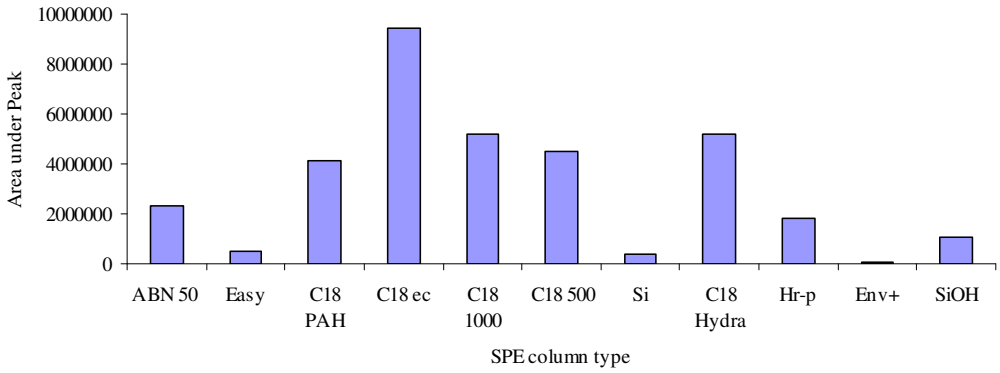

Figure 2.4-a SPE Column Comparative Performance

The area under peak represents the relative abundance of PEITC in the injected sample. Results are presented in the order of testing.

2.4.5 Performance of the C18ec Cartridge

The performance of the C18ec cartridge was assessed over a range of concentrations of PEITC standard in aqueous dilution. SPE of PEITC from the aqueous dilutions was carried out on three separate occasions to assess the reproducibility of the method. A calibration curve for PEITC extracted from aqueous dilution by SPE was constructed and is shown in Figure 2.4-b. It was estimated that the dilution series would result in between 0.9 ng and 92 ng PEITC being injected onto the GC-MS.

The coefficient of determination (R^2) was used to judge linearity and the calibration curve showed linearity over the tested range of concentrations.

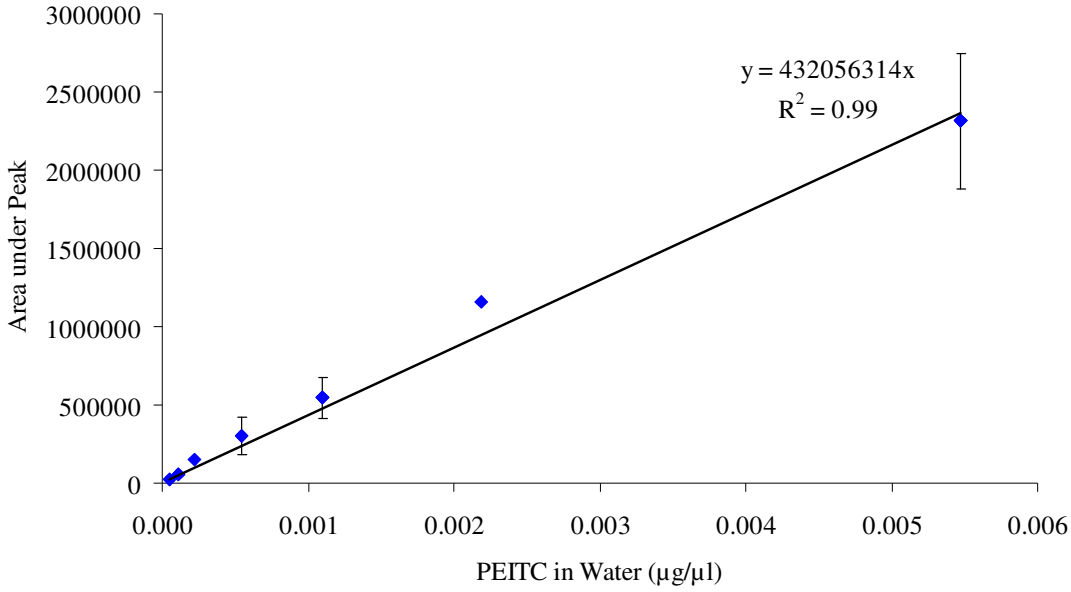

Figure 2.4-b Calibration Curve for PEITC Extracted from Aqueous Dilution

Vertical bars show the standard error where analysis of concentration was repeated on 3 occasions. Other concentrations were analysed on a single occasion. The linear trend line, regression equation and coefficient of determination are shown. The area under peak represents the relative abundance of PEITC in the injected sample.

In order to assess the repeatability of the method a series of repeats of a single concentration of PEITC standard (0.001 µg/µl) in aqueous dilution were extracted by SPE and analysed. These are illustrated in Figure 2.4-c and show good repeatability; all samples within one standard deviation of the mean.

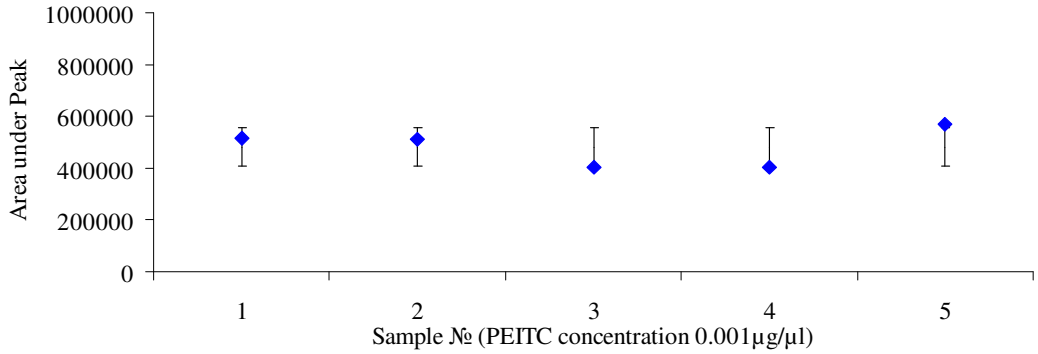

Figure 2.4-c Repeatability of SPE Method

Vertical lines show one standard deviation from the mean of 5 samples. The area under peak represents the relative abundance of PEITC in the injected sample.

In order to assess the efficiency of the SPE procedure a range of PEITC standards prepared in methanol. An aqueous dilution of these standards was made, SPE was undertaken and the extraction analysed alongside the PEITC standards in methanol so that a comparison could be made (Figure 2.4-d). During the SPE process the PEITC standard was diluted with water by a factor of three, then subsequently concentrated by a factor of three during elution with the solvent (9 ml of sample was placed on the SPE column and 3 ml of methanol was used to wash the analyte from the SPE cartridge). However, the efficiency of the SPE method could not be determined by this comparison. GC-MS analysis of the analyte extracted from an aqueous dilution of the PEITC standard consistently showed higher levels of PEITC than the standard in methanol. Even assuming that the SPE method was 100% efficient, there could not be a greater mass of PEITC in the analyte from extraction of the aqueous dilution. The potential causes of this are discussed further (§ 2.6.2).

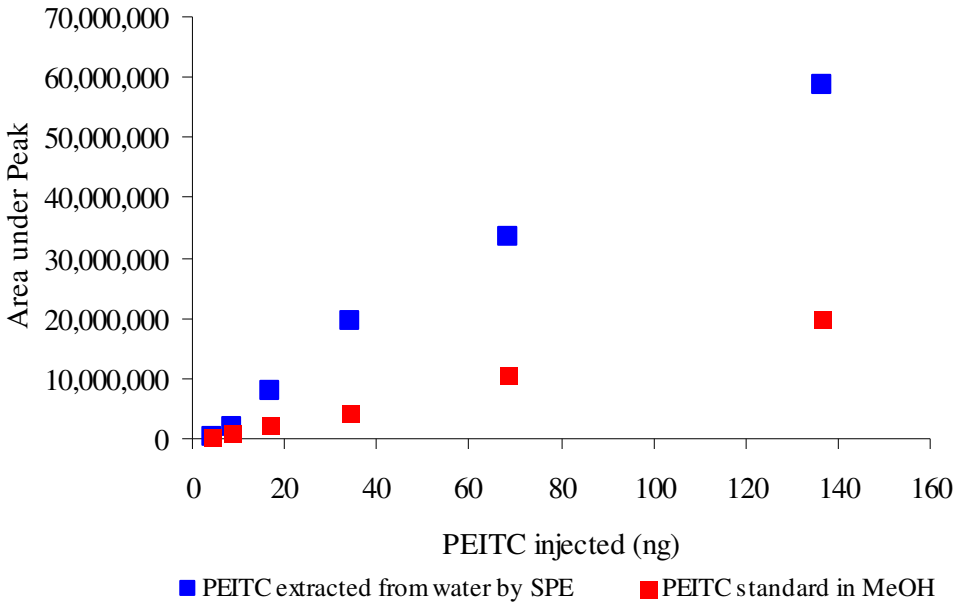

Figure 2.4-d Efficiency of SPE Over a Range of Concentrations

Samples of PEITC standard were extracted from water by SPE and injected onto the GC-MS at the same concentration as standard in MeOH. The area under peak represents the relative abundance of PEITC in the injected sample.

2.4.6 Watercress Wash Water Samples

Having established that it was possible to extract PEITC from an aqueous solution and use GC-MS analysis to identify it, samples of watercress wash water could now be tested. Using the method described in Section 2.4.1, 10g of frozen watercress tissue was washed in 1 litre of water. Solid phase extraction was carried out according to the method described in Section 2.4.3, although for this preliminary test a larger sample volume (50 ml) was additionally tested in anticipation that a low level of PEITC would be extracted from the sample (Figure 2.4-e and Figure 2.4-f.)

The wash water samples were run alongside a PEITC standard. GC-MS analysis showed peaks with a retention time of 8.35 and 8.36 minutes for the wash water samples and the PEITC standard and mass spectral matching confirmed these corresponded to PEITC.

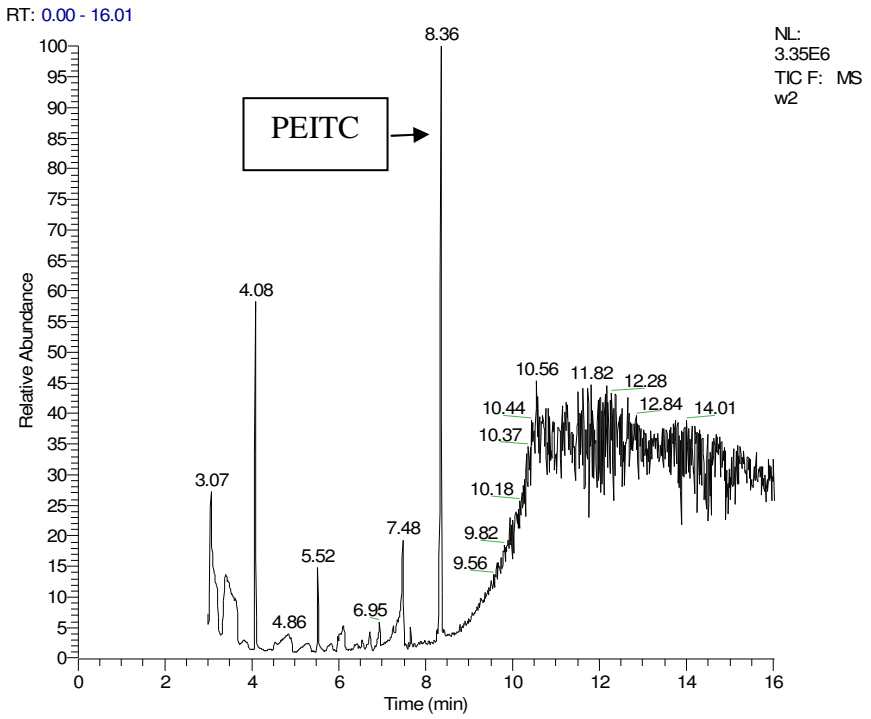

Figure 2.4-e PEITC Abundance Peak – 50 ml Wash Water Sample

The relative abundance peak of 100% for PEITC, with a retention time of 8.36 minutes is indicated.

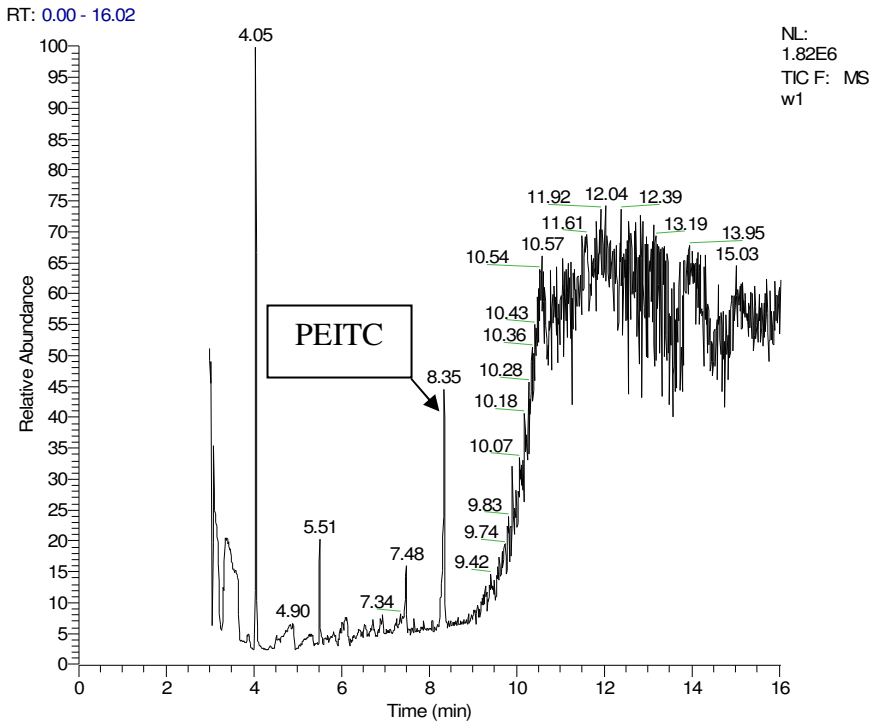

Figure 2.4-f PEITC Abundance Peak – 10 ml Wash Water Sample

The relative abundance peak of 44% for PEITC, with a retention time of 8.35 minutes is indicated. The lower relative abundance is due to the smaller sample volume.

2.5 Quantification of PEITC in Wash Water

2.5.1 Method of Calculation

There are several different methods which may be used to calculate the concentration of a substance present in a sample injected on the GC-MS column. The sample response may be compared with responses of a known concentration of an internal standard (injected along with the sample) or the response of a known concentration of an external standard (prepared at an analogous concentration and injected before or after the sample). The response may also be calculated using a rearrangement of a calibration curve of a standard preparation of the substance to be analysed. Further detail of the calculations used to quantify PEITC in was water samples are given below. For each sample analysed the relative abundance of PEITC or PITC was measured by the area under the peak.

Method of calculation using an internal standard (PITC)

First, the relative response (Response Factor) of a known concentration of a standard of PEITC was calculated comparative to a known concentration of the internal standard PITC (Scott, 2007) [Equation 2.1]

$$\frac{\text{conc. PEITC standard}}{\text{conc. PITC internal std.}} = \frac{\text{area under peak PEITC standard}}{\text{area under peak internal std}} * \text{Response Factor} \qquad [2.1]$$

Then, by rearrangement of Equation 2.1:

$$\text{Response Factor} = \frac{\text{(conc PEITC standard * area under peak internal standard)}}{\text{(conc PITC internal standard * area under peak PEITC standard)}} \qquad [2.2]$$

Once the Response Factor had been calculated, a spike of a known amount of PITC internal standard was then used to calculate the concentration of PEITC in a sample of wash water in Equation 2.3.

$$\text{conc. PEITC in sample} = \frac{\text{area under peak sample * (conc. PITC internal standard) * RF}}{\text{area under peak internal standard}} \qquad [2.3]$$

Method of calculation using PEITC standard calibration curve

The concentration of a range of aqueous dilutions of the PEITC standard after solid phase extraction was plotted against the relative abundance (area under peak) response to produce a calibration curve of PEITC standards in aqueous phase (see Figure 2.4-b). The PEITC concentration of wash water samples was calculated by rearranging the slope equation (y = slope of calibration curve x) of the PEITC standard calibration curve in Equation 2.4.

$$\textit{conc. PEITC in washwater sample} = \frac{\textit{area under peak}}{\textit{slope of the calibration curve}} \quad [2.4]$$

2.5.2 PEITC Analysis of Wash Water Samples

Watercress wash water samples were prepared using a range of different ratios of leaf wet weights in water. Sample A was prepared using 1 litre of water and all other samples (B-F) were prepared using 500 ml water. Solid phase extraction and GC-MS analysis was carried out using the method described in Section 2.4.3. The concentration of PEITC from the wash water samples was calculated using both the PEITC standard calibration curve and the PITC internal standard responses for comparitive purposes and is shown in Table 2.5-a.

Table 2.5-a PEITC Concentration in Watercress Wash Water Samples

Sample ID	Frozen Leaf wet weight (g)	Leaf wt (g) /wash water (L)	Conc. PEITC (µg/µl) ^	Conc. PEITC (µg/µl)*
A	10.00	10.00	0.005	NS
B	1.09	2.18	0.001	NS
C	4.06	8.12	0.004	NS
D	1.10	2.20	0.002	0.006
E	2.03	4.06	0.002	0.007
F	4.01	8.02	0.005	0.013

^ calculated using SPE calibration curve slope

* calculated using internal PITC standard (response factor calculated using internal PITC spike compared to mean of PEITC standard run alongside)

NS - no internal standard (calculation using this method not possible)

The relationship between the weight of leaf washed (per litre of water) and the concentration of PEITC is illustrated in Figure 2.5-a . Both methods of calculation of the PEITC concentration show an increase in the PEITC concentration with increasing leaf weight washed (i.e. an increasing ratio of leaf to water). The SPE calibration method results in approximately a 4-fold increase in µg PEITC per litre with a five fold increase in leaf weight per litre. Calculation using the internal standard method gives higher concentrations of PEITC and a different relationship, although, with only 3 data points, this curve should be treated as less reliable.

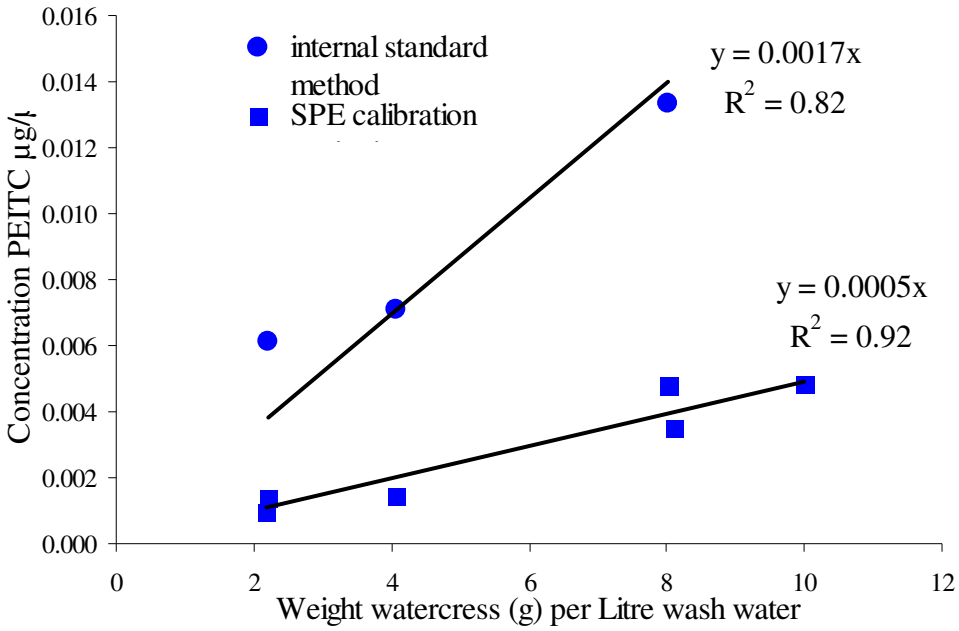

Figure 2.5-a Relationship between PEITC Concentration and Weight of Watercress Washed

Actual (rather than nominal) weights of watercress per litre of wash water are used. The linear trend lines, regression equation and coefficient of determination are shown for each set of data.

2.5.3 Variability of PEITC from Wash Water Samples

In order to assess the variability of PEITC washed from the leaf during sample preparation (i.e. the reproducibility of the sample preparation method) two of the test samples (with different leaf to water ratios) were prepared and analysed on two separate occasions. The second preparation of samples from wash water with 2g leaf tissue and 8g leaf tissue per litre of water resulted in similar concentrations of PEITC

to the first. The mean ± SD (n=2) values for the 2g and 8g samples were 0.0012 ± 0.0002 g/L and 0.0041 ± 0.0006 g/L respectively (also refer to samples B&D and samples C&F in Table 2.5-a), indicating reproducibility of the method. Further repeats would allow the reproducibility to be defined more accurately. Furthermore, an estimation of the concentration of PEITC present per gram of leaf washed was made for each wash water sample (Table 2.5-b). The amount of water used to wash the leaves and the weight of leaf washed was taken into account. The amount of PEITC present in a 5 µl sample placed on the GC-MS column was estimated using a rearrangement of the slope equation [2.5] constructed from calibration of PEITC standard in aqueous dilution.

$$y = 25923x \qquad [2.5]$$

where: y = Area under peak, x = ng PEITC injected onto the GCMS column.

The amount of wash water sample injected was 1/600th of the 3 ml sample collected from SPE process and, assuming 100% efficiency of the SPE process, this was therefore equal to the amount of PEITC in the 10 ml sample put onto the SPE column. By multiplying this according to the total volume of water used to wash the watercress, the amount of PEITC washed per gram of watercress tissue could then be estimated by Equation 2.6.

PEITC (µg) / weight watercress washed (g) = Conc. PEITC per g leaf (µg/g) [2.6]

Table 2.5-b Amount of PEITC Released per Weight Frozen Plant Washed

Sample ID	Wet weight leaf washed (g)	Volume wash water (ml)	PEITC µg/g leaf
A	10.0	1000	497
B	1.09	500	527
C	4.06	500	448
D	1.10	500	696
E	2.03	500	397
F	4.01	500	612
		Mean (± SE)	529 (±45)

2.6 Discussion

2.6.1 Identification of PEITC in Watercress Wash Water

The production of isothiocyanates by cruciferous plants is well documented and their measurement from plant tissues widely reported (§ 2.2). Sections 2.3 to 2.5 have described the development of a method to isolate and measure PEITC from aqueous samples, i.e. the water in which watercress tissue has been washed. The potentially negative effect of PEITC on freshwater invertebrates in the receiving waters below watercress farms has been the subject of much discussion (Worgan and Tyrell, 2005, Natural England, 2009, Newman, 1990b, Dixon, 2009), although it has not been possible to confirm the extent of its presence. The ability to measure PEITC, in particular from samples of watercress wash water, would assist in the monitoring of its release to the receiving waters below watercress farm outfalls.

The method described, using solid phase extraction to prepare samples for analysis using gas chromatography mass spectrometry, was successfully applied to identify PEITC from samples of watercress wash water. PEITC could be consistently identified from wash water samples prepared using small quantities (as little as 1g wet weight) of frozen watercress tissue (Table 2.5-a). The method was additionally made straightforward by requiring only small volumes of wash water sample for the extraction of PEITC. This enabled a relatively rapid sample preparation and extraction process which in view of the volatile nature of PEITC was an important consideration. Analysis using the GC-MS also proved very sensitive and we were able to detect PEITC from samples of PEITC standard prepared at low concentrations (in the order of 0.00001 g/L). Greater sensitivity would potentially allow the identification of PEITC from river water samples, where larger dilution occurred.

2.6.2 Method Reproducibility and Accuracy

In order to assess the reproducibility and accuracy of a specific method, it is important that as many possible sources of variability are removed from the process.

In considering the reproducibility of analysis of wash water samples a number of issues were considered in relation to sample preparation. Sources of variability due to potential within-plant variation (Fahey *et al.*, 2001, Rosa, 1997, Shelton, 2005) were minimised by selecting leaf and only small stems for sample preparation. The use of a single batch of freshly harvested crop stored frozen addressed the issue of PEITC variability due to crop age and environmental growth conditions (Palaniswamy *et al.*, 2003). However, the use of the wet weight of frozen tissue potentially introduced an unknown and possibly variable weight of water in the defrosted sample.

In considering variability which may be introduced due to the equipment, volumetric glassware and calibrated equipment were used where possible to reduce further sources of inaccuracy. Additionally, prior to each analysis of samples using the GC-MS, a blank (methanol only) was injected to assess column bleed. On several occasions there was column bleed evident, although there were no peaks which coincided with the retention time where the PEITC or PITC components were expected, therefore inaccuracies due to column bleed could be discounted.

Using a PEITC standard diluted in water, the method was found to be reproducible over range of concentrations from 0.0005 – 0.0055 g/L (Figure 2.4-b). The analysis of PEITC in wash water samples prepared with the same ratio of wet weight watercress:water (i.e. to the same nominal concentration) also indicated that the method was reproducible.

It was not however possible to measure the recovery rate of PEITC using the solid phase extraction method as analytical standards diluted in water gave consistently higher readings than standards in methanol. This was counter-intuitive, as extracted samples would normally contain lower levels of the component i.e. a proportion of the component would not be retained by the SPE column. This may have been due to the nature of the SPE column and/or the response of the GC column. It is possible that interfering components in the aqueous sample matrix were retained by the SPE column and thus appeared to enhance the PEITC signal picked up by the GC-MS column. The GC response factor for aqueous PEITC standards was much higher than the response factor for PEITC standards in methanol or standards in methanol

which had passed through the SPE column. Furthermore, the polarity of the leaf extracts could have been enhanced by dilution with water, which would enhance the non-polar extraction on the C18 material (Machery-Nagel & Co., 2009). The use of internal standards, which were not reliant on the extraction procedure, enabled the use of the method to establish PEITC concentrations.

In comparison with previously reported values of PEITC directly extracted from watercress tissue (and expressed by weight of leaf), the range of PEITC concentrations from wash water samples (Table 2.5-b), 397-696 µg/g leaf washed, fell within a similar range as those reported by Palaniswarmy (2003), 233-688 µg/g leaf. The watercress used for this study was mature and we would expect that levels of PEITC washed from it would be similar to the larger values found by Palaniswarmy which corresponded to the more mature plants. Cole (1976) reported a lower level in young watercress plants grown in the UK under glass (74 µg/g leaf).

2.6.3 Suitability for Industrial Application

Consideration must also be made as whether the method developed for the measurement of PEITC from aqueous or wash water samples could feasibly be applied to monitor or trace PEITC originating from watercress farming and/or processing.

The cost of carrying out analysis will be a key factor in the way in which a method is applied. The equipment required to prepare the wash water samples and PEITC/PITC standards was not extensive or specialised and mostly constituted widely available laboratory glassware and consumables. The SPE process required more specific equipment, for example the vacuum manifold and disposable (single-use) specialised SPE columns. However, by far the greatest expense was the GC-MS analyses. It would be expensive to run large numbers of samples and cost-benefit analysis would most likely be necessary when considering the feasibility of using this method for example as a tool for PEITC tracing, where many samples may be required. Given that the method appears to be relatively sensitive, it has the potential to be used to establish the levels of PEITC in receiving water downstream of watercress farms.

The decision to use a single batch of frozen watercress to minimise variability between samples was made with the compromise that wash water would be less like that produced from a watercress farm; the process of freezing would maximise the potential for PEITC release into the wash water – a worst case scenario. Method reproducibility was considered of greater significance at this stage of the method development process. An initial trial of analysis of PEITC in wash water from fresh watercress tissue was able to detect PEITC at approximately 15% of that from frozen tissue (Appendix C).

The ratio of leaf weight to water was selected to bear comparison with product to wash water ratios used at the Vitacress Salads Ltd washing and packing facility at Lower Link Farm i.e. at a ratio of 1g leaf per 100 ml water (Vitacress Salads Ltd, 2008b). Furthermore, the same ratio was maintained for wash water prepared for use in the ecotoxicological studies described in Chapter 3 and thus gives an indication of the concentration of PEITC that the organisms were exposed to.

In addition to method specific consideration, there are a number of site specific issues which must be considered in an industrial application of the method. Temperature and time taken to reach the receiving water could both affect the PEITC concentration of an outfall. In the laboratory the sample preparation and analysis was carried out at room temperature, whereas ambient temperature in a watercress bed or receiving water would vary seasonally and daily. The factory wash and packing house at Lower Link Farm operates at an ambient temperature of 5ºC and washes salad leaves with borehole water which has a constant temperature of 10 ± 0.5ºC. At these temperatures (Ji *et al.*, 2005) found the half life of PEITC at pH 7.4 was 108.1 ± 4.3 h ($p>0.001$). Most wash water at Lower Link Farm is also re-used by re-circulation within the wash process line which could increase the input of PEITC but also allow some degradation during this process. PEITC is also likely to be degraded during the wash water flow through the watercress cropping beds.

Calculation of residence times on watercress farm sites prior to discharge to the receiving water is also not straightforward and may be affected by for example, the site locality and size, low flow rates in beds of young watercress seedlings or during harvest operations when flow is stopped. Finally, an assessment of the on-site crop

management activities would need to be carried out prior to taking samples, as variability of PEITC levels in site discharge would be affected by harvesting or other crop manipulation or work within the beds. Further consideration of the reduction of PEITC by the recirculation of factory wash water is given in Section 6.2.3.

2.6.4 Further Work

In order to assess fully the reproducibility of the method, additional wash water samples, prepared according to the method in Section 2.4.1 would need to be analysed. Method reproducibility could also be assessed using results from PEITC standard or a single split sample in several different laboratories. Alternatively, an extension of the trial of sample preparation described in Appendix C could be carried out, using samples washed directly in methanol, rather than water and negating the requirement for SPE.

A timed sequence of analyses of samples prepared and stored under controlled laboratory conditions and using differing light and temperature regimes could be used to establish the stability and degradation rate of PEITC in an aqueous matrix. Samples prepared from fresh leaves could be used for this, although it may be easier to identify the (GC-MS) peaks and thus to establish the rate of degradation if there is a greater PEITC concentration at the start of the sequence.

Analysis of samples of wash water collected on site or downstream of a watercress farm would establish whether levels present in the environment were measurable. Analyses of wash water solutions prepared using other isothiocyanate-producing salad crops could be carried out. For example kale or mizuna, or combinations of watercress and such crops. Gil and MacLeod (1980) noted that relative glucosinolate abundance was altered by incorporating another Cruciferous plant. It is possible that combinations of isothiocyanate-producing plants would have synergistic or antagonistic effect on concentrations of PEITC produced.

2.7 Conclusions

This Chapter has demonstrated that the identification of PEITC from freshly prepared watercress wash water is possible and relatively straightforward using gas chromatography-mass spectrometry (GC-MS) techniques. Using solid phase extraction, PEITC can be isolated from samples of watercress wash water and standards prepared using an analytical grade PEITC standard. The determination of the efficiency of the solid phase extraction methodology was however more problematic, although it was still possible to carry out quantification of PEITC by comparison with an internal standard and with reference to calibration using an external standard.

The concentration of PEITC in wash water samples was found to increase when the ratio of watercress plant tissue to water was increased. A calculation of the amount of PEITC released per gram of plant tissue washed found levels analagous to those reported in the literature. An assessment of the variability of levels of PEITC in standardised preparations of watercress wash water (i.e. with a known ratio of leaf wet weight to water) was also possible. The method was found to be reproducible at the concentrations tested (2g and 8g of leaf washed per litre wash water).

A number of future challenges were identified which would primarily extend the dataset and further establish method reliability, but also would increase the knowledge of PEITC and its fate once released into wash water. The sensitivity of the method indicated that it would be suitable for application to measurement of PEITC from receiving waters, although site specific issues, such as crop management activites and cost of analyses would require consideration prior to implementation.

This Chapter has established that PEITC is present and measureable in watercress wash water. The following Chapters will explore the potential effect that PEITC released into watercress wash water has on the macroinvertebrate *G. pulex*.

3 THE EFFECT OF WATERCRESS-DERIVED PEITC ON *GAMMARUS PULEX*

3.1 Introduction

3.1.1 Watercress-Derived Isothiocyanates

Chapter 2 has described how PEITC is produced by watercress and can be measured in watercress wash water. Isothiocyanates released by watercress have well documented allelopathic and genotoxic properties (Newman *et al.*, 1996, Bialy *et al.*, 1990, Kassie and Knasmuller, 2000, Musk *et al.*, 1995). Glucosinolate-containing plants also have the potential to control terrestrial pests (Brown and Morra, 2005), but previous studies have been largely restricted to terrestrial cultivated species. Isothiocyanates produced by watercress and other crucifers have a role in the plant defence against herbivorous macroinvertebrates such as snails, caddis flies and gammarids (Newman *et al.*, 1996). Few studies have been carried out in relation to the effect of isothiocyanates on aquatic macroinvertebrates. However, although the effectiveness of PEITC as a feeding deterrent has been established (Newman *et al.*, 1996, Newman *et al.*, 1992, Newman, 1990b) and behavioural tests by Worgan & Tyrrell (2005) which showed avoidance of salad wash water by *Gammarus pulex*, the effect on macroinvertebrates of repeated exposure to water in which watercress has been washed (and therefore potentially having artificially elevated PEITC concentrations) is largely unknown. Little is known about the effect of isothiocyanates on *G. pulex* reproductive behaviour and the survival of juveniles.

Low numbers of *G. pulex* have been recorded in the receiving waters of the Bourne Rivulet, below Lower Link farm, where water from both the cropping watercress beds and also the salad washing and processing factory is discharged (Medgett, 1998). Reduction in macroinvertebrate numbers and species diversity is of particular cause for concern due to the status of the watercourse as a chalk stream headwater which has an important role in the functioning of the River Test ecosystem downstream (Furse, 1995). It is also a coarse fishery, once celebrated in print (Plunkett Greene, 1924) as a particularly fine example. Further exploration of the nature of impact on the macroinvertebrate ecology is carried out in Chapter 5.

This Chapter further explores the extent of acute and sublethal effects of PEITC and watercress wash water on *G. pulex* in order to better understand the causes of their low numbers in streams below watercress farms. Particular reference is made to the daily pulsed exposure to potentially elevated levels of PEITC produced by the large area of watercress cropping beds and salad washing and processing facility at Lower Link Farm.

3.1.2 Short Pulse Exposure

Due to the unrecorded, but possibly unstable, nature of PEITC in watercress and salad leaf process wash water and the receiving environment (§ 2.2.3), the majority of endpoints typically used to measure sublethal toxicity may not be suitable due to the test duration, which is often of the order of weeks rather than days. Reproduction tests with the freshwater invertebrate *Daphnia magna* (Strauss) are routinely carried out over a period of 21 days (the time taken to produce about 5 broods) and growth tests with *G. pulex* are also carried out over a period of 21 days. Further detail relating to the stability of PEITC is given in Chapter 2, although it should be noted that a pharmacokinetic study (Ji *et al.*, 2005) found variable stability over a period of 2 to 4 days. At the salad wash process factory at Lower Link Farm, the crop or combination of salad crops being washed changes periodically throughout the working day. Due to this, it is likely that the invertebrate populations within the watercress beds and possibly in the receiving water are exposed to varying pulses of PEITC depending on the variety of salad leaf being processed and/or activity in the watercress cropping beds and possibly also the mix of produce (Gil and MacLeod, 1980). Additionally, the factory only works during the day and the invertebrate populations will therefore be exposed to PEITC from the wash process during this time; overnight the water flow through the beds is maintained by pumped borehole water flow.

There is some literature documenting ‘time limited’ or ‘time to event’ studies following short pulse exposures. Heckmann *et al*. (2005) detects biochemical biomarkers up to seven days following short pulse exposure of *G. pulex* to a pyrethroid insecticide, lambda-cyalothorin; precopulatory behaviour was also significantly impaired and mortality significant. It is also worth noting that Tyrell

(2005) describes an "un-quantified but notable" increase in mortality on transfer to clean living conditions following exposure to PEITC in a sublethal assay. Cold and Forbes (2004) also note this phenomenon and note that despite 100% survival during exposure to pyrethroid insecticide esfenvalerate, effects on survival, pairing behaviour and reproductive output were still detected at least 2 weeks following exposure.

Worgan and Tyrell (2005) devised a 6 hour avoidance assay to establish whether *G. pulex* actively avoided water containing chemicals derived from watercress. Test concentrations were prepared using a known wet weight of blended filtered leaves mixed with deionised water. Avoidance behaviour was recorded for test concentrations prepared with 40 g and 20 g of leaves in 100 ml water ('actual' PEITC concentration was not measured), although lower concentrations did not cause avoidance behaviour.

3.1.3 Disruption of Precopular Behaviour

G. pulex undertake a period of guarding behaviour prior to mating. An adult male takes hold of a female and the pair remains together in precopular position for a few days until the female moults. Mating then occurs before her cuticle hardens and the eggs are laid into a brood pouch. They hatch after several days and leave the brood pouch. The female becomes attractive to males again at, or slightly before, the hatching of the eggs (Hynes, 1955).

Poulton and Pascoe (1990) developed a sublethal behavioural bioassay based on the disruption of precopular pairing. Precopular pairs previously exposed to a toxicant separated faster than unexposed pairs once placed in an anaesthetic. They found the bioassay to be both rapid and sensitive to cadmium. Prenter *et al*. (2004) also found that precopular separation was a sensitive and rapid indicator of stress to raised ammonia levels.

During a project to develop methods to evaluate toxicity to freshwater ecosystems Girling *et al*. (2000) carried out a series of single species laboratory tests and stream mesocosm experiments. They used a range of lethal and sublethal endpoints and

concluded that, for *G. pulex*, those endpoints consistently sensitive were neonate growth, precopular separation and population growth.

Watts *et al*. (2001) used this reproductive behaviour test to determine the effects of vertebrate–type endocrine disrupting chemicals. The ability of males and females to detect each other, form precopulatory guarding pairs and to continue the guarding behaviour was examined. The time for pairs to reform was also monitored; after a 24 hour exposure to the test solution, pairs were separated and then returned to the test solution and re-pairing was noted over a 4 hour period. Although acutely toxic, they did not find re-pairing behaviour was affected at environmentally relevant concentrations, i.e. those that would be found in the natural environment. However, they note that there was evidence (cited Christofferson, 1978, Gleeson, 1980) to support the use of chemical signals in crustacean sexual behaviour and that pheromonal control of mating in *G. pulex* was likely to be dependent on the stage of sexual development.

3.1.4 Study Objectives and Hypothesis

This study has been designed to investigate the effect of watercress-derived isothiocyanates on the juvenile life-stage of *G. pulex* and also its effect on reproducing adults. In particular, the key objectives of this work were to investigate the effects of watercress wash water on juvenile *G. pulex* and adult precopular pairs and to quantify any effects identified in a format relevant to the factory wash processing of watercress carried out at Lower Link Farm. The hypothesis tested was that phenethyl isothiocyanate (PEITC) present in water in which watercress had been washed causes a detrimental effect on *G. pulex*.

Section 3.2 describes the bioassays which were carried out. The results are presented in Section 3.3 and discussed in the Section 3.4 with reference to the implications for populations of *G. pulex* existing in the receiving waters below watercress farms as well as for the producers and processors of watercress.

3.2 Materials and Methods

3.2.1 Approach

A series of ecotoxicological tests was carried out to establish the acute response of juvenile *G. pulex* to watercress wash water with the aim of establishing the median effective concentration (EC_{50}) for acute juvenile mortality. Subsequently, a series of tests was carried out using adults to establish whether there was behavioural disruption of the reproductive process. The study also aimed to quantify any behavioural disruption in a context relevant to the watercress farming and salad wash process at Lower Link Farm. Thus reproducing adults were exposed and subsequently re-exposed to pulses of watercress wash water in a laboratory simulation of the process operation at the farm.

3.2.2 Preparation of Test Solutions

Watercress Wash Water

A single batch of mature (i.e. ready for harvest) watercress was harvested from the Vitacress Warnford site. It was briefly and very gently washed in tap water to remove coarse debris and separated into 100 g batches. It was not thoroughly washed to minimise handling damage to leaves and any subsequent loss of PEITC from the crop. These were then frozen at -80 ºC to store for tests and prevent further hydrolysis of glucosinolate to PEITC. Freezing also caused complete cell lysis and would ensure hydrolysis of glucosinolate to PEITC when test solutions were made. A single batch was used due to potential variability in glucosinolate concentrations (and therefore potential amount of PEITC which may be released) in crops grown under different conditions (Engelen-Eigles *et al.*, 2006, Palaniswamy *et al.*, 1997).

Watercress wash water was prepared using the same method as for the analysis of PEITC in Chapter 1 (§ 2.4.1). Test solutions were prepared either by washing a measured (wet) weight of frozen watercress leaf/stem in media water (see Plate 3.2-a) or by using analytical grade PEITC to make a solution.

Plate 3.2-a Wash Water Preparation

a) Watercress leaves and stems weighed, b) plant material added to the media, c) course debris removed using a 250 µm mesh. Note that plates a) & b) show fresh plant material, although frozen tissue was used in this study.

Media (dilution) water was prepared by vigorously aerating tap water for more than two hours to remove the chlorine. To prepare the watercress wash water, frozen watercress leaves & stems (large stems were excluded) were weighed using a Mettler AJ50 balance and the weighed watercress added to a measured volume of media water. The watercress was weighed and prepared from frozen to minimise the loss of PEITC and for ease of handling. The media water/plant mixture was stirred once, i.e. a stirring rod making one revolution of the beaker (except for the acute tests where the watercress was 'washed' for 30 minutes) and then the leaf and stem debris was filtered out using a 250 µm mesh. The resulting wash water was used as the test solution. It was assumed that the freezing process had caused complete lysis of cell walls and thus complete and immediate hydrolysis of glucosinolate to PEITC.

PEITC Solution

PEITC ($C_6H_5CH_2CH_2NCS$, molecular weight 163.24 AMU) is heat and moisture sensitive (Sigma-Aldrich, 2009) and required dilution with analytical grade methanol. A stock solution of 1µL/L PEITC in methanol was prepared and stored in the laboratory refrigerator. Test solutions were made on the day of the test by preparing a dilution of the PEITC stock with aerated media water (i.e. chlorine free). The dilution of PEITC was based on the comparison of levels recorded by GC-MS analysis of analytical PEITC solutions carried out in Chapter 2. Also with reference

to the results from crushed watercress solutions prepared by Tyrrell (2005), who was able to measure PEITC in the order of 1000 parts per million from samples of crushed watercress at a ratio of 20 to 40 g of leaf in 150 ml water.

3.2.3 Test Organisms

G. pulex were used as the test organism to be exposed to watercress wash water prepared from harvested watercress. Sensitive life-stages, i.e. juveniles and precopular adult pairs were used. Juveniles were used because they are generally more sensitive compared with adults or larger organisms because they have a larger surface/capacity ratio. A larger amount of test chemical may be absorbed per amount body mass. They have a relatively higher respiration rate and higher metabolic activity per unit body weight. *G. pulex* is relatively straightforward to maintain in laboratory culture. Culture techniques and acute toxicity test methods are described by Welton and Clarke (1980) and McCahon and Pascoe (1988). They determined that 1day old juveniles, prior to their first moult, were optimal.

G. pulex were collected from the River Meon at Funtley Mill, Hampshire (NGR SU556089). They were acclimatised to laboratory conditions in a constant temperature room at 14 ± 2 ºC, with a photoperiod of 8 hours daylight, 16 hours dark under cool white fluorescent tubes (mean bench-top illumination of 800 lux), in glass tanks with tap water media which had been vigorously aerated for more than two hours to remove all chlorine (see Plate 3.2-b). They were fed a diet of alder leaves (*Alnus glutinosa* (L.)) pre-soaked in river water and ten percent (by volume) media changes were made every two days for a period of two weeks. The breeding population was then maintained under these conditions.

Precopular pairs were used for sublethal tests as the interruption of reproductive behaviour would be indicative of an unsustainable population. The use of sublethal data would also provide a greater level of sensitivity and in applying the results to the process at Lower Link Farm would afford a greater degree of protection within the receiving water. Additionally, by using different endpoints from previous studies, we could assess for effect on *G. pulex* throughout their life history.

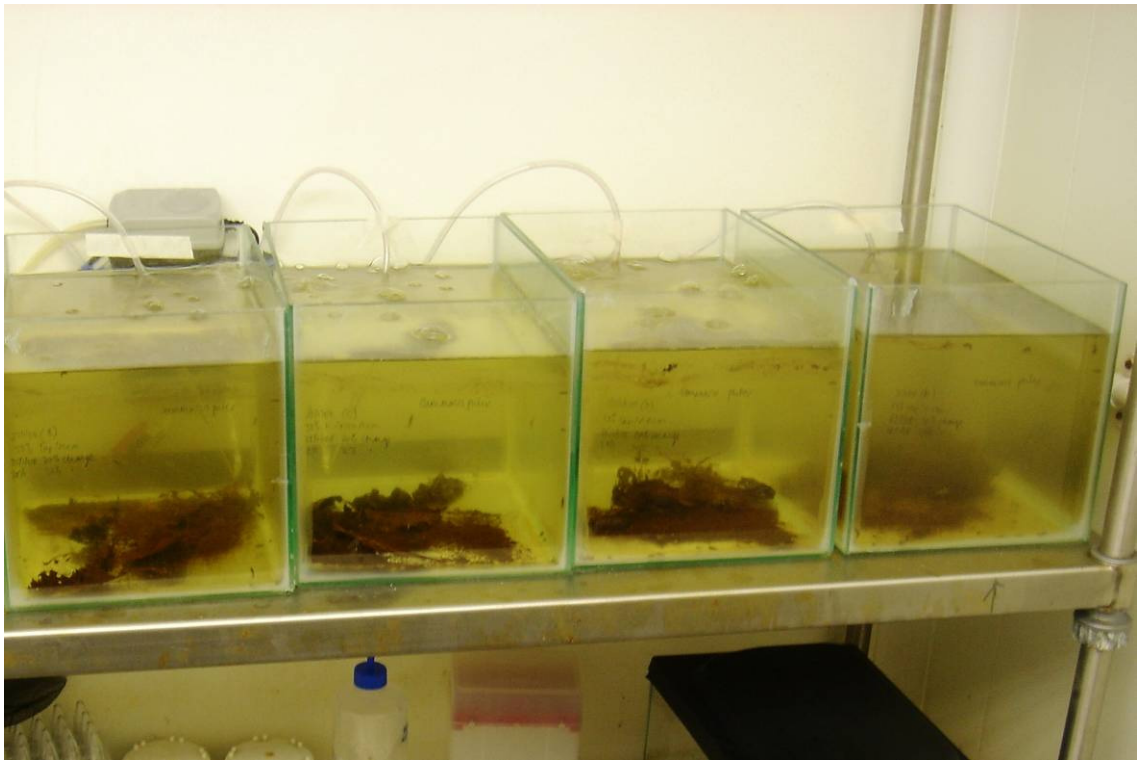

Plate 3.2-b Breeding Population of *Gammarus pulex*

Initial trials resulted in immediate separation of control precopular pairs due to handling stress when they were transferred to and from the holding vessel media. In fact several methods (Cold and Forbes, 2004, Malbouisson *et al.*, 1994, Sexton, 1928) employ physical stimulation as a technique to isolate males from females. Of a number of different methods examined for the transfer of precopular pairs (e.g. use of a wide bore pipette, a sieve, a spoon or emptying out media), the advantage due to minimising handling stress was compromised by other factors such as the time taken or the potential dilution of the test solution by media water. The use of the wide bore pipette was chosen because it caused minimal handling stress and transfer of media water but also did not impractically prolong the transfer of organisms to the test solution.

3.2.4 Quality Control

Although not a formally accredited test method, the tests were carried out as far as possible according to quality control methods prescribed by laboratory standard ISO 17025 (International Organisation for Standardisation, 2005). Daily temperature checks were carried out to ensure the constant temperature room remained within an

acceptable temperature range. Equipment used was calibrated using United Kingdom Accreditation Service (UKAS) approved methods (e.g. balance, Finn-pipettes, water quality meters, timers) and calibrated volumetric glassware was used. Solvent and media controls were carried out for tests using PEITC test solutions and media controls carried out for tests using watercress wash water test solutions. All control organisms were subject to the same handling stress as the test organisms. Water quality validation criteria for dissolved oxygen (>60% ASV), pH (constant to within 0.5 unit), conductivity (<10% change) were also assessed for each test. No adjustment or correction of test solutions was required as validity criteria were met on all occasions.

3.2.5 48 Hour Acute Juvenile Test

Four acute tests were carried out to assess the toxicity of the watercress wash water to *G. pulex* juveniles. Adult precopular pairs were isolated from the cultures into holding vessels containing aerated media for maximum period of 7 days and fed with alder leaves. Juveniles produced from these pairs were used in the tests and were less than seven days old at the start of the test. The acute tests were carried out in 10 ml volume cell wells (six well, non-pyrogenic, polystyrene multidishes) which had been pre-soaked for 24 hours in reverse osmosis water to pre-leach them. Five juveniles were placed in each cell well and four cell wells per concentration were used. The test vessels were covered with lids for the duration of the test to minimise evaporation of test solution and potential loss of PEITC.

For the initial (range-finding) acute test a nominal concentration range between 0 and 10 g watercress washed in 100 ml aerated media was selected, plus a media control. Based on the result of the range-finding test, subsequent tests could be conducted using a narrower concentration between 0 and 0.5 g per 100 ml media due. The wet weight of watercress used to make each test solution was recorded and the weighed watercress was washed in the media in a glass beaker for a 30 minute period. The test solutions were prepared using a slightly different method from the sublethal tests; to ensure enough PEITC was present and ensure a measurable response, a more thorough and longer wash process was used. Each leaf/media mix was stirred once on mixing, after 15 minutes and immediately prior to filtration.

After 30 minutes each leaf/media mix was poured through a 250 µm mesh to remove the coarse leaf debris from the test solutions. Test solutions were pipetted into test vessels (cell wells) and juveniles randomly assigned. Aerated media was used for the controls and the same number of test organisms assigned as for each test concentration. Water quality parameters (pH, temperature, conductivity and hardness) were recorded at test start and end. All the tests were prepared and carried out under the same environmental conditions as the cultures were maintained at. The test endpoint recorded was immobilisation and was recorded at 48 hours. Test organisms were considered immobile if they did not move within 15 seconds following gentle agitation of the test vessel even if there was still movement of the pleopods.

3.2.6 Two Hour Time to Pair Separation Test

As part of their mating behaviour *G. pulex* form precopulatory pairs for several days, separating once fertilisation has taken place (Pascoe *et al.*, 1994, Watts *et al.*, 2001) (Plate 3.2-c).

Plate 3.2-c ***Gammarus pulex* Precopulatory Pairs**

Initial observations of precopulatory pairs in wash water were made with a view to carrying out the precopulatory separation (GaPPs) test described by Pascoe *et al.*

(1994). This bioassay exposes precopulatory pairs to test solution for a one hour period, followed by an enforced separation (mechanically or using an anaesthetic solution) and records the time taken for pairs to reform. However, during the one hour exposure to watercress wash water, pairs were already separated and after two hours the majority of pairs were often separated. Therefore a variation of this method was used.

Precopulatory pairs were exposed to a single dose of watercress wash water for a two hour period. The concentration of watercress wash water test solution selected was guided by the ratio of leaf to water washed in the salad washing and processing factory at Lower Link Farm; accordingly a concentration equivalent to 1g watercress per 100 ml wash water was selected. The endpoint used was time to separation of pairs and was recorded at 15 minute intervals. It was not possible to take more frequent readings due to the time taken to transfer test organisms at the start of the test and the minimum time taken to make readings by one person. Glass crystallising dishes covered with a watch glass were used as the test vessel, with 150 ml of test solution and 5 precopular pairs added to each test vessel.

3.2.7 Precopular Re-exposure Test

A series of re-exposure tests were also conducted to elucidate responses of precopular pairs to pulsed exposures experienced *in situ* due to on-site operations at Lower Link Farm. The farm wash process at the farm operates daily from 0730 to 1700 h on weekdays and 0630 to 1600 h at the weekend. Outside these hours the discharge to the East Rivulet consists of borehole water from bed flow only. Consequently, there is a period every 24 hours where there are very low (ambient) levels of PEITC (or none at all) present in the discharge. During the processing hours the wash lines are changed at frequent intervals throughout the day. For example, on 10 June 2008 there were 43 different product lines washed and packaged (i.e. mixed or single leaf salad bags). Each product contained up to five crops (out of a total of 39 different crops washed) and a varying proportion of watercress in the total weight washed (28,260 kg) (Vitacress Salads Ltd, 2008a). This illustrates the extremely variable nature of the discharge to the East Rivulet.

Re-exposures were carried out in a laboratory simulation of the variable nature of the wash and process factory water. At the end of the two hour precopular separation test (§ 3.2.5), the test organisms were removed to clean water and left to re-pair over a period of 48 hrs. The re-paired organisms were then re-exposed to fresh test solution as per the first test.

3.3 Results

3.3.1 Acute Tests

Immobilisation of organisms in wash water was compared to that in the control and the relationship between dose and magnitude of the effect was established. Nominal concentrations were used for the analyses as it was not possible to test for the PEITC concentration in the test media (§2.6.4). Data were analysed using ToxCalc v5.0.32 environmental toxicity data analysis software (Tidepool Scientific Software, 1994). The proportional data were arcsine square root transformed and, depending on the format of the data, either maximum likelihood probit analysis, maximum likelihood logit analysis or linear interpolation was used to calculate the EC_{50} (the concentration of the test substance which produced a response in 50% of the test organisms). An example concentration (dose) – response curve is illustrated in Figure 3.3-a.

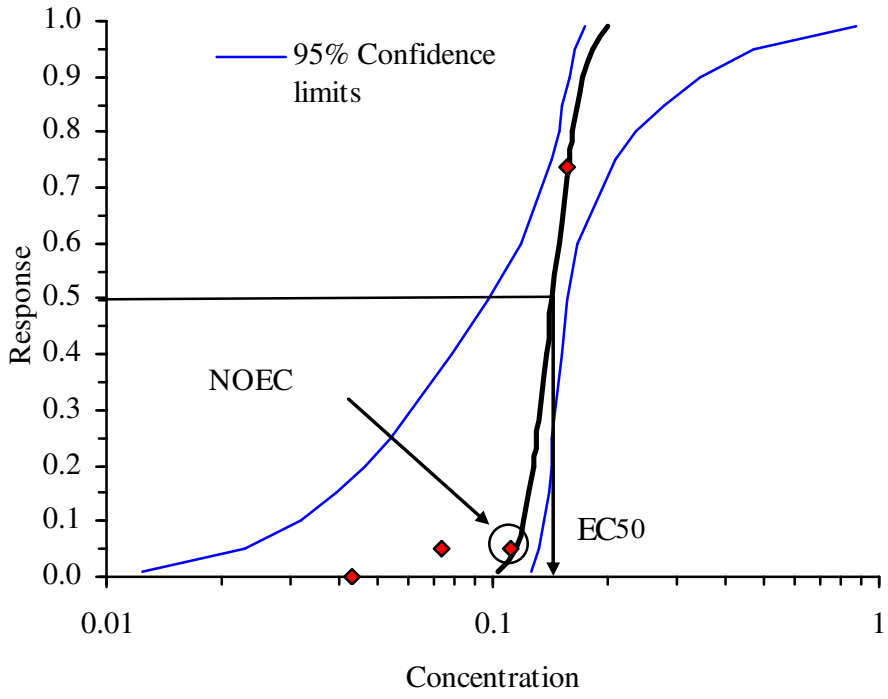

Figure 3.3-a Example of Calculation of the EC_{50} Value and NOEC

At each test concentration (log scale) the proportional response of the test organism is plotted (red diamonds). Software generated 95% confidence intervals (blue lines) and a response curve (black line) are used to establish the EC_{50}. The NOEC, established by hypothesis testing, is circled.

Hypothesis testing was used to establish the No Observed Effect Concentration (NOEC), i.e. the highest concentration of a test substance that has no statistically significant adverse effect on the exposed organisms.

A summary of the 48 h acute juvenile test results is given in Table 3.3-a. The acute juvenile *G. pulex* 48 h EC_{50} to watercress wash water was between 0.1 and 0.5 g leaf per 100 ml water. It was only possible to establish the EC_{50} for the first (range-finding) test by linear interpolation as it fell below the lowest concentration. Juveniles exposed to watercress wash water (1 g leaf per 100 ml water) and monitored were all immobilised within 1 hour. The NOEC was found to be between 0.1 and 0.2 g leaf per 100 ml water. The NOEC for Test 3 fell within the 95% confidence limits calculated for the EC_{50} value. For the final test the NOEC was greater than the EC_{50} values established for other tests.

Table 3.3-a Summary of 48 h Acute Juvenile Test Results

Test ID	EC_{50} (95% CL) (g watercress in 100ml water)	NOEC	Statistical test used (1-tailed, α =0.05)
Acute 1	0.23 (linear interpolation)	<0.46 (lowest conc)	Steel's many-one rank
Acute 2	0.14 (0.13-0.16) (Trimmed Spearman Karber)	0.10	Steel's many-one rank
Acute 3	0.14 (0.10-0.16) (Max.likelihood-Probit)	0.11	Dunnett's Test
Acute 4	0.46 (Max. likelihood-Probit)	0.22	Steel's many-one rank

3.3.2 Sublethal Tests

Initial Exposures

Five tests were carried out with watercress wash water as the test solution, although one had control failure, possibly due to cross contamination, and is not reported here. Test organisms from two of these were re-exposed to freshly prepared watercress wash water, one at test end plus 24 hours and the other at test end plus 48 hours. Five tests were carried out using a PEITC solution as the test solution although one had control failure and is not reported here. Test organisms from two of the tests were re-exposed, one at test end plus 24 hours and the other at test end plus 48 hours.

Both the watercress wash water and the PEITC solution disrupted reproductive behaviour. A summary of the proportion of pairs separated during each test exposure is presented in Appendix D. The mean (± SE) values for each test solution and the controls are presented in Figure 3.3-b. There was immediate separation of at least one pair in all the PEITC test solutions (i.e. by the first 15 minute reading). There was separation of at least one pair in all the watercress wash water test solutions after 45 minutes. There was a steady increase in number of pairs separated over the course of the two hour test, to 70% or greater in all wash water test solutions (maximum 95%, mean 84%) at the test end. The pattern of response for the PEITC solution was very similar (maximum 100%, mean 85%) at test end.

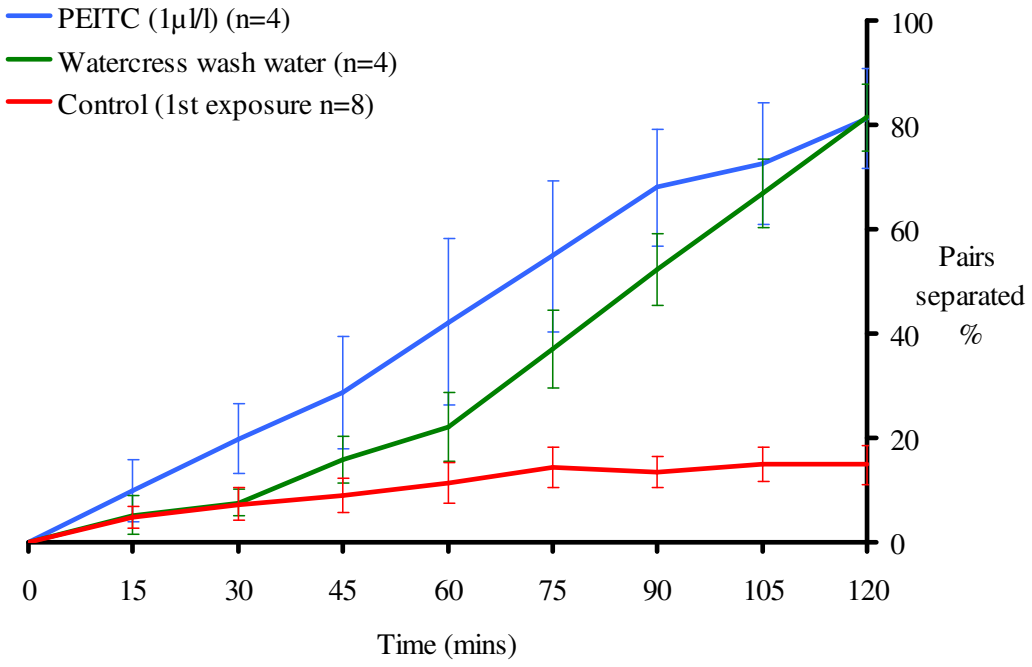

Figure 3.3-b Mean Cumulative Proportion of Pairs Separated

Precopular pairs were exposed at Time=0, the mean of n tests (carried out of separate occasions) over the course of the 2 hr exposure is shown for each test substance and the control. Vertical bars show standard error.

The ET_{50} (i.e. the exposure duration at which 50 % of precopular pairs had their natural behaviour disturbed and separated) was calculated by hypothesis testing for each test using ToxCalc v5.0.32 environmental toxicity data analysis software (Tidepool Scientific Software, 1994). The proportional data were arcsine square root

transformed and the ET_{50} calculated using Maximum Likelihood-Probit or Logit analysis. A summary of the ET_{50} values for all tests is presented in Table 3.3-b. The time taken for 50% of pairs to separate after a single exposure to watercress wash water was between 77 and 106 minutes (n=4) and for PEITC solution (nominally 1µl per litre water) was between 40 and 119 minutes (n=4).

Table 3.3-b Summary of ET_{50} Values

Sample	ET_{50} (minutes)	95% Confidence Intervals
WW1	77 *	73-85
WW2	106 *	103-110
WW3	89	78-102
WW5	84	72-93
P1	48	38-56
P2	119	108-133
P3	85	77-92
P5	40	20-56

* Calculated using Logit model – all others with Probit.

Re-exposure Tests

Data from the re-exposure tests can be examined in several ways:

- comparison of the rates of separation during the two exposures,
- comparison of the 2-hour proportion of pairs separated,
- comparison of the ET_{50} from the initial exposure and the re-exposure,
- analyses of the proportion of organisms that re-pair following return to clean water.

On re-exposure to freshly prepared watercress wash water and PEITC solution at the same concentration as the first exposure, pair separation was observed in a similar manner as for the first exposure, however it occurred sooner. The two wash water re-exposures are illustrated in Figure 3.3-c, superimposed on the mean (± SE) proportion of pairs separated for the first exposures. Test WW5r was carried out after the exposed *G. pulex* had spent 24 hours in clean water and WW2r carried out after 48 hours in clean water. In re-exposure WW5r, the proportion of pairs separated after two hours was greater than the mean (+SE) of all the first exposures.

Similarly the re-exposures to PEITC solution, tests P5r and P3r, are illustrated in Figure 3.3-d. Test P5r was carried out after the exposed *G. pulex* had spent 24 h in clean water and P3r carried out after 48 h in clean water. Once again the pair separation occurred sooner and for re-exposure P3r resulted in an overall greater proportion separation after two hours. Figure 3.3-e shows a comparison of the proportion of pairs separated after each re-exposure compared to the initial exposure. The control 95% confidence intervals for the initial exposures are shown. At the end of the two hour re-exposures the proportion of pairs separated was higher than for the initial test in both PEITC tests and the wash water tests, although there was no significant difference.

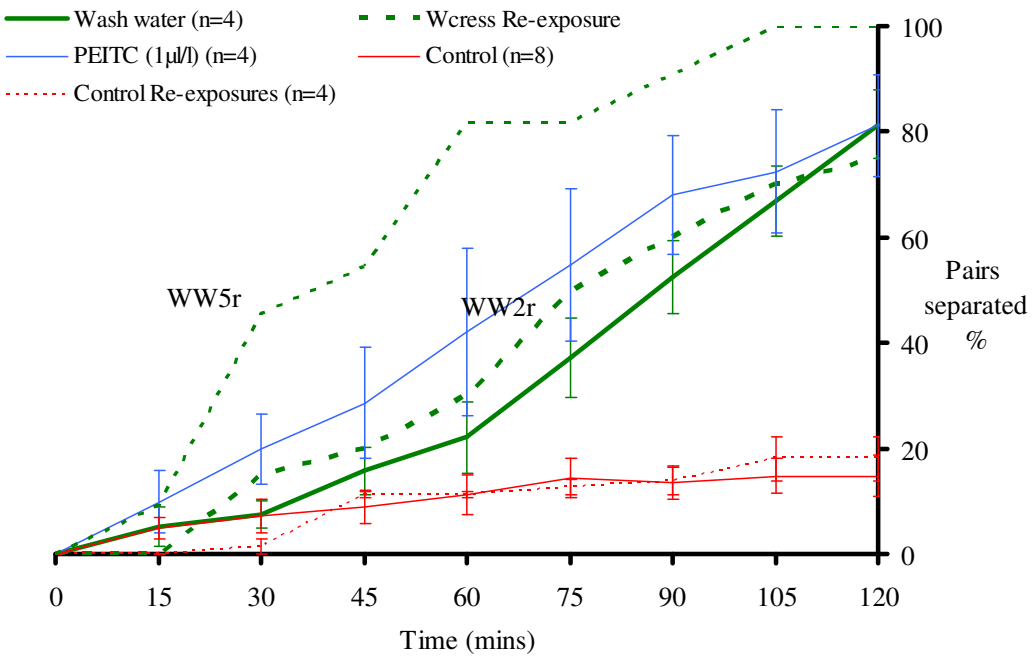

Figure 3.3-c Cumulative Proportion of Pairs Separated – Watercress Wash Water Re-exposures

Solid lines show the mean response for n initial exposures. The initial wash water exposure (green) is emboldened for comparison with re-exposures (green dotted lines) to wash water on 2 separate test occasions; Test WW5r after 24h in clean water and Test WW2r after 48h in clean water. Control re-exposures (red) follow a similar pattern to initial exposure. Vertical bars show standard error.

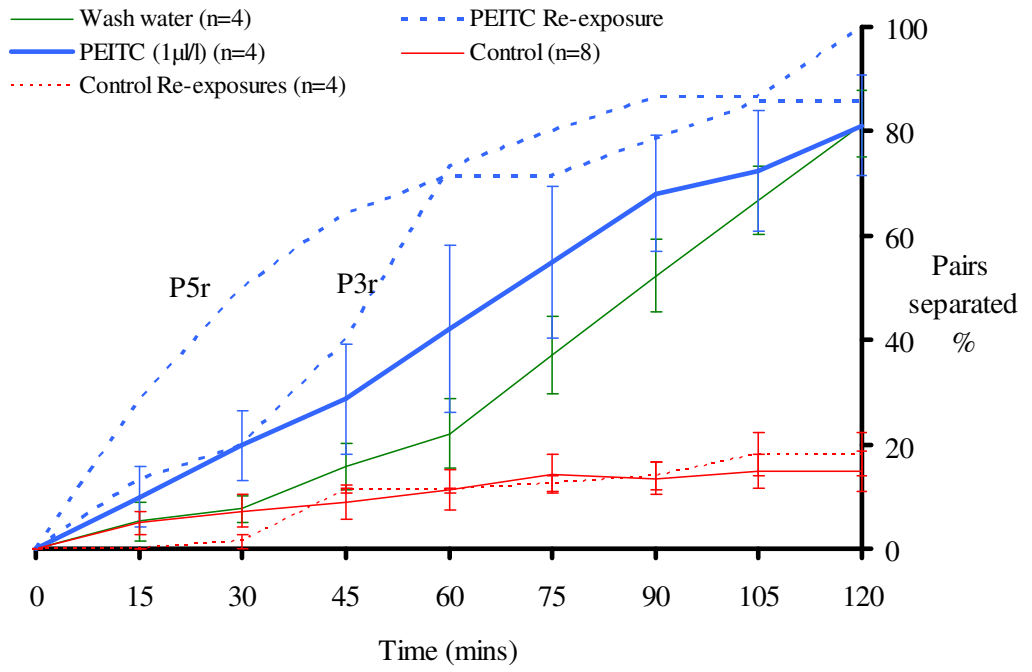

Figure 3.3-d Cumulative Proportion of Pairs Separated - PEITC Re-exposures

Solid lines show the mean response for n initial exposures. The initial PEITC exposure (blue) is emboldened for comparison with re-exposures (blue dotted lines) to PEITC on 2 separate test occasions; Test P5r after 24h in clean water and Test P3r after 48h in clean water. Control re-exposures (red) follow a similar pattern to initial exposure. Vertical bars show standard error.

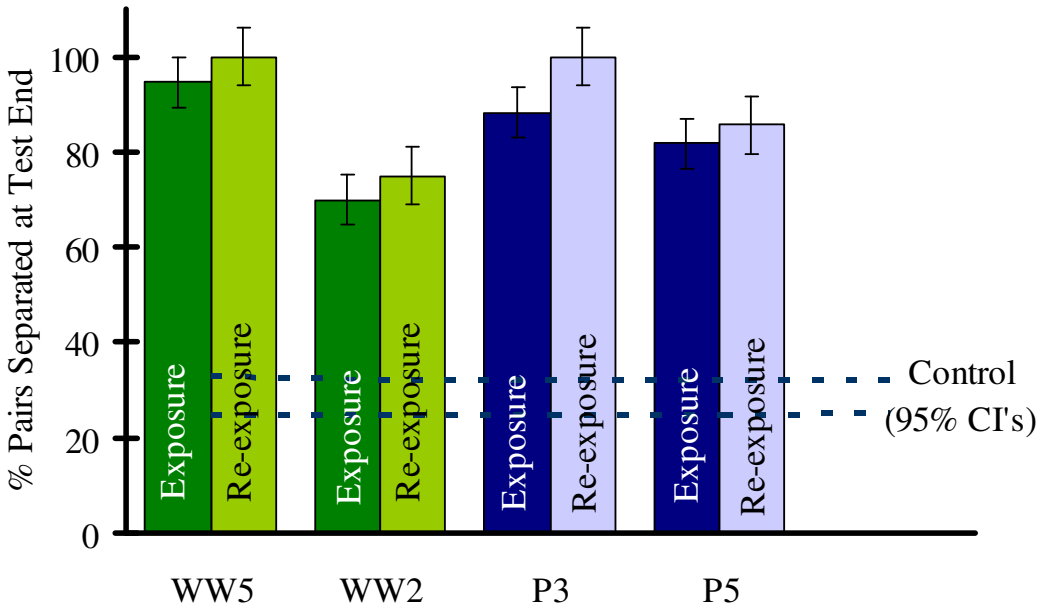

Figure 3.3-e Proportion of Pairs Separated at Two Hour Test End

Wash water exposures are shown as green and PEITC exposures are shown as blue. The 95% upper and lower confidence intervals for the initial control exposures are shown as dotted lines for comparative purposes.

The ET_{50} (95% CI) values for pairs re-exposed to watercress wash water were 87 (77-106) and 41 (27-51) minutes. The ET_{50} (95% CI) values for pairs re-exposed to PEITC solution were 54 (41-64) and 40 (19-53) minutes. These were compared to the ET_{50} values for the initial exposures and are presented in Figure 3.3-f. For all re-exposures the ET_{50} was reduced, i.e. pair separation occurred sooner. Only two re-exposures were carried out so a statistically robust assessment of the variability could not be made.

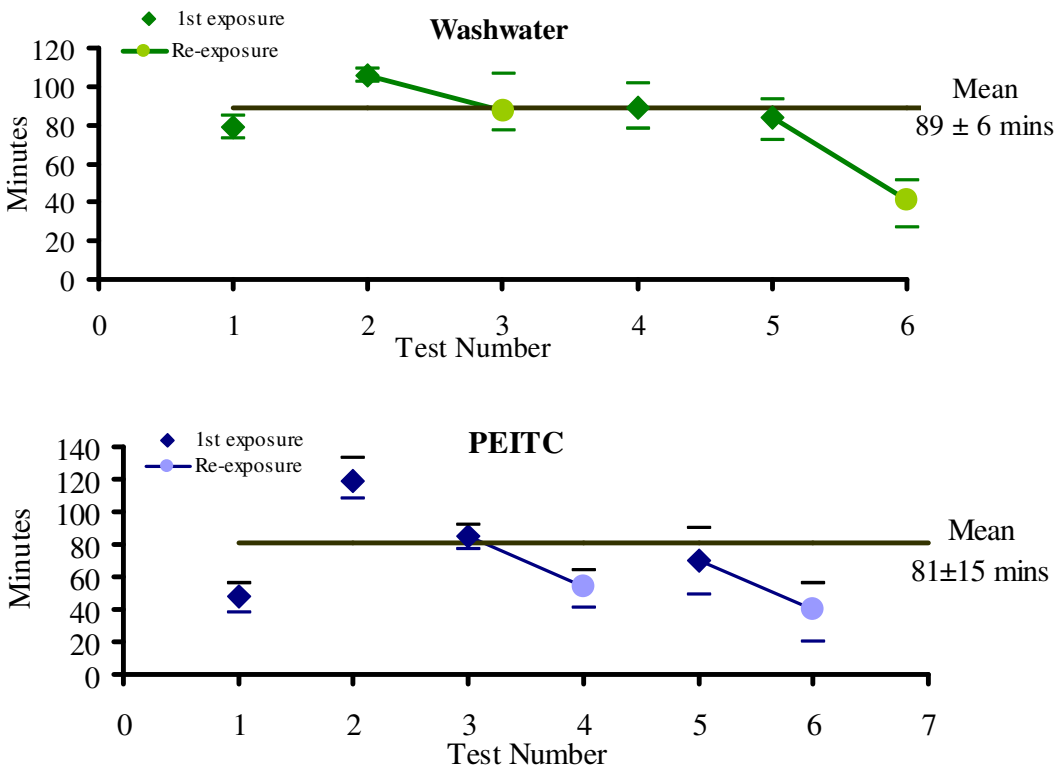

Figure 3.3-f ET_{50} Values for Exposures and Re-exposure Tests

The upper graph shows the ET_{50} value for each wash water exposure (green diamond) and re-exposure (green circle). The lower graph shows ET_{50} value for each wash water exposure (blue diamond) and re-exposure (blue circle). Re-exposures are linked to the initial exposure by a solid line. Horizontal bars show the 95% upper and lower confidence intervals. For each graph the mean ET_{50} (± SE) value for the initial exposures is shown as a solid black line.

The rate of pairs re-forming was assessed for organisms returned to clean water at the initial test end. Figure 3.3-g shows the proportion of pairs re-forming after a return to clean water at test end compared with the mean control proportions achieved. The proportion of pairs present at the start of the first exposure was taken

as 100%. For test P3, data were recorded after 24 and 48 hours; on all other occasions the proportion of pairs re-formed after either 24 or 48 hours was recorded.

In all instances except one (Test WW3), where the proportion of pairs re-forming was recorded, the number of pairs was greater after a period in clean water than at the end of the test exposure. After return to clean water there was generally a proportion of *G. pulex* that were unable to re-pair and the mean control pair re-formation achieved was 75% (n=6). However, on a single occasion (test WW5) control re-formation was 100%; the two control pairs that had separated during the initial test were able to reform in the following 24 hour period.

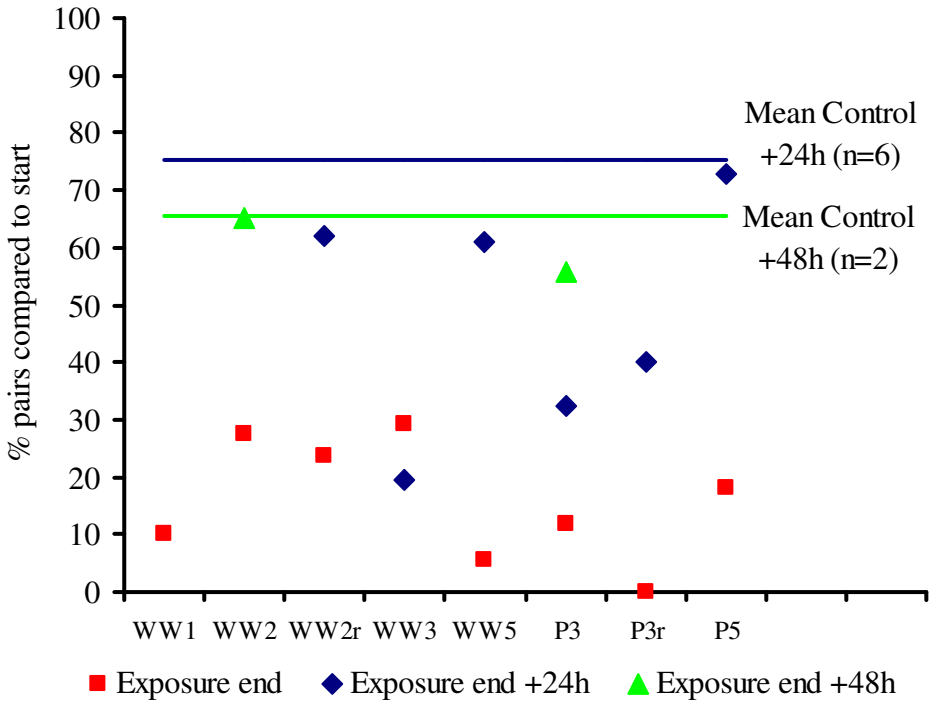

Figure 3.3-g Pairs Re-Forming After Return to Clean Water

The proportion of pairs re-forming after transfer to clean water at the initial exposure test end is shown for each separate test occasion. The mean control re-pairing for n separate tests is shown for comparison, after 24h in clean water (blue line) and after 48h in clean water (green line). Wash water exposures (WW); PEITC exposures (P); proportion of pairs remaining at initial exposure end (red square); after 24h in clean water (green triangle) and after 48h in clean water (blue diamond). NB. Re-pairing was not recorded after test WW1.

3.3.3 PEITC Concentration in Wash Water

The watercress wash water test solutions were prepared at a nominal concentration of 1g leaf washed in 100 ml water for the sublethal tests in this study. A range of concentrations between 0 and 10 g leaf per 100 ml water was used for the acute tests. Although analysis of the test solutions for PEITC was not carried out, GC-MS analyses of watercress wash water reported in Chapter 2 may be used to give an indication of the amount of PEITC that the test organisms were exposed to.

The amount of PEITC released per weight of leaf estimated in Section 2.5 gives a mean value of 529 ±45 µg/g leaf washed. Therefore, adult precopular pairs were exposed to PEITC at an estimated concentration of 5.3 ± 0.5 mg/L PEITC. Juveniles were exposed to an estimated range of concentrations between 0 and 53 ± 5 mg/L for the preliminary range-finding test. For subsequent tests the estimated concentration range was between 0 and 2.6 ± 0.2 mg/L PEITC.

3.4 Discussion

3.4.1 Sensitivity of *Gammarus pulex* to PEITC and Watercress Wash Water

The use of *Gammarus* spp. for ecotoxicological testing at both acute and sublethal levels of sensitivity has been well documented and evaluated (Maltby *et al.*, 2002, Taylor *et al.*, 1993, Welton and Clarke, 1980) and includes the specific use of a precopular separation test (Pascoe *et al.*, 1994). Protocols for acute testing with *G. fasciatus*, *G. pseudolimnaeus* and *G. lacustris* are available within the United States Environmental Protection Association test methods collection (USEPA, 1996). Johnson *et al.* (2004) recognise the importance of appropriate bioassay choice, design and quality assurance/quality control measures in effluent assessment and control. The choice of *G. pulex* as the test organism in this study was influenced by the exceptional impact on Gammaridae recorded in the receiving water downstream of Lower Link Farm (see Chapter 5).

The 48 hour acute juvenile *G. pulex* toxicity tests resulted in EC_{50} values ranging between 0.14 to 0.46g leaf per 100 ml water. The lower EC_{50} value however, was extrapolated to below the No Observed Effect Concentration (NOEC) of the final test. The NOEC depends heavily on the sample size and concentration pattern used and represents a non-significant result of a statistical test, therefore it does not mean that there is no effect (Sparks, 2000). It is also possible that although the test vessels used for the acute tests (small volume polystyrene multi-well dishes) were pre-leached, there could have been some adherence by the organic toxicant and consequently the EC_{50} values may have been underestimated. Due to the small working volumes used in these tests, very small quantities of leaf were used in the test solution preparation; which may have detrimentally influenced the precision and accuracy of the preparation method used. 48 hour acute EC_{50} values recorded by Newman *et al.* (1990b) using adult *G. pseudolimnaeus* exposed to frozen watercress leaf discs ranged from 475 to over 1000 mg (wet)/L, over 100 times greater. This may be explained by the use here of the more sensitive juvenile life-stage, a more sensitive species or the mode of action of the toxicant; it being more available to juveniles than to feeding adults.

Due to the unknown degradation pathway of PEITC in watercress wash water, which may depend on temperature, pH (Ji *et al.*, 2005) and/or the presence of other members of the family Cruciferae (Gil and MacLeod, 1980) and the volatility of glucosinolate breakdown products (Bones and Rossiter, 1996), a rapid test endpoint was preferred. A sublethal test using the endpoint scope for growth (SfG) has been reported (Naylor *et al.*, 1989, Maltby *et al.*, 1990a), but requires an exposure duration of 14 days. Due to the volatility of the compound used in this study, a continual dosing system would have been necessary and was not practicable for this study. It was possible to achieve a precopular separation endpoint over a short period of exposure to wash water solution and the response was also recorded throughout the duration of the two hour exposure period. This response was similar for the watercress wash water solution, the re-exposed organisms and the PEITC solution, although for the re-exposures occurred sooner.

The mode of action of PEITC from watercress wash water has not yet been established, although many studies have documented the relationship between terrestrial herbivorous invertebrates and glucosinolate producing crops (Koritsas *et al.*, 1991, Lambdon and Hassall, 2001, Roessingh *et al.*, 1992, Rowell and Blinn, 2003) and the use of chemoreceptors in adaptive behaviour. Watercress wash water has elicited a response in juveniles (this study), adults (Worgan and Tyrell, 2005), feeding adults (Newman *et al.*, 1992) and reproductive adults (this study). Therefore, although the ingestion of PEITC may cause an acute response, it is possible that detection of PEITC by chemoreceptors or its metabolism within cells may also be eliciting the sublethal behaviour that has been recorded.

The effect of re-exposing precopular pairs to watercress wash water and PEITC solution was analysed using four different methods. The graphical comparison of rates of separation during the two hour exposure (Figure 3.3-c and Figure 3.3-d) illustrated that the effect was seen more quickly in organisms already exposed to the toxicant. This was supported with resultant lower ET_{50} values for exposures to both watercress wash water and PEITC than for the initial exposures i.e. the effect would be seen in half of the population more quickly than for the first exposure. The two hour proportion separated showed that overall the sensitivity of the pre-exposed organisms was however not significantly increased.

3.4.2 Practical Implications

Exposure to watercress wash water and PEITC produced a physiological response measurable both in juvenile and reproductive adults. The behavioural response seen in reproductive adults was carried out at a single concentration and it is not clear from this work whether fluctuations in their response would be altered by a change in the dose regimen. It is possible that with an increase in the exposure duration and/or if the dose was increased beyond a certain level, the separation of reproductive pairs may become a toxic response leading to adult mortality. There will, therefore, be implications for the sustainability or survival of populations of *G. pulex* in the receiving water below watercress farm discharges where exposure to PEITC is at similar doses to those used in this study.

The reversibility of the behavioural response may also depend on the exposure duration and dose. Returning organisms to fresh water at test end allowed the interrupted reproductive behaviour to recommence; at the dose tested, the separation was due to a transient effect. However, it is important to note that the opportunity for male *G. pulex* to fertilise females is time limited to a few hours after the female moults (Hynes, 1955). The mate guarding behaviour thus ensures access to the female when she's receptive. In relation to the process on site at Lower Link Farm, the repeated disruption by daily pulses of discharge of watercress wash water would reduce the opportunities for males to fertilise and therefore, over a long period, reduce the reproductive success of the population. The farm processing plant washes at a ratio of 1g leaf to 50 ml water (Vitacress Salads Ltd, 2008b) and isothiocyanate producing crops make up approximately 50% of product washed, therefore at the concentration of 1g leaf per 100 ml water *G. pulex* were exposed at environmentally relevant concentrations.

Analysis of the number of pairs re-forming showed there was an inconsistent increase in pair re-forming over a 48 hour period and even in controls 100% re-pairing was not generally achievable. The number of pairs re-forming were also subject to the natural pattern of the reproductive cycle (Hynes, 1955) and thus a proportion would naturally separate anyway. It is interesting to note that separation of re-exposed pairs (see Figure 3.3-c and Figure 3.3-d) occurred sooner in the tests

which were carried out after 24 h rather than 48 h in clean water, even thought this was not reflected in an overall greater proportion separation at the end of the two hour period or a much lower ET_{50}.

Where low diversity or abundance is noted in the macroinvertebrate populations of chalk stream receiving waters below watercress farms, the potential effects due to PEITC should therefore be considered. Watercress producers are required to meet consent conditions for a variety of water quality parameters such as suspended solid load and biological oxygen demand (BOD) (see Table 1.2-a). The contribution of PEITC induced effects should also be examined (§ 6.4).

3.4.3 Wash Water Sample Preparation

The method of preparation of watercress wash water test solution was based on the salad wash process at Lower Link Farm and reference to the levels of PEITC recoverable from the watercress wash water using Gas Chromatography Mass Spectrometry (GC-MS) techniques in Chapter 2 was made. Sources of variation were minimised where possible (§ 2.6.2), PEITC was consistently measurable where small quantities of leaf were washed and the method found to be reproducible. This was particularly important for the nominal concentrations prepared for the acute test where very small quantities of leaf were used.

Where the wash water was prepared using larger quantities of watercress, both leaf and stem were used and variability may have been introduced by different glucosinolate content in each part of the plant. Although a comparison of PEITC present in stem and leaves has not been made, Gil and Macleod (1980) showed there were different levels of PEITC produced from *N. officianale* seeds and leaves and Rosa (1997) described significant variation between glucosinolates present in the roots and aerial parts of Brassica seedlings. Newman (1990b) also reported that toxicity of frozen watercress roots to *Gammarus pseudolimnaeus* was similar to the leaves.

Six wash water preparations made using the same methodology as that used for precopular separation test described in this study (§ 3.2.1) were analysed by GC-MS

(§ 2.5.3). They were found to contain between 0.9 and 3.9 mg/L PEITC and showed an increasing trend ($R^2 = 0.94$) with leaf weight washed, i.e. the greater the leaf weight washed, the higher the level of PEITC measured even at the same leaf weight to water ratio. Precopular pairs were exposed to wash water prepared using comparatively large leaf weights due to the volume of wash water required. Therefore the levels of PEITC they were exposed to were likely to be in the order of 5 mg/L PEITC (§ 3.3.3).

3.4.4 Test Limitations

It was only possible to carry out re-exposure tests when there were enough precopulatory pairs after the return of test organisms to clean water and as discussed in Section 3.2.1, the rate of re-pairing was not always consistent and it became apparent that complete control pair re-formation was not possible. The use of much larger numbers of pairs in the initial tests would have resulted in more pairs becoming available for re-exposure. However, this was governed on a practical basis by the facilities and manpower available for test set up. Similarly, a longer time period in clean water may have increased the numbers of pairs available for re-exposure. A compromise was made between practicability and relevance to field simulation at the farm where re-exposures occur within 24 hours. Re-exposure tests were carried out where at least 3 replicates of 3 pairs were possible as well as control replicates, although this was less than recommended by standardised acute test methodology such as Environment Agency (2007) acute single concentration D*aphnia magna* test where 6 replicates and 20 organisms are prescribed. It should be recognised that the use of a larger number of pairs would have increased the statistical robustness of the method.

3.4.5 Further Work

Further testing with freshly collected samples of salad wash water, taken directly from the wash lines at Lower Link Farm would provide a direct link to the crop washing process and its effect in the Bourne Rivulet. Tests could also be carried out using wash waters prepared from watercress crops grown and harvested at different

times of the year or with different compositions of isothiocyanate producing crops to investigate synergistic effects (Gil and MacLeod, 1980)

It would additionally be beneficial to increase the number of sublethal tests carried out with PEITC solution and watercress wash water to assess the level of variability in the *G. pulex* response and confirm the reproducibility of the test. A further series of re-exposure tests could also be carried out, including re-exposures after two tests with the same organisms. This approach may be limited by the number of test organisms that re-pair (for statistical validity), although the number of pairs reforming could also be used as an endpoint in itself. The reliability of the short term sublethal test could also be evaluated by further tests to establish the natural background variability against which the stress-induced precopular separation can be measured (Maltby *et al.*, 2002). An estimate of the 'natural' re-pairing rate for the population could be made by artificially separating control organisms prior to a period in clean water.

To increase the statistical robustness of the methodology, further testing could be carried out with larger initial numbers of pairs. This would enable a full assessment of the method variability and would also introduce the potential to carry out further re-exposures or a re-exposure series. Other endpoints could be used for the assessment of risk for example, monitoring the pairs remaining after they are removed from the test exposure to record if juveniles are produced and their numbers. Other measures commonly used to determine a measure of acceptable risk in the receiving environment are the ET_{10} (exposure time at which 10% of the population are affected), the No Observed Effective Concentration (NOEC) and Lowest Observed Effective Concentration (LOEC). In order to calculate NOEC and LOEC a dose response approach to testing must be used, rather than single concentration. However an acute to chronic ratio (ACR) could also then be calculated using the geometric means of the NOEC and LOEC, which could be used to estimate chronic sensitivity where known data for acute response was known.

The pair separation test method could be carried out using an organic reference toxicant e.g. 3, 4 Dichloroaniline (3,4 - DCA). Published data are available for *G. pulex* sensitivity to this compound, against which a comparison could be made.

Experimental constraints (limitations due to time and equipment availability) during this study made it unfeasible to carry out reference toxicant testing alongside the test solution.

3.5 Conclusions

This Chapter has shown that the secondary metabolite PEITC produced by harvested and processed watercress has a sublethal effect on *G. pulex* breeding pairs. It also has an acute effect on juveniles less than 7 days old. These effects are evident at concentrations similar to those produced by the leaf washing process at Lower Link Farm on the Bourne Rivulet.

Re-exposures of *G. pulex* precopular pairs to PEITC in watercress leaf wash water did not appear to illicit a significantly different separation response, although all re-exposures had a lower ET_{50} and responded more quickly during the exposure. The organisms did not appear to acclimatise to PEITC or become less able to withstand its effect. Further tests and re-exposures would establish if this was a consistent finding.

The adaption and extension of a more commonly used reproductive pair separation methodology; i.e. to re-expose organisms to freshly prepared test solution, reflected more accurately the exposure pattern experienced by organisms in the receiving environment. This novel use was considered important to the relevance of the particular situation in the Bourne Rivulet below the discharge from Lower Link Farm.

The mode of action of the toxicant has not been confirmed, although behavioural effects are evident. The similar response seen in both PEITC solution and watercress leaf wash water solution would indicate that PEITC is the causative agent.

4 MITIGATION OF IMPACT ON *GAMMARUS PULEX* OF FARMED WATERCRESS AND ITS WASH WATER

4.1 Introduction

4.1.1 Context

There are over 60 hectares of watercress farms in southern England many of which are located on the headwaters of chalk streams. Chapter 1 described the very varied nature and size of watercress cultivation operations that exist in England. Most farms, both large and small, operate within an voluntary industry standard Code of Practice (Assured Produce, 2006) and are subject to statutory requirements with respect to water quality and environmental protection (§ 1.2.4, Legislative Requirements).

A link between the isothiocyanates (primarily PEITC) produced by watercress when the plant tissue is damaged and effect on *Gammarus pulex* has been established in Chapter 3. The production of isothiocyanates can be triggered either by grazing invertebrates or during farming operations; growing, harvesting and washing the crop. Lower Link watercress farm at St Mary Bourne, Hampshire is the largest commercial operation in England by area of watercress beds cultivated (18 ha). In addition to watercress production, the farm operates a salad washing and processing factory and this is therefore an additional source of isothiocyanates. There are a number of mitigation measures in place at Lower Link Farm to protect the receiving water from impact due to the farm operations. Several of these have been designed specifically to address potential impact on macroinvertebrates in the receiving water by isothiocyanates. This Chapter will explore further the process and practice at the farm and evaluate the success of mitigation measures in place in relation to ecotoxicological effect specifically on *G. pulex*.

4.1.2 Isothiocyanates

Chapter 2 has described the production of isothiocyanates by Cruciferous plants as secondary metabolites in response to tissue damage. This mechanism probably evolved as a self defence mechanism against grazing invertebrates, but also occurs

when cultivated watercress crops are harvested, washed and processed. Isothiocyanates are produced when the stable and water soluble precursor glucosinolate, which is stored in the plant tissues, is hydrolysed by the action of an enzyme. The enzyme, myrosinase, is released when the plant is wounded either as a result of invertebrate grazing or other physical damage for example, during farming operations. Isothiocyanates, in particular PEITC, are also recognised as important in providing health benefits for people when consumed.

Isothiocyanates appear to provide the defence from grazing that the plant requires, but allow macroinvertebrates to thrive amongst stands of the plant. They have a small and localised effect on invertebrates in the natural state and watercress occurs as a common macrophyte, alongside a diverse community of macroinvertebrates in healthy chalk streams. However, Chapter 3 has described how PEITC and wash water prepared from watercress has an adverse effect on the juvenile life stage and *G. pulex* adult reproductive behaviour.

There are many interrelated problems involved in the analysis of effects due to isothiocyanates present in the wash water discharge from the factory at Lower Link Farm. The complex nature of the discharge must be considered. The wash water composition will vary due to the constantly changing crop lines and salad mixes being processed. The levels of the glucosinolate precursor present within cruciferous crop tissues will vary as a result of environmental conditions during growth. There may potentially also be synergistic (Gil and MacLeod, 1980) or antagonistic mixture effects of different cruciferous crops. Not least is the difficulty in measuring PEITC from wash water samples taken at the factory outfall as there is currently no reliable or accredited test to measure PEITC in water (Chapter 2).

4.1.3 Biological Impact on the Bourne Rivulet

Biological surveys carried out over the last two decades in the Bourne Rivulet, Hampshire (Medgett, 1998, White and Medgett, 2006, Murdock, 2007) have shown that there has been a measured and significant effect on macroinvertebrate populations in the water up to 1.8 km downstream of Lower Link watercress farm. Recent surveys (Everall and Bennett, 2007, Medgett, 2008, Murdock, 2009) have

shown an improvement in the number and diversity of pollution sensitive taxa from samples taken downstream of the outfall on the East Rivulet. Further description of the macroinvertebrate community of the Bourne Rivulet is given in Chapter 5, along with changes in populations of pollution sensitive and pollution tolerant taxa that have taken place over the last two decades.

In addition to the acute and sublethal ecotoxicological effects on *G. pulex* described in Chapter 3, unpublished ecotoxicological studies (Marsden, 2006, Worgan and Tyrell, 2005) have indicated that the processing factory wash water at Lower Link Farm exhibits acute and sublethal toxicity to *G. pulex.* Marsden (2006) also concluded that despite the toxicity of the factory wash water to *G. pulex*, phenethyl isothiocyanate levels were not high enough to elicit sustained avoidance from the harvested beds.

4.1.4 Mitigation Measures

Since 1995 Vitacress Salads Ltd has made change to the process and practice at Lower Link Farm and these are detailed in Table 4.1-a. These measures were put in place in response to the reported poor biological quality downstream of the farm, results of studies on the potential impact of watercress farm discharges (such as Roddie *et al.* (1992) and Natural England (2009)) and to maintain the farm discharge within its water quality consent conditions. The specific effect of PEITC was not the initial driver for these changes.

Changes to the farm management practice and the mitigation measures installed were initially included to mitigate effects of high sediment load in the farm discharge. The high sediment load arose primarily from the bed clearing process and Lower Link Farm was required to meet the discharge consent condition (20 mg/L) for suspended solids in the farm discharge. The presence of large amounts of plant matter in the discharge was addressed by the installation of a finer parabolic screen (see Plate 4.1-a).

Plate 4.1-a Parabolic Screen, Lower Link Farm

Plant matter in the discharge was not only an aesthetic issue as it also had the potential to obstruct the watercourse and was perceived to provide an additional source of PEITC. Subsequent changes to chemical use, both as inputs to the growing crop (e.g. fertiliser) and for washing the salad leaves were made. Notable amongst these measures was the reduction and cessation of chlorine use to wash the salad. This, it was anticipated, would lead to a significant improvement in the biological quality in the receiving water. However macroinvertebrate populations in the Bourne Rivulet did not show signs of recovery and there was a perceived need to reduce levels of PEITC in the farm discharge.

In response to initial studies suggesting the adverse effect of wash water on *G. pulex* (Worgan and Tyrell, 2005, Marsden, 2006), pressure from fishery interests using the watercourse and continued poor biological surveys, a system to recirculate salad wash water was installed. The wash water was recirculated through a series of watercress beds, which was intended to act in a manner similar to a reed bed and allow the dissipation of PEITC prior to wash water discharge to the receiving water.

Table 4.1-a Water Quality Improvements at Lower Link Farm (1995-2009)

Date	Description
1995	Suspended solids settlement tank installed to comply with 20 mg per litre suspended solids consent.
April 2003	Sludge blanket detector fitted to tank to alert to sediment removal requirement
April 2004	5mm drum replaced by a 2 mm parabolic screen to remove leaf matter from salad wash outflow. Two suspended solids settlement chambers also added.
July 2005	Settlement tank (which also contained dechlorinated wash water) discharged through E block watercress beds
October 2005	Permanent electric pump system installed to pass settlement tank effluent through E block watercress beds
November 2005	East Rivulet channel de-silted.
March 2006	Volume of ammoniacal nitrogen used in liquid fertiliser reduced by 80%.
June 2006	Second parabolic screen added to double the capacity.
June 2006	Chlorine use (& de-chlorination) reduced by 80%. Chlorine-free wash water added to primary rinse water directed via parabolic screens.
July 2006	Chlorine use ceased (20% of product washed treated with Citrox, directed to foul sewer).
January 2007	Watercress bed and factory discharges de-culverted to create 95 m of chalk stream on site. Project completed April 2007
February 2007	Turbidity sensor with telemetry alert installed to the East Rivulet discharge.
March 2007	Ammoniacal nitrogen eliminated from fertiliser regime.
November 2007	Recirculation system installed to allow all parabolic screen wash water discharge to flow through B & C blocks of watercress beds prior to discharge to the East Rivulet channel.
July 2007	Citrox used ceased. Salad leaf washed using only spring water.
Aug 2007-March 2010	Additional blocks of watercress beds included in factory wash water recirculation system.

Adapted after (Murdock, 2007)

Consequently, the watercress and other salad leaf are currently washed in spring water only. This wash water passes through a 2 mm parabolic screen and a settlement tank before being re-circulated back through a series of watercress beds and then discharged to the East Rivulet. Figure 4.1-a shows the basis for the current on-site water use and discharge scenario.

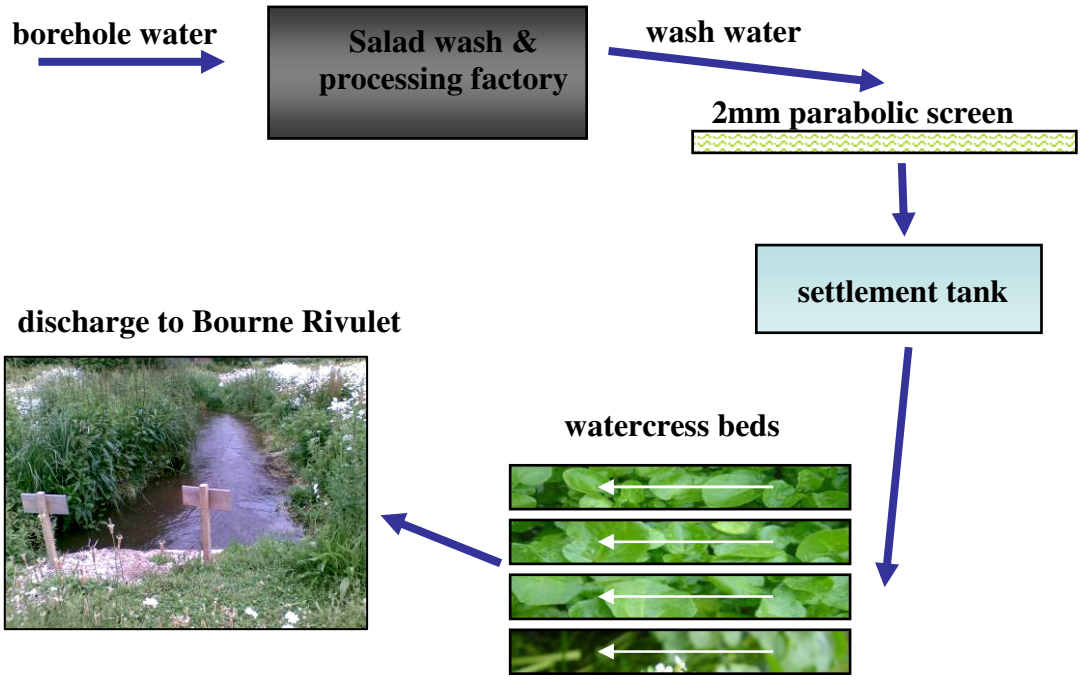

Figure 4.1-a Lower Link Farm Process Water Treatment and Discharge

4.1.5 Study Objective and Hypotheses

Section 4.1.4 describes how the effect on the biological community on the receiving water below Lower Link farm was severe enough to warrant mitigation measures to be put in place at the farm. Arising from this, therefore, was the need for an empirical test of the attempt to mitigate this. In particular, the most recent measure to install a system to re-circulate the salad wash and processing factory discharge back through a series of watercress beds. The key objective of this study was to assess the success of this mitigation measure.

In Chapter 3, the effect of watercress wash water and PEITC on *G. pulex* was assessed under controlled laboratory conditions. Conducting bioassays *in situ* provides a link between the results gained under very specific conditions in the laboratory and those present in the constantly changing environmental conditions present in the receiving water.

The study tested two hypotheses. Firstly, that the salad wash water discharge significantly reduces the survival of *G. pulex*. Secondly, that the re-routing of salad

wash water through the watercress beds is successful at mitigating this effect by providing additional residence time prior to discharge of wash water to the receiving water.

4.2 Method

4.2.1 Methodological Approach

Gammaridae play a key role in the community structure in chalk stream headwaters. The fall in numbers or absence of *G. pulex* in the Bourne Rivulet has been consistently noted by biological surveys carried out over the past decade. Therefore, *G. pulex* is particularly suitable as the test organism for this study, as it is the organism that is recorded as affected in the receiving water, i.e. it is relevant to the area of concern (Pereira *et al.*, 2000). Sensitivity to pollutants is also an important criterion in determining the suitability of a test organism. Girling *et al*. (2000) found that tests with *G. pulex* were sensitive to a wide range of toxicants and had endpoints that were consistently sensitive.

Factors to be considered, which support the use of *in situ* testing, were the transient nature of PEITC and the variability of the wash process. In stability studies carried out by Ji *et al.* (2005) the half life of PEITC was found to vary from 56 to 108 hours depending on temperature and pH. This would preclude most of the published bioassays (e.g. 10 day acute or 30 day sublethal testing regimes) and semi-static or 'continual dosing' systems were unavailable to us. *In situ* testing was preferred as it would be very difficult to replicate the variability of wash water produced by salad wash process in a laboratory. For example, during a single week, there maybe more than 40 different lines (i.e. combinations of leaf) washed, most of which contain one or more isothiocyanate producing species. Some combinations are washed in very large quantities and every day of the week and others in much smaller quantities on only a single day.

The effective use of *Gammarus* spp. as ecotoxicological test organisms, both *in situ* and *ex situ*, is well documented. A number of *in situ* assays have been developed for use with *G. pulex* (Naylor *et al.*, 1989, Veerasingham and Crane, 1992). Maltby *et al*. (1990b) successfully applied the 'Scope for Growth' assay to field deployments. Walker (2006) describes the most widely used *in situ* bioassay as the Scope for Growth assay, which Naylor (1989) found to be more sensitive than the acute 24 h

test. Crane *et al*. (1995) describes a battery of *in situ* bioassays, used to assess water quality in an agricultural catchment and they found that *G. pulex* mortality and feeding rate bioassays provided useful information which complemented the macroinvertebrate survey data. Matthiessen *et al*.(1995) carried out an *in situ* caged *G. pulex* exposure to a carbofuran insecticide runoff during a heavy rainfall event and showed the recorded impact was analogous to subsequent laboratory tests.

In situ bioassays are carried out under natural conditions and include environmental variables which may affect the behaviour of contaminants and consequently their toxicity. As such they will integrate the effects of varying exposures to pollutants in the environment. They have the advantage of directly measuring the toxic effects of bioavailable substances on aquatic organisms and consider both known and unknown hazardous substances, including degradation products (Den Besten and Munawar, 2005). *In situ* tests are also important for validating laboratory tests and in extrapolating their results to the field (Pereira *et al.*, 2000). Den Besten and Munawar (2005) also described how they may be used to provide a compromise between the desire to test *in situ* and the use of an environmentally relevant endpoint or may be used as a diagnostic tool to trace toxicity effects back to their source.

The use of *in situ* feeding and growth assays were considered. However, Maltby (2002) reported that results of the *in situ* Scope for Growth feeding assay would be affected by temperature differences. Due to large temperature differences above and below the watercress beds, this bioassay was not considered appropriate in this case. Deployment of a 28-day *in situ* feeding and mortality bioassay was also too long in relation to the undisturbed window of opportunity allowed by the watercress bed management system (described in detail in § 4.2.3) which was generally in the order of 14 days.

A series of 7-day acute ecotoxicological tests using caged *G. pulex* were carried out *in situ* at the watercress farm. The test length was constrained by the harvesting regime, which limited the number of suitable similar locations available for testing at the same time. A longer test would not have allowed replicates or control deployment with a watercress crop of similar age. Caged *G. pulex* studies (unpublished) have been carried out both in the watercourses on the farm site and in

the stream below the farm, although none have previously used the water carriers feeding and draining the watercress beds. The cages were placed at locations in the water carriers in order to compare the survival of *G. pulex* in watercress wash water (i.e. with PEITC present) and in borehole water (no PEITC). Cages were also placed at water carrier locations where flow had passed through watercress beds to enable comparison and measurement of the effectiveness of this as a mitigation measure.

4.2.2 Test Organisms

The test organisms used were adult *G. pulex* (approximately 6-9 mm length). They were collected prior to the test from coir matting placed in the western arm of the Bourne Rivulet downstream of the watercress farm (NGR SU 427 490, refer also to Figure 4.2-b). Organisms with visible parasites were not selected as they are known to affect the behaviour of *G. pulex* and reduce fecundity (Pascoe *et al.*, 1995, Bollache *et al.*, 2002). The Bourne Rivulet flows through the watercress farm in a channel and this reach (below the farm) receives discharge from the watercress beds fed by borehole water. However, an Environment Agency survey had found that "the invertebrate community found in the western arm of the Bourne in 2006 was typical of a chalk stream of its size and proximity to source" (White and Medgett, 2006). This has since been supported by Murdock (2007) who reports a Biological Monitoring Working Party (BMWP) score of 179, i.e. 'very clean' Environment Agency water quality grade and the highest River Ecosystem classification (RE1) and was therefore considered a suitable source of *G. pulex*.

In previous *in situ* caged *G. pulex* studies carried out at Lower Link Farm (Marsden, 2005, 2006, Tyrell, 2005) test organisms were collected both from other representative chalk streams in Hampshire and Dorset and from the western arm of the Bourne Rivulet downstream of the watercress beds. It is possible that the organisms collected downstream of the watercress beds would have a different response to those collected elsewhere due to fluctuating environmental conditions resulting from flow though the watercress beds. For example, borehole water temperature is relatively constant when entering the bed, but for young plants a very slow flow rate is used and on sunny summer days the water temperature may increase considerably (~10ºC) by the time the water leaves the bed. Similarly, the

potential presence of PEITC in the outflow from harvested crops may also affect the local population. Any latent effect due to their exposure to PEITC (for example, a vulnerability or an acclimatisation) could bias the results of trials using these organisms.

In order to measure any potential difference the response of *G. pulex* sourced from the River Meon at Funtley, Hampshire (NGR 556 089) was compared with that of *G. pulex* from the Bourne Rivulet immediately downstream of the borehole fed watercress beds at Lower Link Farm. The only commercial watercress farm on the River Meon is located approximately 10 miles upstream of Funtley at Warnford. The bioassay was carried out according to methodology described in Section 4.23 which was used for all the test deployments. The Mann Whitney Rank Sum test was used to compare the mean 7-day *in situ* survival of *G. pulex* sourced from the River Meon with *G. pulex* collected from the Bourne Rivulet. There was no statistically significant difference in the survival of the organisms from each river placed in the wash water fed carriers ($T=61$, $p=0.505$). There was no statistically significant difference in the survival of the organisms from each river placed in the borehole fed carriers ($T=60$, $p=0.442$). Kruskal-Wallis One way ANOVA on Ranks was used to compare all test locations where mortality was recorded, i.e. the carriers upstream of the watercress beds. (The test organisms placed in the carriers downstream of the both borehole and wash water fed watercress beds exhibited no mortality). There was no statistically significant difference between the responses of organisms from the two river sources ($H=3.417$ with 3 degrees of freedom, $p=0.332$).

4.2.3 Test Deployment

The study took place during the months of May, June & July in 2007 and 2008. This time frame was chosen to represent the worst case scenario, as these months include the peak production period at the farm and the periods of maximum growth rate of the crop.

Cages constructed from Durapipe (50 mm length and 37 mm internal diameter) were used for the study. Mesh panels (250 µm) were attached at each end to allow free flow of water through the cage. A preliminary study using 1 mm mesh panels was

unsuccessful because of a build up of silt within the cages. The cages were secured to heavy tiles to maintain their alignment into the flow and prevent them being washed away (see Plate 4.2-a). A large bore pipette was used to transfer organisms to minimise damage to their appendages.

Plate 4.2-a Arrangement of Cages on Tiles

The test organisms were provided with alder (*Alnus glutinosa*) leaf and were fed to excess during the course of each *in situ* deployment. The leaves were pre-conditioned by soaking in organically rich water, for a minimum of 10 days, to encourage the growth of surface bacteria and fungi (Naylor *et al.*, 1989).

In the absence of formal guidelines for *in situ* testing with *G. pulex*, the Organisation for Economic Co-operation and Development guidelines (OECD, 2004) for conducting laboratory based 48 hour lethal tests with the freshwater aquatic invertebrate *Daphnia* spp. were referred to. This recommends at least 20 test organisms per concentration. Environment Agency guidance (Environment Agency, 2007) for conducting laboratory based single concentration tests with juvenile *D. magna* recommend at least six replicates of each control and six replicates of each test sample. *In situ* test were carried out using 24 organisms at each test location (with eight replicates at each location). Three randomly selected *G. pulex* were placed in each of eight cages, along with alder leaf food and the cages also randomly

assigned to each location. The cages were deployed in the carriers i.e. the channels supplying water to and removing water from the beds (see Plate 4.2-b). Initial trials using caged *G. pulex* placed within the watercress beds failed due to an inadequate depth of water to maintain adequate flow within the tubes and to buffer large water temperature increases on sunny days.

Plate 4.2-b Cages in Carrier below Watercress Bed

One deployment was made at the top of the watercress bed and a second at the lower end of the bed, once the wash water had passed through the bed, as illustrated in Figure 4.2-a. The location selected for deployment was a watercress bed receiving maximum salad wash water flow (i.e. minimum dilution by borehole water) and which would not be harvested during the deployment. A test control was carried out with *G. pulex* exposed at similar locations above and below a watercress bed that had only borehole water (i.e. no salad wash water) flowing through it.

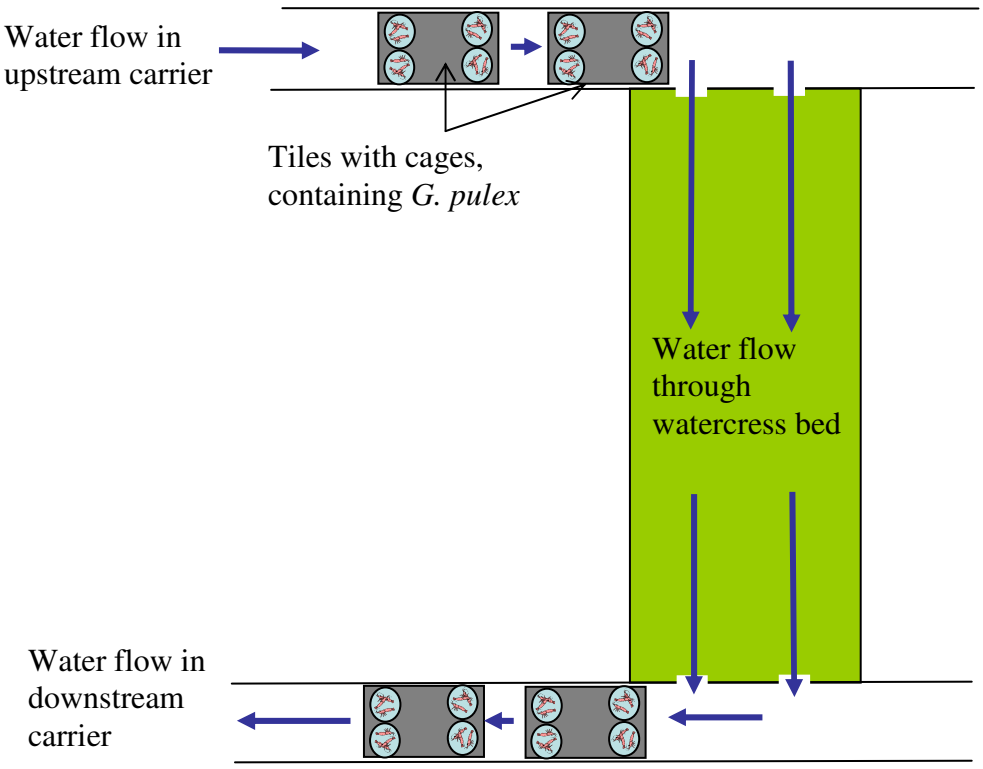

Figure 4.2-a Schematic of Experimental Set-up

The cages were deployed in the carriers for a number of reasons, although primarily because the flow rate in the carriers is more consistent than at other locations. The watercress beds are managed daily and individually according to the requirements of the crop. For example, water flow within the beds is increased with increasing age of the crop. Fertiliser is applied directly to all the watercress beds similarly, rather than the carriers, although an *ad hoc* application of calcium nitrate was made to borehole carriers during Test 8. For each deployment the choice of bed was made with reference to a number of factors. The crop should be at least 14 days old (from planting); by this age the flow through the beds had been increased and the crop provided greater cover, so as to buffer water temperature increase through the bed. Additionally, the liquid fertiliser regime would be consistent for all tests. The beds sharing the same downstream carrier should not be harvested during the course of the test, to minimise the potential contamination of downstream organisms with PEITC from freshly harvested crops. Ultimately the choice of beds was dependent on the harvesting, cleaning and planting schedules determined by the farm. There

were occasions where little choice was possible and Figure 4.2-b shows that a variety of bed locations were used throughout the duration of the study.

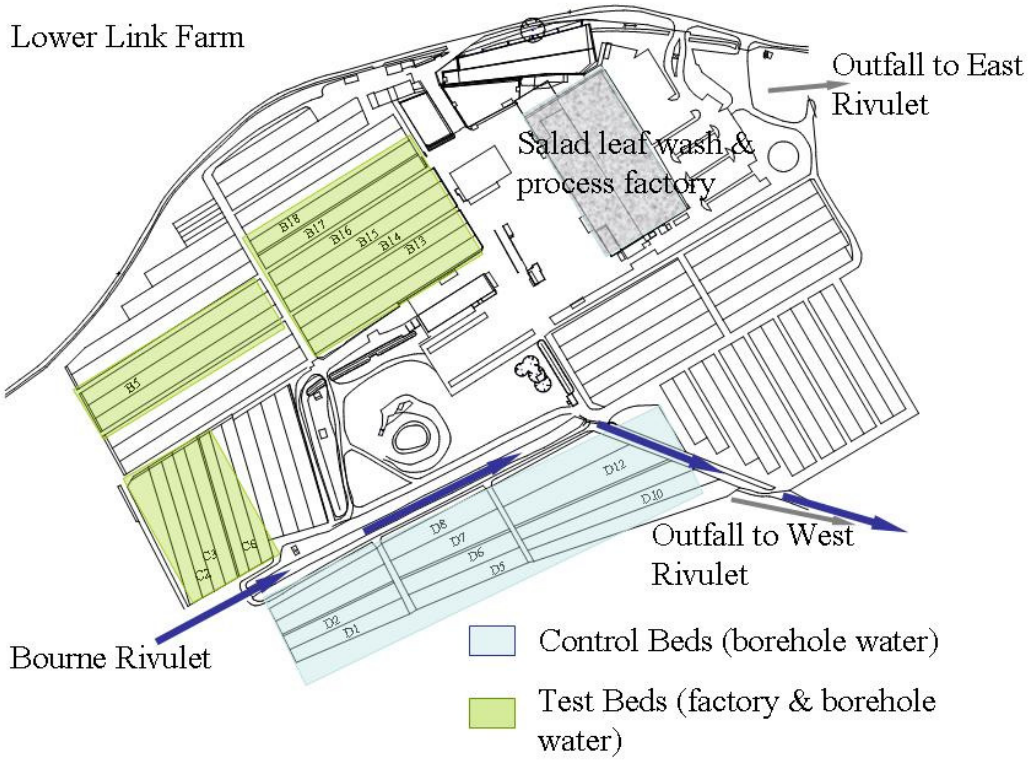

Figure 4.2-b Location of Watercress Beds used in Study

4.2.4 Test Endpoint and Measurements

The mortality of *G. pulex* was measured at the end of the exposure. The test endpoint measured was 7-day adult *G. pulex* mortality (as immobilisation). Test organisms were considered immobile if they did not move within 15 seconds following gentle agitation, even if there was still movement of antennae or thoracic limbs (which maintain water flow over the gills). This is in accordance with Environment Agency guidance (Environment Agency, 2007) and similarly McMahon and Pascoe (1988) reported immobilisation as the "failure to respond to mechanical stimulation".

The water quality parameters; dissolved oxygen content, pH, conductivity and temperature were recorded at each location on deployment and when the test ended.

Water quality measurements were made using calibrated WTW hand-held meters (Oxi 330i and pH/Conductivity 340i). Water quality validation criteria used for the laboratory based bioassays (§ 3.2.4) were used as a guide to assess the level of water quality fluctuation in the deployment locations. These were defined as dissolved oxygen (>60% ASV), pH (constant to within 1 unit), conductivity (<10% change). However, water quality cannot be adjusted to meet the criteria as in the laboratory and the nature of *in situ* testing is to assess the effect of all environments variation. Ultimately, test validation was based on a control or survival rate of > 90%. Thus, if the water quality criteria were outside these limits, but the control mortality was <10%, the test would be accepted.

4.3 Results

4.3.1 Water Quality

The dissolved oxygen content of the watercress wash water supply was consistently high, with the majority of readings above 100% Air Saturation Value (ASV). The minimum recorded was 82.8% ASV (20 Jul 08, Test 3). At all other locations the dissolved oxygen was consistently above 80% ASV, except 25 Jun 07 (Test 1) below wash water bed 70.3% ASV, although all organisms survived at this location and this was not considered to affect the test result.

The temperature recorded in the borehole supplied carriers was consistently between 10.5 ºC and 12.7 ºC. The temperature recorded in the wash water supply was also relatively consistent (between 10.6 ºC and14.4 ºC) compared with the downstream carriers where temperature recorded varied considerably more (11.8 ºC to 24.0 ºC). Flow within the watercress beds varied because of changes in the ambient environmental temperature. The maximum temperature increase between the carrier upstream and downstream was 11.8 ºC (control bed, Test 6) and 12.0 ºC (wash water bed, Test 5). Mortality at these locations was low (<10%) and therefore temperature was not considered to have affected the test results.

The downstream carrier locations were also subject to the greatest pH variability, although the difference recorded between upstream and downstream carriers was less than one pH unit for all test occasions and was not considered to have affected test results. For the borehole supplied beds the pH mostly increased during flow through the bed, whereas for the salad wash water supplied beds the pH was mostly decreased.

The conductivity measurements recorded for all locations were consistent, with median values in the order of 530 µS/cm. The across-bed variation was less than 50 µS/cm on the majority of occasions. An extremely high measurement (973 µS/cm) in the carrier below the control bed; a difference of 435 µS/cm from the above bed reading) was made at the same time that fertiliser pellet application was

taking place in the beds. However, for this test location (11 Jun 08, Test 6) all organisms survived, i.e. none were immobile. During test 8 there was an *ad hoc* application of calcium nitrate to the carriers above the control beds and although there was some control mortality at this location it was only 6.3% and was not considered to affect the test results.

4.3.2 Proportion Immobilisation

The proportion of organisms immobilised on each test occasion is summarised as a percentage of the total number of organisms deployed at each location in Table 4.3-a. Individual data for each test deployment are included in Appendix E. Results from Test 3 were discounted as they had control mortality above 10% and are not shown. During this test alone there had been an unusually high rainfall and localised flooding. The location of the borehole fed carrier for this test (D2, see Figure 4.2-b) was at the foot of a bank and it may have received significant amounts of run-off on this occasion, from unidentified material contained in re-used fertiliser bags which were located at the top of the bank during the test. The bags were not present during the other tests.

Table 4.3-a Summary of *Gammarus pulex* Immobilisation at each Location

	% Immobilisation			
	Borehole fed control	**Below control bed**	**Wash water supply**	**Below wash water bed**
Test 1 (25 Jun 07)	0	0	13	0
Test 2 (13 Jul 07)	8	8	46	13
Test 4 (14 May 08)	4	0	21	0
Test 5 (4 Jun 08)	0	3	7	7
Test 6 (11 Jun 08)	2	4	2	4
Test 7 (18 Jun 08)	0	4	10	2
Test 8 (25 Jun 08)	6	2	33	4
Test 9 (02 Jul 08)	9	2	10	6
Mean	4	3	18	5
STDEV	4	3	15	4
SE	1	1	5	1

Immobilisation was greatest in the wash water supply on 6 out of 8 test occasions. On one occasion (Test 6) the effect measured in the wash water supply was less than the effect in the control (borehole supply) and the below control bed locations. Also on a single occasion (Test 5) the effect measured in the wash water supply was less than the effect in the below wash water bed location. Acceptance criteria for the tests were met. During these tests alone (Tests 5 and 6) it was noted that the wash water supply was receiving additional borehole water flow and although it was not possible to quantify, it was possible that this provided greater dilution of the wash water supply than during the other tests.

The mean immobilisation for all tests is presented in Figure 4.3-a. The mean immobilisation was greatest in the wash water supply (18 %) and consistently less (between 3 and 5%) for the other three locations. However, the standard error for data from the wash water supply was higher than the other locations; organisms were immobilised on every test occasion in the wash water supply but the extent of this effect was variable.

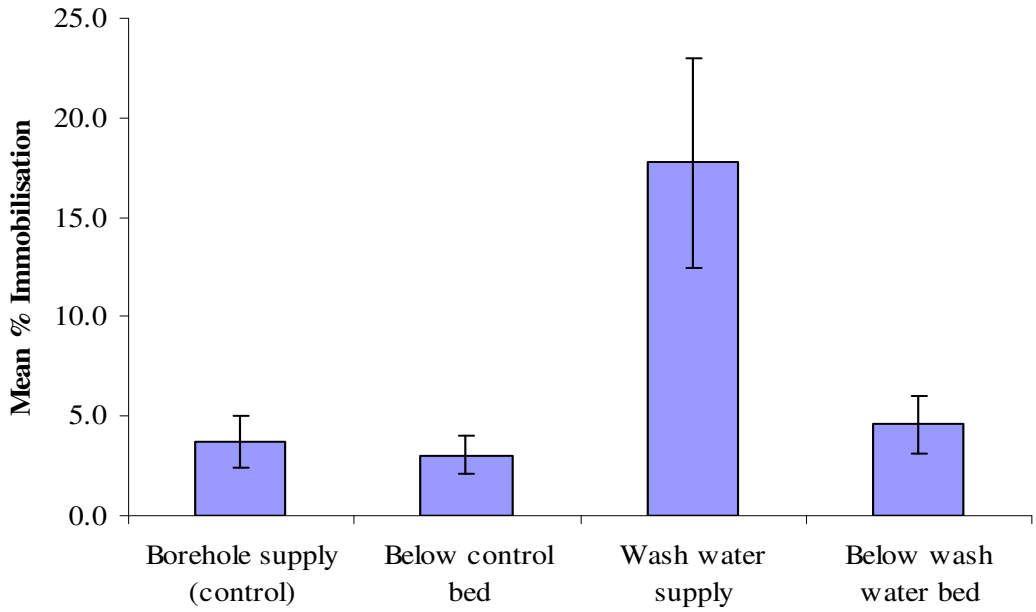

Figure 4.3-a *Gammarus pulex* Mean Immobilisation

The mean proportion immobilisation for the 8 test occasions is shown for each deployment location. Vertical bars show standard error.

4.3.3 Significance Testing

The response of organisms from the test locations in carriers upstream of the watercress beds was compared with that of organisms in carriers below the watercress bed. The hypothesis tested was that the response in the downstream location was not significantly different from that upstream. The between sites response was also tested and the results are presented in Table 4.3-b. Significance testing on individual exposures was not carried out as statistical assumptions (normality and variance) were violated. The data were tested for normality using the Kolmogorov-Smirnov distribution (data had normal distribution, $p>0.2$). One Way Analysis of Variance (ANOVA) with pairwise multiple comparison procedures (Holm-Sidak method) was used to compare effects at each location.

Table 4.3-b Comparison of Response at Test Locations

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Significant? (P<0.05)**
Wash water u/s *vs*. wash water d/s	0.12	2.203	0.0426	Yes
Control u/s *vs*. control d/s	no statistical difference (p=0.546)			No
Wash water u/s *vs*. control u/s	0.135	2.446	0.0282	Yes
Wash water d/s *vs*. control d/s	no statistical difference (p=0.404)			No

The effect recorded in the wash water downstream of the watercress bed was not significantly different from the control (borehole water) downstream. The effect recorded in the wash water upstream of the bed (i.e. before 'treatment') was significantly different (i.e. higher) from the control upstream (borehole supply) and from the wash water downstream (i.e. after 'treatment').

Two way ANOVA on ranks was used to test for significance of difference in response between sites (i.e. borehole supplied and wash water supplied beds) and within sites (i.e.upstream and downstream). A pairwise multiple comparison procedure (Holm-Sidak method) was used to interpret the main effects where significant interaction was determined. The results are shown in Table 4.3-c. There was a statistically significant difference between the responses of organisms in the wash water supply carrier from those in the carrier below the bed on four test occasions (Tests 4, 7, 8 and 9).

Table 4.3-c Difference in Response; Between-Site and Within-Site

Date (Test)	Borehole x Washwater	Upstream x Downstream	Site x (U-stream/ D-stream)
25 June 2007 (1)	f=2.196, p=0.15	f=2.196, p=0.15	f=2.196, p=0.15
13 July 2007 (2)	f=2.309, p=0.14	f=2.962, p=0.096	f=1.616, p=0.214
14 May 2008 (4)	f=6.405, p=0.014	f=15.647, p=<0.001	f=6.405, p=0.014
04 June 2008 (5)	f=2.18, p=0.149	f=0.314, p=0.579	f=0.127, p=0.724
11 June 2008 (6)	f=0.0107, p=0.918	f=0.905, p=0.345	f=0.0001, p=0.993
18 June 2008 (7)	f=2.449, p=0.123	f=0.612, p=0.437	f=5.51, p=0.022
25 June 2008 (8)	f=13.775, p=<0.01	f=18.658, p=<0.01	f=9.632, p=0.003
02 July 2008 (9)	f=13.775, p=<0.001	f=18.658, p=<0.01	f=9.632, p=0.003
Tests performed with significance level= 0.05			

4.3.4 Weight of Isothiocyanate Containing Crops Washed

The weight of different crops and their combinations varied each day and it was possible to examine the weekly weight of crops washed during each test. Of all the isothiocyanate producing crops washed during the study, the amount of watercress was by far the greatest being between 86 and 94% of the total isothiocyanate containing crops by weight. It is interesting to note that the week that the highest proportion immobilisation of *G. pulex* was recorded (Test 2), the greatest weight of PEITC containing crops was washed that week, with the greatest weight of watercress and the highest daily weight of watercress washed (17,100 kg).

Pearsons Product Moment correlation indicated a positive relationship between weight of watercress washed (kg) during a test and the % immobilisation recorded in the wash water carrier during the test ($r = 0.671$, $p = 0.0476$), although linear regression was not possible as the constancy of variance of data requirement was violated. During Tests 5 and 6 the wash water supply was diluted with additional borehole water (§ 4.3.1) and a possible reduction in effect due to this can be clearly seen in Figure 4.3-b. Although the dilution with borehole water could not be quantified and therefore the extent to which this affected the results cannot be predicted, if data from these two tests are not included, the correlation is much stronger ($r = 0.94$, $p = 0.00164$).

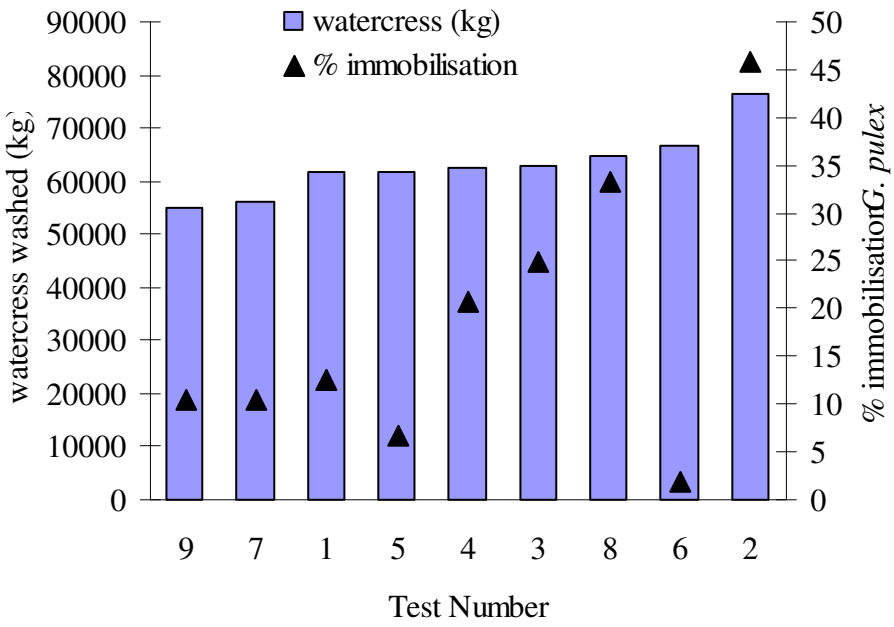

Figure 4.3-b Relationship Between Weight of Watercress Washed and *Gammarus pulex* Immobilisation

For each *in situ* test the weight of watercress washed during the test exposure is shown (blue bars). Proportional mortality (as % immobilisation) of test organisms at the end of each test exposure is shown (black triangles) to illustrate the relationship.

Linear regression predicts an association between the weight of watercress washed (kg) and the % immobilisation of *G. pulex* with Equation 4.3:

$$\% \text{ immobilisation} = -88.015 + (0.00176 * \text{watercress (kg)}) \quad [4.3]$$

This results in a coefficient of determination (R^2 value) of 0.883. Analysis of Variance also gives a high F statistic (F = 37.0, p = 0.002) indicating a strong association.

4.4 Discussion

4.4.1 Effect of Wash Water on *Gammarus pulex*

The aim of this study was firstly to assess whether the salad wash water discharge would significantly reduce the survival of *G. pulex*; and secondly to assess whether the re-routing of salad wash water through the watercress beds is successful at mitigating this effect. The test organisms were deployed on nine occasions throughout two seasons of peak watercress growth and harvesting. During these periods Vitacress Salads Ltd experiences maximum demand from its customers for washed and packaged watercress and other salad crops. The methods employed sought to use this as a 'worst case scenario' and were carried out *in situ* as the processes and environmental factors which may affect the concentration, bioavailability, toxicity, fate and distribution of contaminants in the salad wash water discharge are difficult to replicate under laboratory conditions (Pereira *et al.*, 2000). The re-routing of the wash water back through watercress beds may be considered a similar action to the use of wetlands for waste water treatment. There are numerous studies in which the success and scale of such treatment systems has been examined (Kassenga *et al.*, 2003, Ahn and Mitsch, 2002, Nuttall *et al.*, 1997, Price and Probert, 1997).

The results showed that the immobilisation effect on *G. pulex* deployed in 'untreated' salad wash water discharge in the carrier channels at the top of the watercress beds was significantly higher than for test organisms deployed in 'treated' water which had passed thorough the watercress bed or other control locations with no salad wash water. If PEITC was the causative agent, the results indicated that residence time in the watercress bed allowed it to dissipate, reducing the recorded effect on *G. pulex* and thus was a successful mitigation measure. This could possibly occur either through ultraviolet action or adsorption onto sediment in the water, the substrate or both (Murdock, 2008b). The time taken for water used in the factory washing process, which then passes via watercress cropping beds to reach the discharge outfall, is estimated to be approximately two hours (Vitacress Salads Ltd, 2010) (§ 6.2.3). Variability of flow within the watercress beds (Casey and Smith,

1994) due to the age of the crop (§ 4.2.3) could alter the residence time. However, the water carrier system at Lower Link Farm combines flow from a block of several beds, most likely to contain crops of differing ages which would counter this.

4.4.2 Experimental Variables

The results from each test location revealed that the mean immobilisation of *G. pulex* in the salad wash water supply (17.7 SE ± 5.3 %) was more variable than at the other locations (below wash water bed 4.6 SE ± 1.5 %). This could be due to a number of factors. The PEITC concentration in watercress leaves (§ 2.2.3) varies with the age of the plant and the environmental conditions under which they are grown (Palaniswamy *et al.*, 2003). Although the selection of watercress bed carriers for test locations was made within a range of pre-set criteria, the crop age varied between 14 and 41 days old (median age was 22 days and mode was 21 days). A degree of variation in the crop age was inherent due to the flexibility of farm management practice on site.

The weight of PEITC-containing crops processed changed from day to day (Vitacress Salads Ltd, 2008a), depending on customer demand and crop availability; the annual peak factory production occurs around the last week of May each year. Although analysis of data detailing relative and absolute weights of PEITC containing crops washed at the factory during the study revealed a correlation between weights of watercress washed during a test and *G. pulex* mortality, potential variability due to dilution of wash water with borehole water appeared to weaken this relationship.

The water carriers were not consistently supplied with 100% salad wash water and were supplemented with borehole water outside factory operating hours (i.e. overnight) and depending on site management practices (e.g. when greater flow in the watercress beds was required). The effectiveness of using watercress beds as a treatment to mitigate the effects of salad wash water to *G. pulex* may be enhanced by the amount of 'dilution' with borehole water that it receives in the carrier at the top of the bed. On site at Lower Link Farm there was no set pattern for augmentation with borehole water; individual bed flows were altered as required by the farm

manager, and there was no method of recording the dilution of factory wash water by borehole water supplied to upstream carriers.

The random selection of test organisms and the use of control deployments should have minimised natural variation within the population inherent due to factors such as moult cycle and diurnal rhythms (Blockwell *et al.*, 1998), parasitic infection (Pascoe *et al.*, 1995) or individual sensitivity to toxicants (Taylor *et al.*, 1993). It is possible that although test organisms were selected randomly, the sample population (6-9 mm length) may have included a higher proportion of smaller younger males and larger older females. However, in a comparison of male and female *G. pulex* selected from precopular pairs on the basis of length (approximately 6-9 mm), Pratt (2008) found that although the mean dry weight of males (8.63 SE ± 0.25 mg, n = 69) was greater than females (4.38 SE ± 0.15 mg, n = 58), there was no significant difference in feeding rate or mortality between sexes after 7 days in salad wash water discharge.

The monitoring of water quality parameters revealed that although there were large changes in temperature (and to a lesser extent conductivity and pH) within the watercress beds at all deployment locations on some occasions, these fluctuations did not have an acute effect on organisms deployed in the control locations, in particular downstream of the watercress beds. It should be noted that these measurements were not continual, i.e. they were only taken at test deployment and test end, so they must be taken as an indication rather than actual measurement of water quality throughout the test. It is very likely that, for example, higher temperatures were reached in the carriers below watercress beds holding a younger crop (i.e. providing less cover) on very hot sunny days.

4.4.3 Ecotoxicological Effect on the Receiving Water

The results indicate that the ecotoxicological effect on *G. pulex* in the Bourne Rivulet East channel below the Lower Link Farm discharge would be reduced by the redirection of salad wash water to additionally flow through the watercress beds. The *in situ* test provided a more 'realistic' scenario than the laboratory tests, although at an acute rather than a more sensitive sublethal level, as the effects

recorded were the inclusive result of the toxicant and other environmental factors. The *in situ* test measured biologically relevant toxic effects to representative organisms (Den Besten and Munawar, 2005). The variability of mortality seen in the wash water could have been due to the sensitivity of the test or the variability of levels of isothiocyanates in the wash water or a combination of both.

Within the watercress industry in southern England, the scale of watercress production and processing of harvested salad crops at Lower Link Farm may be unique. Biological disruption of the same magnitude as recorded in the Bourne Rivulet is not reported in the receiving waters immediately below other watercress farms. Many other smaller watercress growers harvest and bundle the watercress crop on-site on a much smaller scale than at Lower Link Farm. In general, other watercress farms do not operate large salad pack houses on site and thus do not have associated additional wash water discharge to the chalk stream receiving waters. However, during periods of peak watercress harvest, when large pulses of PEITC may be washed into the receiving water, it is possible that ecotoxicological effects may be evident in the downstream macroinvertebrate populations.

4.4.4 Further Work

During the study it was noted that a large amount of sediment was present in the carrier supplying re-routed wash water to the watercress beds. A series of sediment tests (either *in situ* or under controlled laboratory conditions) may reveal whether the contaminants (insoluble isothiocyanates) are being adsorbed within the sediments. Additionally, the hypothesis that sediments bought in on crops imported or grown at other sites may include other toxicants could be tested.

Drift netting in the carriers below beds during harvesting to investigate the drift response of *G. pulex* living in the watercress beds to freshly released PEITC could be carried out. However, difficulties arising due to low flows, when the bed flow is reduced as far as possible to zero during harvesting to prevent sediments being washed from the bed, would need careful consideration.

Early life stage testing was not used *in situ* even though it is more sensitive due to impracticability in carrying out the test in this instance. The adult organisms used were collected from the field on the day of the test which would not be possible with juveniles. Juveniles could be cultured in the laboratory for transport to the site for testing, although other confounding factors, e.g. the culture temperature and light regime would need to be considered, along with the effect of sediment within the test cages and the food source for the organisms. Difficulties may also arise in assessing the organism status in the test cage at the test end; it may be difficult to see them.

4.5 Conclusions

The primary objective of this Study was to assess the success of the recirculation system installed on-site at Lower Link Farm as a mitigation measure to reduce the effect of its discharge on the macroinvertebrate community in the receiving water downstream of the farm. The system installed recirculates salad wash water, from the salad washing and processing factory, through a series of watercress beds prior to its discharge.

With the use of caged deployments of adult *G. pulex*, the study was able to address the hypothesis that the salad wash water discharge significantly reduces the survival of *G. pulex*. It demonstrated that there was an effect on the survival of organisms in locations supplied by wash water from the process and packaging plant on site at Lower Link Farm.

The second hypothesis, that the re-routing of salad wash water through the watercress beds is successful at mitigating effect due to the salad wash water, was also addressed. The extent of the effect of salad wash water on *G. pulex* was variable, but overall it was reduced to levels comparable to control levels (recorded in borehole only fed beds) after the wash water had been fed through the watercress beds. Accordingly, we can conclude that the recirculation of process wash water through the watercress beds is an effective mitigation measure to reduce its effect on the survival of *G. pulex*. The results indicated that this is achieved by providing additional residence time to allow degradation of the toxicant along with additional dilution prior to discharge of wash water to the receiving water.

These conclusions have also been supported by an increase in the quantity and diversity of the macroinvertebrate population in the receiving water below the watercress farm process outfall, in particular since the recirculation system was put into practice in 2006 and this is further discussed in Chapter 5.

5 LONG TERM CHANGES IN MACROINVERTEBRATE COMMUNITIES BELOW A WATERCRESS FARM

5.1 Introduction

5.1.1 Watercress and Chalk Spring Water

Producing and processing watercress could not take place without reliable and plentiful supplies of high quality water. Historically, watercress production has been associated with the ideal conditions provided by chalk streams in southern England (Berrie, 1992). Watercress is grown in shallow gravel beds fed by springs and boreholes which provide a constant flow of relatively warm winter and cool summer water. It has been cultivated commercially for approximately 200 years and there are currently 60 Ha (planted area) of watercress beds in the UK on chalk headwaters, streams and rivers (Department for Environment Food and Rural Affairs, 2009a) (see Appendix A). The vast majority of these (approximately 90%) are located on or upstream of a chalk river Site of Special Scientific Interest (SSSI) (Natural England, 2009).

5.1.2 Chalk Rivers

England has the largest chalk river resource in Europe, reflecting the distribution of chalk geology from Dorset to Kent and up to Norfolk, Lincolnshire and Yorkshire. Chalk rivers arise from springs where the water table of the highly porous chalk aquifer reaches ground level. The majority (80%) of discharge originates from the chalk aquifer with little overland flow (Mann *et al.* 1989 in (Mainstone, 1999), therefore they generally have a stable hydrological regime. Peak flows may be sustained for long periods resulting in riparian soils becoming waterlogged. An ephemeral 'winterbourne' section may be present which only flows during the summer months when there has been sufficient winter rainfall recharge of the aquifer. The ground water chemistry is also relatively stable with a high alkalinity and conductivity. The constant temperature of water rising from the chalk springs maintains a river water temperature of around 11ºC which generally protects against seasonal extremes.

A typical chalk river channel has a shallow cross-section and sinuous form, a low occurrence of pools and riffles and infrequent gravel shoals or exposed substrates (Sear *et al.*, 1999). Due to a relatively low hydraulic energy and thus low levels of suspended solids the waters are generally very clear. Levels of phosphates and nitrates are highly dependent on anthropomorphic activities.

Chalk rivers are designated as UK Biodiversity Action Plan habitat (UK Biodiversity Reporting and Information Group, 2008). Mainstone (1999) describes the characteristic assemblage of plants and animals in chalk rivers as generally taken to be that of a "low-intensity meadow dominated catchment with a high water table and frequent inundation of riparian and floodplain areas" rather than the valuable but spatially limited original woodland carr habitat.

Aquatic macrophytes are important in contributing to the overall health of the chalk stream system for example, by oxygenating the water, helping to cycle nutrients, providing refugia and breeding sites, providing the air-water link to enable invertebrates to complete their life-cycles, stabilising the substratum, supplying colonising surfaces for microscopic organisms and providing structural diversity to the watercourse. Beds of water crowfoot (*Ranunculus* spp.) are the characteristic macrophytes of chalk streams and reaches of the River Avon and River Itchen are scheduled as priority habitats under the Habitats Directive (1992). *Ranunculus* spp. are associated with a different assemblage of other aquatic plants, such as water-cress *Rorippa nasturtium-aquaticum*, water-starworts *Callitriche* spp., water-parsnip *Sium latifolium* and *Berula erecta*, water-milfoils *Myriophyllum* spp. and water forget-me-not *Myosotis scorpioides*.

An abundant and diverse macroinvertebrate community is supported with many specialised and rare species (e.g. the fine-lined mussel, *Pisidium tenuilineatum* and the mayfly *Paraletophlebia wemeri*) (Hampshire Biodiversity Partnership, 2000). *Gammarus* spp. and *Cottus gobio* (bullhead) have been identified as keystone species (Woodward *et al.*, 2008) with the potential to exert disproportionately powerful effects on the community structure and ecosystem processes. Gammaridae, which exist in chalk streams in very large numbers, are the principal detritivore and dominate the prey assemblage.

There are few chalk river reaches in the UK that remain unaltered by human intervention, i.e. are shaded by trees, for example alder (*Alnus glutinosa*) or willow (*Salix* spp.) carr and with a floodplain formed of a ill-defined channels (Ladle and Westlake, 1976). Chalk rivers and their floodplains are generally highly managed and characterised by local land use patterns, as well as their use for angling and other leisure pursuits. Pressures and potential impact are detailed in Table 5.1-a.

Table 5.1-a Pressures and Potential Impacts on Chalk Rivers (Environment Agency, 2004b)

Pressure	Specific Aspects	Potential Impacts
Abstraction	Drinking water supply, industry, fish and watercress farms, irrigation	Low flows, reduced pollutant dilution, sedimentation, excess algal growth, loss of species, wild fish entrapment
Effluent Discharge	Sewage, industrial effluent, fish and watercress farms, endocrine disruptors, increased temperature	Organic, nutrient and toxic pollution; loss of species, excess algal growth, reduced population size
Agriculture	Livestock: bank damage, polluted run-off (organic matter, nutrients, sediment). Arable: drainage, polluted run-off (nutrients, sediment, herbicides, endocrine disruptors)	Damage to aquatic & wetland habitats & sensitive species, reduced water quality, accelerated run-off, reduced groundwater recharge
Flood defence, land drainage, poor water level control	Channel and bank engineering, weed cutting, dredging, hatch operation	Damage to aquatic and riparian species and habitats
Development	Urban development: construction, polluted run-off	Habitat loss, poor water quality, higher water demand, fish passage obstruction
Fisheries Management	Weed cutting, riparian management Fish stocking and removal	Habitat loss, reduced flow velocity & gravel scour, fish community change, risk of disease spread
Recreation	Walking, canoeing and boating	Disturbance
Non-native & invasive species	Escape and spread of farmed fish, crayfish, mink & non-native plants	Loss of native species and habitats

5.1.3 Chalk Stream Headwaters

Headwaters (in general i.e. not just chalk streams) have been defined on the basis of their physical characteristics as “reasonably low stream order or relatively small

stream width and catchment area" (Clarke *et al.*, 2008) or by location, "a watercourse within 2.5 km of its furthest source as marked with a blue line on Ordnance Survey (OS) Landranger maps with a scale of 1:50,000" (Furse, 1995). In Britain, headwaters probably represent >70% of the total length of flowing waters (UK Biodiversity Reporting and Information Group, 2008). Mainstone (1999) describes perennial chalk headwaters as "first order streams, below the perennial head that dry out only in exceptional circumstances" and chalk winterbournes as "those that have a naturally dry periods each year (except in unusual circumstances)".

Physical and chemical characteristics of headwaters vary greatly according to their location, altitude, geology, and surrounding land-use and faunal communities are most influenced by local hydraulic conditions (Sear *et al.*, 1999). They are generally excluded from protected areas such as SACs and SSSIs/ASSIs, but play an important role in the overall functioning of the river ecosystem downstream (Furse, 1995).

Macroinvertebrate and plant species typical of chalk stream upper reaches have been suggested by Mainstone (1999) drawn from a number of studies of southern chalk rivers (Appendix F). There are differing expectations for the ecology of chalk headwaters with respect to downstream reaches (Environment Agency, 2004b) and Sear *et al*. (1999) found that faunal assemblages vary between upper, middle and lower chalk stream reaches and are most influenced by the local conditions. In a review of 11 studies of longitudinal changes in macroinvertebrate diversity, Clarke *et al.* (2008) found evidence to support the prediction that there is low species richness in headwater streams. A comparison of the expected with observed species diversity or taxonomic richness of a chalk stream headwater would enable us to establish whether management practices had any effect on the biological 'quality' in terms of species diversity and abundance.

5.1.4 Impact of Watercress Farming on Chalk Stream Ecology

Actual and potential impact to chalk stream ecology below watercress farms is well documented. In a comprehensive study of the impacts due to both small and intensive scale watercress production, Casey and Smith (1994) described changes in

the water chemistry downstream of watercress beds due to the addition of nutrients (potassium and phosphate) and the depletion of nitrate by the growing crop. Casey (1981) found that the watercress beds of a commercial operation were responsible for 39% of the total reactive phosphate throughput of a chalk stream headwater. High concentration of reactive phosphate in the bed outflow raised the stream reactive phosphate concentrations, the effects of which were not measured, although could possibly alter the structure of the plant communities in the stream.

A sustained stream flow has been described where there is a pumped borehole supply and Casey (1981) reported that up to 90% of the summer flow could be provided in this way in years of low natural discharge. There was a contribution of large amounts of suspended solids and increased levels of fine organic sediment due to bed clearing (Casey and Smith, 1994, Fewings, 1999), although more recently the use of sediment traps and settlement tanks/ponds has reduced this impact.

Roddie *et al.*(1992) found impact on *Gammarus pulex* feeding rates caused by the addition of zinc to watercress crops to control crookroot. Crookroot is propagated by zoospores which penetrate the watercress root cells and is the vector for watercress yellow-spot and chlorotic leaf spot viruses. More recently, the practice of using zinc to control crookroot is much reduced (and no longer used at Lower Link Farm). Cultural control techniques, such as increased flow of water to wash away the zoospores and regular planting of the beds with clean young plants, are recommended to counter the proliferation of crookroot (Assured Produce, 2006).

In a survey of operational practice on watercress farms in Hampshire, Fewings (1999) described the potential effects of produce preparation. Watercress was often found to be washed in chlorinated water, the disposal of which presented a risk. In a review of environmental impact of watercress farming on English chalk rivers, Natural England (2009) concluded that further work is required to explain the effects seen in invertebrate populations in watercress beds and discharge streams, in particular in relation to phenylethyl isothiocyanate (see Chapter 2). Recommendation was also made to investigate the levels of PEITC released during harvesting operations and whether there was a link to reduced or absent populations of *G. pulex* (Fewings, 1999).

The unusual macroinvertebrate assemblages present below watercress farms has been a problem recognised and well documented for the river below Lower Link Farm, both as a series of routine monitoring surveys (Medgett, 2008) or one-off investigations (Everall and Bennett, 2007). This Chapter describes the macroinvertebrate community of the Bourne Rivulet and the changes that have taken place over the past two decades. This section of the Bourne Rivulet is a winterbourne headwater and anthropogenic pressures associated with abstraction, effluent discharge, agriculture and fisheries management are particularly relevant. In addition part of the Bourne Rivulet (the East Rivulet channel) is maintained wholly by water used at Lower Link Farm in the production and processing of watercress and other salad leaf crops. This Chapter also compares the temporal and spatial variation in macroinvertebrate population with changes made to the watercress farm management practice (i.e. measures taken in an effort to improve biological status of the Bourne Rivulet).

5.2 Study Location and Method

5.2.1 The Bourne Rivulet

The Bourne Rivulet, although not designated, is a tributary of the River Test which is a SSSI along it entire length. The River Test is described as a classic chalk stream within which are found nationally rare as well as nationally scarce macroinvertebrate species (Environment Agency, 2004b). The Bourne Rivulet rises from chalk springs at Hurstbourne Tarrant and flows through Stoke, St. Mary Bourne, Lower Link watercress farm and Hurstbourne Priors before entering the River Test (Figure 5.2-a).

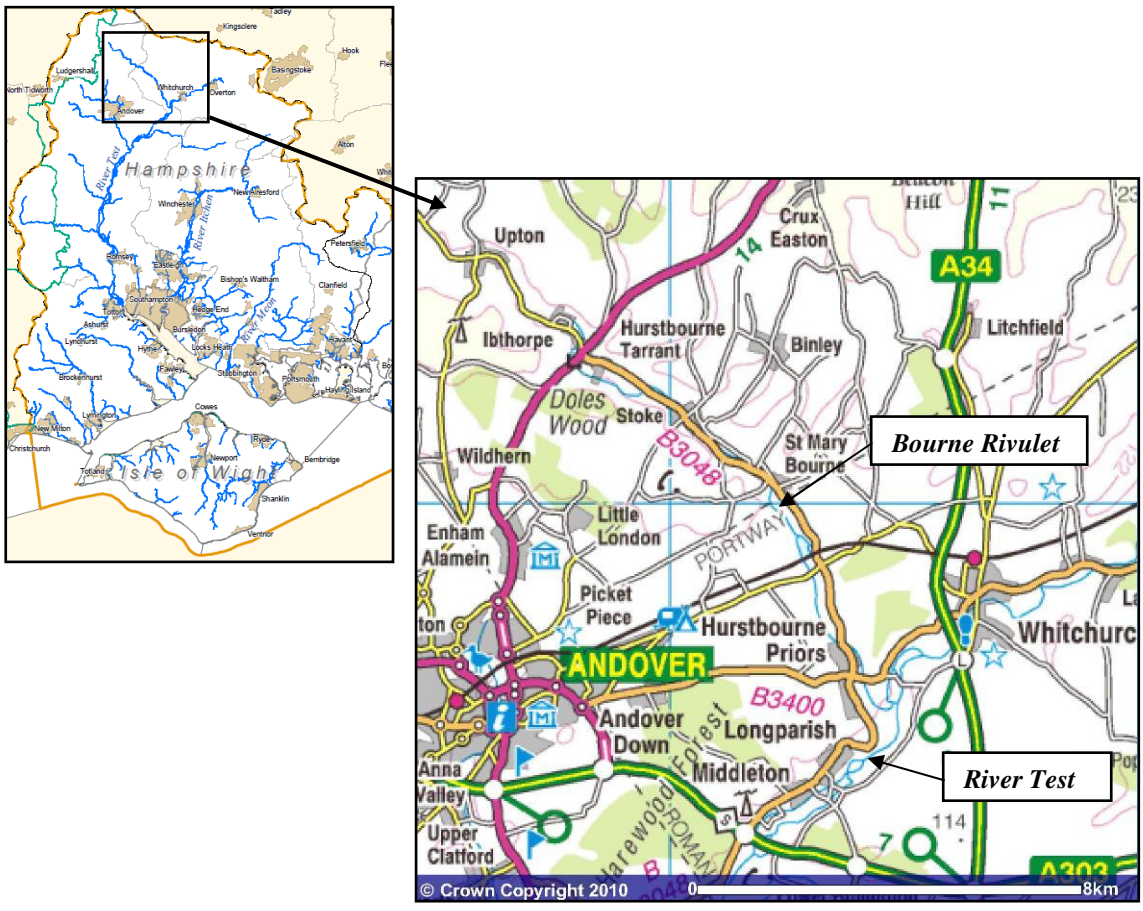

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Figure 5.2-a Bourne Rivulet Location Map

It is approximately 15 km in length, although in the environs of Lower Link Farm it is a headwater winterbourne and often dries for a few months during the summer and autumn when rainfall levels have been low. It flows through the farm in canalised form, emerging on the west side (Figure 4.2-b). Flow from a number of watercress beds discharges to this channel.

Historically, this section of the winterbourne was characterised by numerous springs, water meadows and drains (Ordnance Survey, 1872). The East Rivulet channel was cut off when the first watercress beds were built at Lower Link Farm at the beginning of the 20th Century. Since then ground water has been abstracted to supply the watercress beds and the flow in the East Rivulet has been relatively constant. It is maintained by the spring water discharge from the watercress beds as well as farm process effluent and site storm discharge. Therefore, it does not dry in low flow periods, as does the West Rivulet. Its confluence with the West Rivulet is approximately 250 m downstream of the farm.

As it is fed from a chalk aquifer the pH of the water is neutral to alkaline and relatively constant in temperature (~11ºC) throughout the year. The Bourne Rivulet is classified by the Environment Agency as River Ecosystem 1 (RE1); the highest level of water quality standard, i.e. the water is suitable for drinking water abstraction and for supporting high class game and course fisheries.

The flora and fauna of the Bourne Rivulet are characterised by taxa that are able to withstand periods of low flow or drying. Typical groups found in the Bourne Rivulet include Gammaridae, Trichoptera (caddis), Ephemeroptera (mayfly) and Elmidae (riffle beetles). Immediately below the watercress farm on the Bourne Rivulet there is a difficulty in determining the ‘natural’ condition of the receiving water as a baseline to measure against, not only because anthropogenic disturbance has taken place in particular over the last century, but also due to the maintenance of flow in ephemeral sections by discharge from the factory and pumped borehole water flow discharged from the watercress beds. The macroinvertebrate community below the watercress farm has historically differed from others in southern English chalk rivers; although Gammaridae were present, their numbers were relatively low.

The Bourne Rivulet is a designated salmonid water (under the EC Freshwater Fish Directive 78/659/EEC) and the stream is a managed trout fishery. Chalk streams represent major salmonid habitat in lowland Britain (Mainstone, 1999) and smaller fish (e.g. lamprey spp., stone loach (*Noemacheilus barbatulus*) and bullhead (*Cottus gobio*)), also of ecological importance, can occur in the smallest headwaters and beyond the perennial head. The Environment Agency carries out monitoring of fish stocks; the most recently reported was carried out in November 2006. A perceived decline in the numbers of larger 3+ brown trout since the early 1980s had been reported by fishing interests on the river and the survey (Gent, 2006) investigated whether the watercress farm and salad-processing unit at Lower Link Farm was having a measurable effect on the brown trout (*Salmo trutta*) population downstream. Brown trout were not caught during the survey in either the East or West Rivulet, despite predictions indicating that the habitat was suitable for fry. Although carried out following de-silting works of the East Rivulet and a second successive drought year, the report concluded that species found in the Bourne Rivulet were typical of a chalk stream fish community, with the exception that no stone loaches were found. The report also concluded that the growth rates of brown trout were average when compared to other chalk streams. No further fish surveys have been carried out despite a recommendation for repetition of an annual basis. There are however anecdotal reports of brown trout and bullhead caught since new chalk stream habitat was created at the East Rivulet discharge on the watercress farm (Cain Bio-Engineering Ltd, 2009).

Management of the Bourne Rivulet is carried out by the riparian owners. The farm manager at Lower Link Farm routinely carries out weed clearance to encourage flow through the farm and prevent flooding upstream and siltation within the stream. This is considered necessary, in particular, during the early spring and summer months when rapid plant growth coincides with seasonal peak flows. The overhanging bank-side vegetation is also cut back and removed, again to prevent blockage of the stream. There is some poaching by cattle on the western banks of the West Rivulet (land not controlled by Vitacress Salads Ltd).

5.2.2 Changes in Farm Management Practice

In response to the reported poor biological quality downstream of the farm, the results of studies on the effect of watercress farm discharges (Natural England, 2009, Roddie *et al.*, 1992) and to meet its water quality consent conditions, a series of improvements to the farm process and practice on-site at Lower Link Farm have been made (Table 5.2-a.).

Table 5.2-a Key Changes in Farm Management Practice (1995 to 2007)

Description	Resultant Effect
Suspended solids settlement tank installed to take water from bed clearing operations. Settlement tank later discharged through watercress beds to allow further settlement. Sludge blanket detector fitted to alert to fill level. Settlement chambers installed above outfall and turbidity sensor with telemetry alert installed to the East Rivulet discharge. East Rivulet channel de-silted.	Removal of silt and fine organic particles which would otherwise block stream bed gravel interstices.
5mm drum replaced by 2 mm parabolic screens to remove leaf matter from salad wash outflow.	Removal of allochthonous input which could accumulate downstream restricting flow and artificially increase watercress proportion in the plant community.
Volume of ammoniacal nitrogen used in liquid fertiliser reduced by 80%and subsequently eliminated from fertiliser regime.	Potential for eutrophication of the receiving water associated with inputs of nitrogen removed.
Reduction and elimination of zinc chloride used to control crook root disease.	Reduction and removal of potential toxicity to biological communities in the receiving water
Chlorine use to wash product (& de-chlorination) reduced by 80%, subsequently ceased. Citrox used to treat 20% of product, directed to foul sewer, use subsequently ceased. Salad leaf washed only with spring water.	Removal of potential toxicity to biological communities in the receiving water.
Watercress bed and factory discharges de-culverted to create 95 m of chalk stream on site.	Additional chalk stream habitat created.
Recirculation system installed to allow all parabolic screen wash water discharge to flow through watercress beds prior to discharge to the East Rivulet. Subsequent expansion of this system to include additional watercress beds.	Reduce and potentially remove the effect on macroinvertebrates of increased levels of PEITC in the wash water discharge.

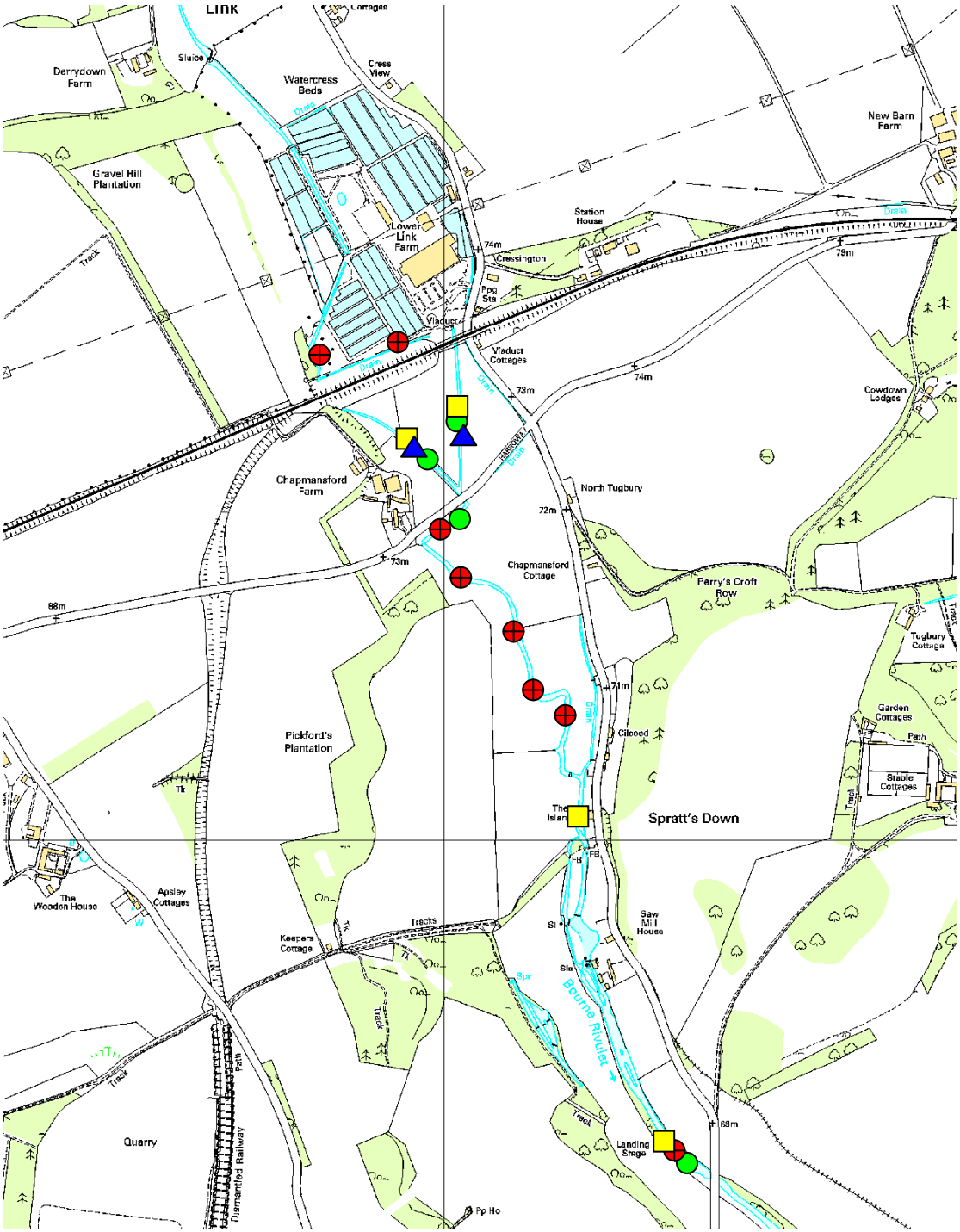
Consequently there are no chemicals used during the processing operation; the watercress and other salad leaf are currently washed in spring water only. This wash water passes through a 2 mm parabolic screen and settlement tank before being re-circulated back through a series of watercress beds and then discharged to the East Rivulet. The only chemicals used at the farm are fertilisers applied to the watercress beds and fungicides remaining on seedling plugs when transplanted to the beds (detail of farm operations are given in § 1.2.6).

5.2.3 Macroinvertebrate Data

A long term data set exists for macroinvertebrate samples taken from the Bourne Rivulet, due to additional monitoring which has been carried out in response to an unexplained poor biological quality below the watercress farm. A summary of all surveys carried out is given in Table 5.2-b and sampling locations are illustrated in Figure 5.2-b. The Environment Agency has been conducting routine surveys at locations on the Bourne Rivulet downstream of Lower Link Farm since 1989. Four mid-reach sites have been routinely sampled; the West and East branches of the Bourne Rivulet 200 m downstream of the watercress farm and after their confluence the Bourne Rivulet 1.1km and 1.9km downstream (White and Medgett, 2006, Medgett, 2008, 1998). More recently, invertebrate samples have been taken at various sites on and below the watercress farm as part of B.Sc. and M.Sc. project work (Marsden, 2005, 2006). Vitacress Salads Ltd also commissioned regular surveys at a number of locations around the farm outfalls and downstream (Murdock, 2007, 2008a, 2009). A further survey was carried out downstream of the farm (Everall and Bennett, 2007).

Table 5.2-b Summary of Biological Surveys, Bourne Rivulet (1989-2009)

Author	**Site Names**	**Sampling Notes**
Environment Agency (2009)	East Rivulet 200m d/s, West Rivulet 200m d/s The Island 1.1km d/s, Ironbridge 1.9km d/s	3 min kick sweep Nov 89-May 09 archive dataset
Murdock (2009)	West Rivulet above Viaduct, New channel head of Eastern Rivulet, Middle of Eastern Channel	3 min kick sweep (May 09)
Murdock (2008a)	Bourne Rivulet above Viaduct, New channel head of Eastern Rivulet, Middle of Eastern Channel, 500 m u/s of gauging station (Malyons Land), New channel taking discharge from cress beds	3 min kick sweep (May 08)
Medgett (2008)	East Rivulet 200m d/s, East Rivulet 200m d/s The Island 1.1km d/s, Ironbridge 1.9km d/s	3 min kick sweep (May 04, Sep 05, Mar 06, Apr 06, Apr 07, Nov 07)
(Everall and Bennett, 2007)	West Rivulet 5-50m u/s confluence, East Rivulet 5-50m u/s confluence, Chapmansford 0.25km d/s, Ironbridge 1.8km d/s	Surber 0.1m^2, 0.5mm mesh, 5 random samples - *G. pulex* counts, central 2/3 alley, No NGR's, 3 min kick sweep at each site Sep07
Murdock (2007)	EA Control site, Discharge from Cress Beds 550m, 700m & u/s gauging station, Middle Eastern Arm, New Site, Ironbridge	3 min kick sweep (Mar 07, May 07)
White & Medgett (2006)	West Rivulet 200m d/s, East Rivulet 200m d/s The Island 1.1km d/s, Ironbridge 1.9km d/s	3 min kick sweep May & Oct 04, Sep 05, Mar & Nov 06, Apr & Nov 07
Marsden (2006)	East Rivulet, West Rivulet, Recirculation Channel, Beds Only channel, Downstream	Surber 883 cm2 250um mesh, 15 secs, No NGR's 16 random samples, central alley of 20m section
Marsden (2005)	East Rivulet (at outfall) West Rivulet (at outfall)	no NGR's same method used as Marsden 2006
Medgett (1998)	West Rivulet 200m d/s, East Rivulet 200m d/s The Island 1.1km d/s, Ironbridge 1.9km d/s	3 min kick sweep



Key

- Murdock (Environ UK) (2007, *11 sites*, 2008 *5 sites*)
- White & Medgett (Environment Agency) (2004-2007, *4 sites*)
- Everall & Bennett (Aquascience) (2007, *4 sites*)
- Marsden (2006, *2 sites*)

Figure 5.2-b Biological Survey Locations

There are a number of difficulties which arise with the cross comparison of survey data provided by different sources. These include the different methods used, survey location identifiers, surveyor bias and the time of year that the sample was taken. The Environment Agency data set was the largest and most complete. In order to establish whether survey data from other sources could also be included in the analyses a series of criteria was applied.

The Environment Agency three minute kick sample is an inclusive methodology intended to give a broad representation of fauna based on apportioned sampling of habitats. Macroinvertebrate identification is to family level. This methodology was used by all samplers except Marsden, who employed a Surber sampler (for 15 seconds). Surber samples give densities of organisms in discrete patches and therefore data from Marsden have not been included. The use of BMWP/ASPT biotic scores (§5.2.4) is also tailored to the use of 3 minute kick sweep sampling. Difficulties also arise when comparing site locations, which although appearing to have a similar location by description, are prone to subjective interpretation. Everall and Bennett and Marsden did not report site locations with National Grid References (Table 5.2-b) and therefore data from them have not been included in the long term data set.

In order to establish whether to include ENVIRON UK Ltd data, a comparison of samples taken from the same sites, in the same season (September and November 2007 respectively) and using the same methodology was made. The number of families identified from the West Rivulet revealed that only 52% (21 out of 40 families) were present in both samples, although an additional 14 families were identified by Environment Agency that were not present in the ENVIRON UK Ltd sample. Similarly at East Rivulet and The Island sites, only 38% of families were identified in common and the Environment Agency identified many additional families (20 out of 34 and 18 out of 32 respectively). Therefore ENVIRON UK Ltd data were not included in the long term data set.

Pre-2000 data were originally recorded by the Environment Agency in the form of abundance category and was supplied (Environment Agency, 2009) as converted to

notional counts; in most cases the geometric mean of the number of individuals (Table 5.2-c).

Table 5.2-c Category Conversion used for Environment Agency Pre-2000 Abundance Data

Abundance category	Number of individuals	Notional count (Geometric Mean for Range)
1	1	1
2	2-10	3
3	11-100	33
4	101-1000	333
5	1001-10000	3333
6	>10000	33333

Post-2000 data were originally recorded as counts. Where abundance category data was required for analyses, notional counts and individual counts were converted accordingly. It should be noted that there were no samples taken for a three to five year period as follows:

- East Rivulet, between April 1999 and June 2003,
- Ironbridge, between June 1999 and April 2002,
- West Rivulet, between June 1999 and June 2003,
- The Island, between June 1999 and January 2004.

5.2.4 Analyses

Multidimensional Scaling

Comparisons of the four Environment Agency routinely sampled sites were made at a community level using multidimensional scaling (MDS) with the statistical software Community Analysis Package v4.0 (Seaby *et al.*, 2007). The similarity between sites was assessed in relation to the time periods when changes in farm management practice had taken place to assess whether any correlation was evident.

Multidimensional scaling allowed visualisation of relative community structure by placing the most similar samples closest together. The software constructed a similarity or dissimilarity matrix between the samples and a set of coordinates in p-

dimensional space was then assigned to each sample using Principal Coordinates Analysis. The Bray-Curtis distance between the samples using the starting coordinates was calculated (Bray Curtis measures the % difference of a given distance between abundant species as contributing the same as between rare species). The original dissimilarity between the sites was compared with the Bray Curtis distances by calculating a stress function, i.e. a measure of the ability of the ordination to position similar samples together; positions were adjusted to minimise the stress (the smaller the stress function the closer the correspondence). The software used Kruskals's least squares monotonic transformation to minimise the stress and the program was designed to find an optimal two-dimensional representation of the data.

Biotic Indices

Biotic scores are commonly used as a measure of biological quality of receiving waters. The Biological Monitoring Working Party (BMWP) classification system was developed for use in national river pollution surveys and is based on benthic macroinvertebrates (Hawkes, 1997). A score is given for each family based on its pollution tolerance, ranging from 1 for pollution tolerant *Oligochaeta* to 10 for pollution sensitive families such as *Ephemerellidae* (mayfly) and *Leuctridae* (stonefly). In order to reduce the influence of sampling effort the Average Score Per Taxon (ASPT) may be calculated by dividing the BMWP Score by the number of BMWP scoring taxa (Ntaxa). A decrease in the ASPT score is indicative of organic pollution or low oxygen levels and a decrease in Ntaxa indicative of toxic pollution or habitat disruption.

Temporal changes were investigated by analysis of the long term trends using BMWP, ASPT and Ntaxa biotic scores for two sites; West and East Rivulet. In order to interpret the effect of changed in farm management practice, data from surveys undertaken from 1995 onwards were compared to baseline data from surveys undertaken between 1989 and 1995. This was prior to changes made at Lower Link Farm to improve the farm discharge quality. Improvements began with the installation of a suspended solids settlement tank in 1995 and subsequently included habitat creation at the outfall culverts and clearance of the East Rivulet channel. Further details of improvements are given in Section 4.1.3. The

comparison therefore gives an indication of the relative change in the macroinvertebrate population as a result of these changes.

Macroinvertebrate Abundance

In order to investigate more specifically the variation in macroinvertebrate assemblage at sites immediately downstream of the watercress farm, a sub-sample of families was made. The Species Diversity and Richness v4.0 (Seaby and Henderson, 2006) statistical software package was used to establish the families which were consistently found in higher numbers at both East and West Rivulet sites. Six families were identified to be used for the analyses; three pollution sensitive (high BMWP scoring) families, *Ephemerellidae* (mayfly) score 10, *Limnephillidae* (cased caddis) score 7 and *Gammaridae* (gammarids) score 6 were selected along with three pollution tolerant (low scoring BMWP scoring) families; *Glossiphonidae* (leeches) score 3, *Chironomidae* (non-biting midges) score 2 and *Oligochaeta* (true worms) score 1. The West Rivulet site was used as a control site (i.e. unaffected by factory process outfall) for comparison with the East Rivulet site below the factory outfall. The abundance category of each of these families was compared for samples taken between November 1989 and November 2008. Archive hydrological data (annual flow data for the River Test at Broadlands) (Centre for Ecology & Hydrology, 2009) were cross referenced to further investigate seasonal irregularities in macroinvertebrate populations. The long term Gammaridae count from Environment Agency samples taken at three sites (East Rivulet, The Island and Ironbridge) downstream of the watercress bed and factory discharge to the East Rivulet channel was additionally analysed.

5.3 Results

5.3.1 Multidimensional Scaling

The similarity between sites was initially related to changes in farm management practice by making a comparison between macroinvertebrate counts in samples taken prior to any change in farm management practice (1989 to1994) with all those following (1995-2009). The stress value plotted against the number of dimensions established the optimum number of dimensions to be two. A plot of stress versus number of iterations (maximum 200) showed an approximately asymptotic decline in stress with iteration number and additional iterations were not considered necessary to further minimise stress.

Figure 5.3-a shows that, prior to 1995 when water quality improvements were initiated at Lower Link Farm, samples from the East Rivulet were clustered and dissimilar to all other sites. After this date, Figure 5.3-b shows that the majority of East Rivulet samples were more similar to samples from Ironbridge and The Island. The samples most dissimilar (i.e. those which ordinate furthest from Ironbridge and The Island samples) were those taken in 1995 and 1996; the first samples taken after initial improvements to reduce the sediment load of the discharge.

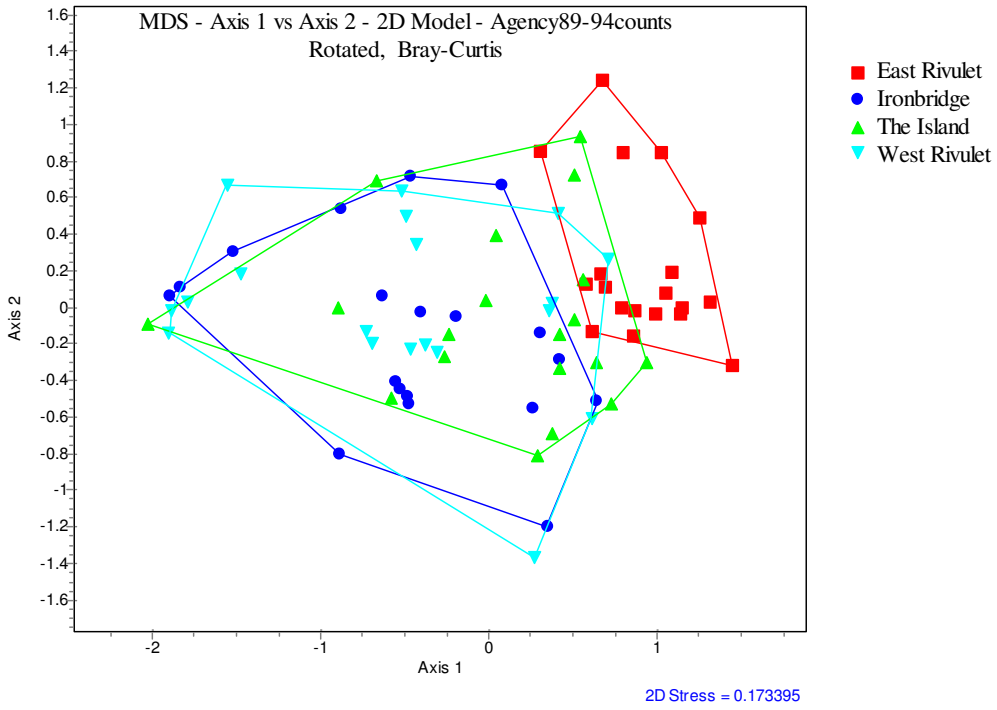


Figure 5.3-a Comparison of Macroinvertebrate Counts Before Discharge Quality Improvement (1989-1994)

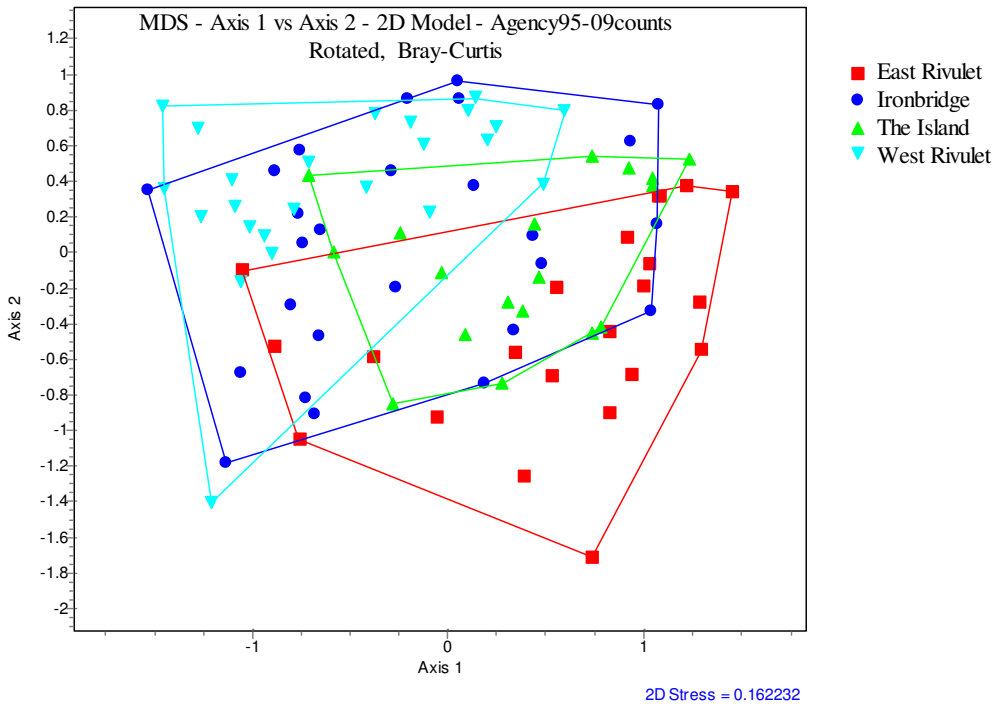


Figure 5.3-b Comparison of Macroinvertebrate Counts After Improvements to Discharge Quality (1995-2009)

The entire data set (1989-2009) was then expressed as presence or absence of families by site; East & West Rivulet, The Island and Ironbridge (refer to Figure 5.2-b for sample locations). Samples were initially separated into groups according to time periods when discharge management/quality was changed. However, due to the number of changes which occurred between April 2004 and October 2006, there were limited (or no) data sets within some of the groups and this was impractical. Subsequently, groups were chosen to represent periods of major change at the farm and these are detailed in Table 5.3-a.

Table 5.3-a Periods of Water Quality Improvement, Lower Link Farm

Period	Management changes
Pre 1995	Prior to changes to management practice
Jan 1995 – March 2004	Settlement tank installed in 1995 (this took sediment laden flow diverted from the beds during harvesting and bed washing operations). Application of zinc chloride to crops reduced and then ceased.
Apr 2004 – Oct 2006	Several significant changes were introduced during this period. A 2mm parabolic screen was installed to remove leaf debris. Two suspended solids settlement chambers were installed. Settlement tank discharge was routed back through a block of watercress beds prior to discharge. The East Rivulet was de-silted. Ammoniacal nitrogen in liquid fertiliser was reduced by 80% & subsequently ceased. A second 2 mm parabolic screen was added. Chlorine use for leaf washing was ceased. The East Rivulet discharge was de-culverted to create additional chalk stream habitat. A turbidity sensor was installed.
Post Nov 06	Wash water discharge was re-routed back through watercress beds before discharge.

Discharge to the West Rivulet would have potentially been improved by the diversion of the harvest and watercress bed wash (i.e. during watercress bed cleaning) flow to the settlement tank, the reduction and cessation of use of ammoniacal nitrogen use in fertiliser and the reduction and cessation of application of zinc chloride to crops to control crook root disease. All other improvements

would have potentially affected the discharge to the East Rivulet and The Island and Ironbridge sites downstream. MDS was carried out, with determination of stress values and number of dimensions as previously described. Figures 5.3-c to 5.3-f illustrate, for each site, the similarity of samples from the selected time periods.

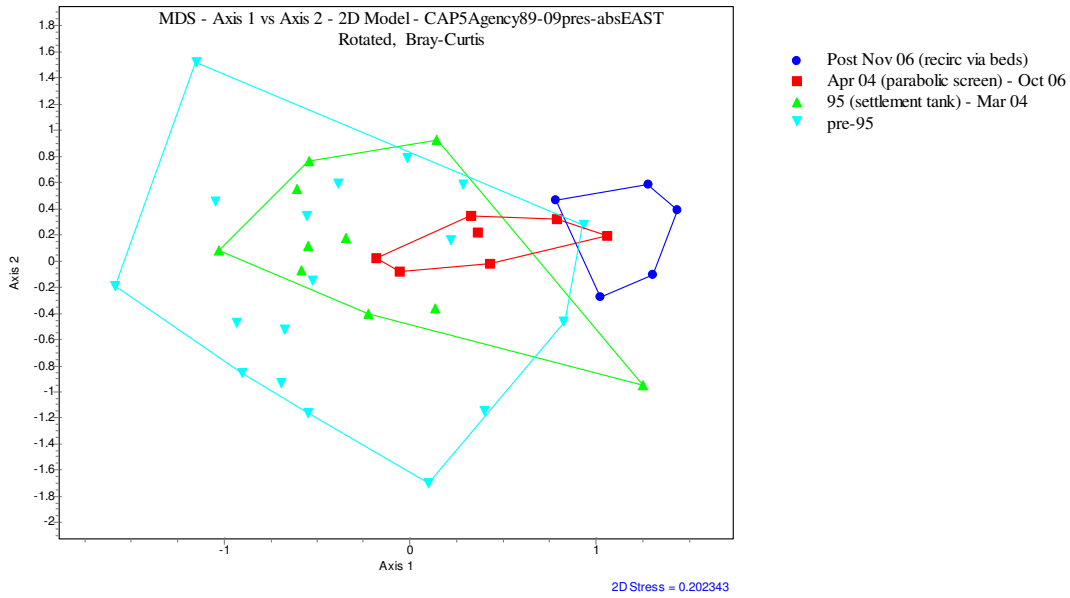


Figure 5.3-c East Rivulet (1989-2009) Macroinvertebrate Presence-Absence

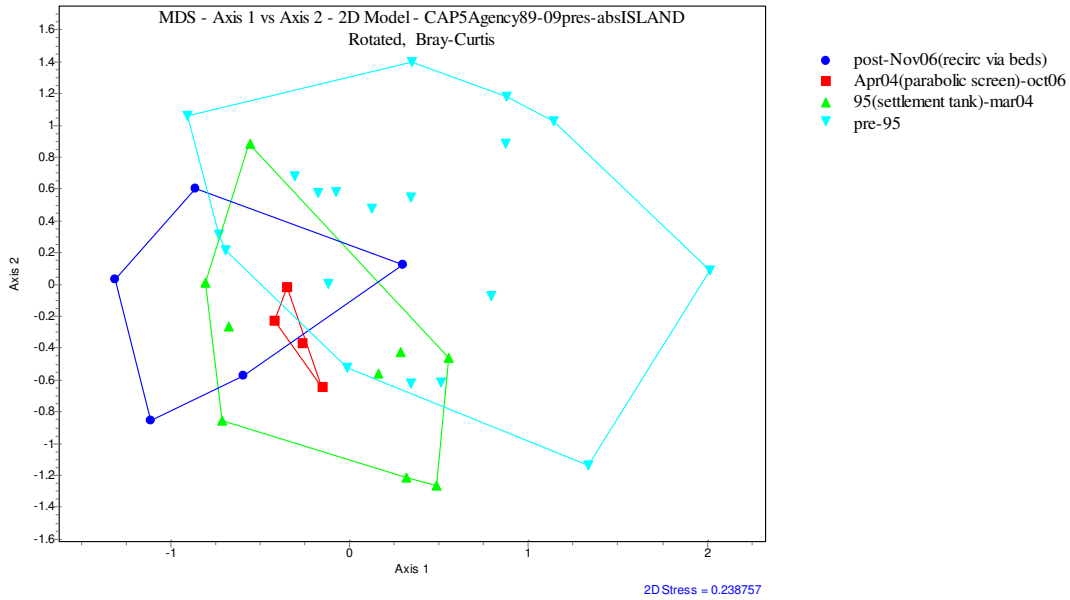


Figure 5.3-d The Island (1989-2009) Macroinvertebrate Presence-Absence

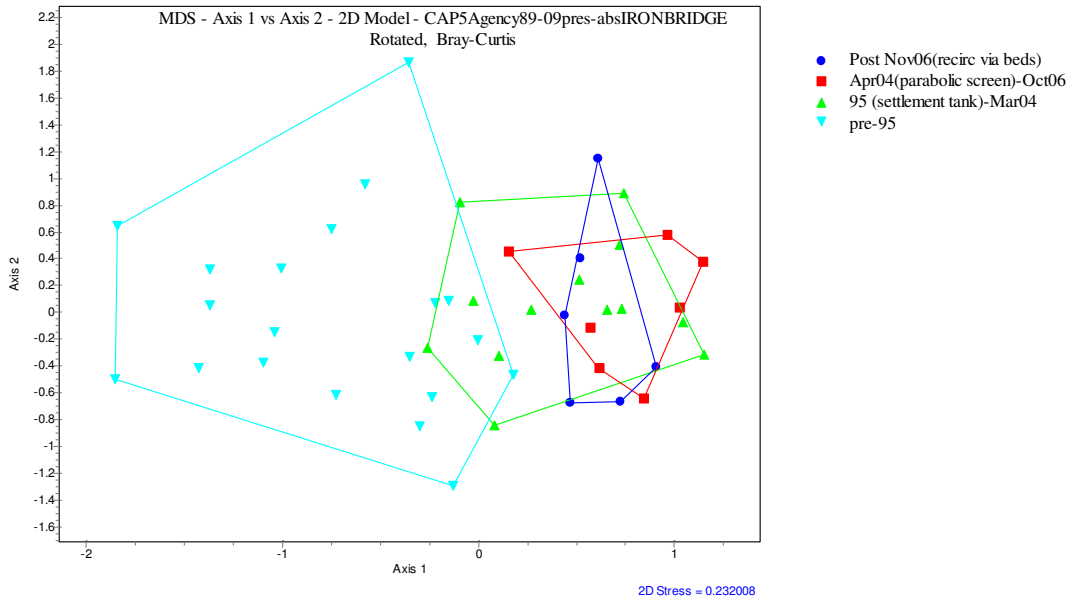


Figure 5.3-e Ironbridge (1989-2009) Macroinvertebrate Presence-Absence

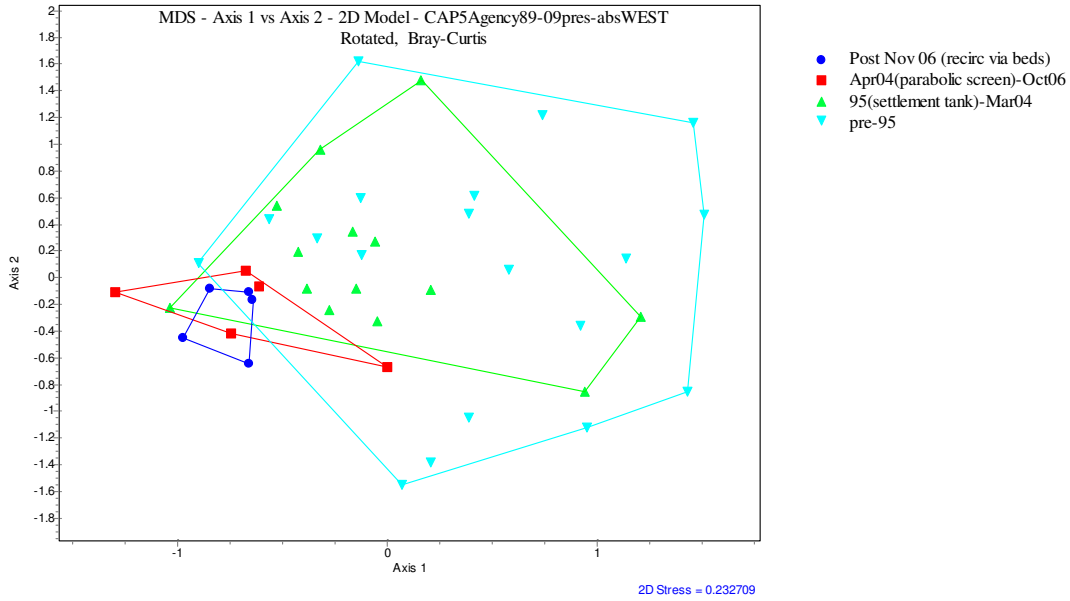


Figure 5.3-f West Rivulet (1989-2009) Macroinvertebrate Presence-Absence

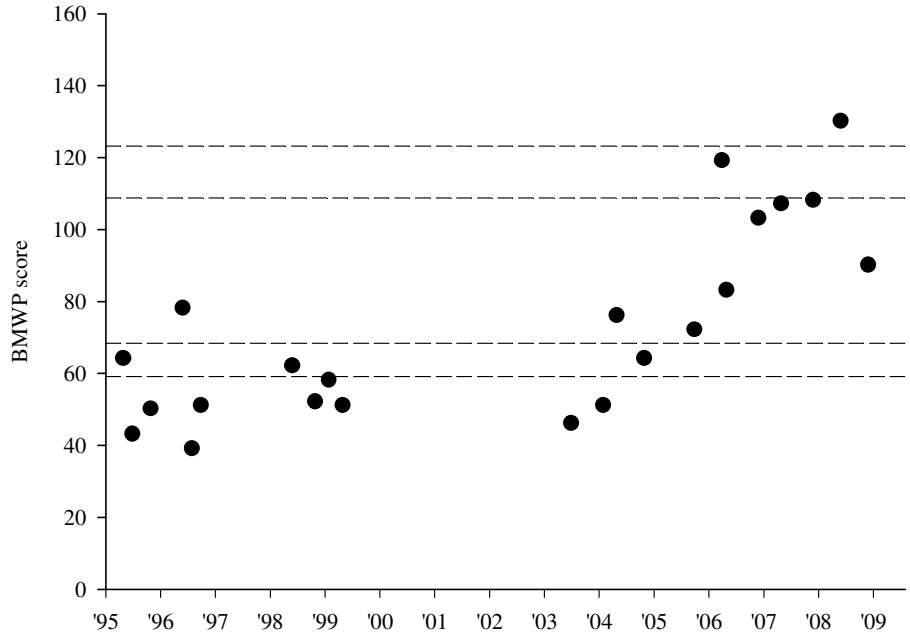
Subdivision of the temporal data based on changes in farm management practices reveals that there was no clear dissimilarity in the East Rivulet samples (Figure 5.3-c) until after the re-circulation of discharge effluent back through the watercress beds. The Island and Ironbridge sites are approximately 900m and 1.8 km downstream of the East Rivulet site, however MDS plots (Figures 5.3-d and 5.3-e) did not indicate a similar change in sample composition after recirculation of wash

water discharge. Samples from these locations show more distinct clustering for the time periods after the settlement tank was installed in 1995. Samples from the West Rivulet (Figure 5.3-f) showed clustering of similarity after the series of measures beginning in April 2004. The West Rivulet only receives discharge from a small number of watercress beds (i.e. no factory discharge). Therefore the only water quality improvement measure which would have had an effect on West Rivulet macroinvertebrate populations was the significant reduction/elimination of ammoniacal nitrogen from fertiliser which took place March 2006/2007 respectively. This concurs with the MDS analysis.

5.3.2 Biotic Scores

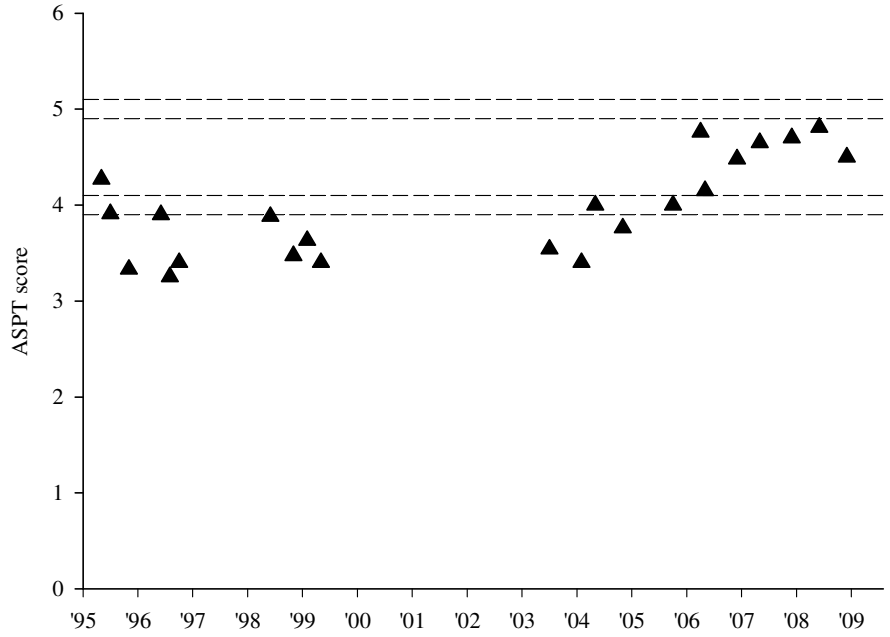
BMWP scores for samples from the East Rivulet are shown in Figure 5.3-g. The lower lines represent the mean BMWP (± SE) for East Rivulet samples (1989 to1995), prior to changes made at the farm to improve discharge water quality. Scores show an increasing trend after these changes were introduced, although between 1995 and 2004 they are mostly below the pre-1995 mean BMWP (-SE) level. The increase is most marked after 2006 when scores represent good biological quality and are analogous to the BMWP scores recorded from the control site, represented by West Rivulet mean BMWP (±SE) scores (upper lines). ASPT scores for samples from the East Rivulet are shown in Figure 5.3-h. A similar increasing trend as for BMWP scores is seen. However post-2006 ASPT scores, although approaching, have not increased to the West Rivulet (control) site levels (as represented by the mean ASPT (±SE) scores (upper lines). Ntaxa for samples from the East Rivulet were also analysed (Figure 5.3-i.). Once again, these show an increasing trend and this is more closely analogous to the BMWP scores than the ASPT scores.

In all three cases, there was no consistent increase in biotic score to levels found in the West Rivulet (represented by the mean biotic scores for 1989-1994) until after 2006 when the salad wash water recirculation system was commissioned (Table 5.3-a).



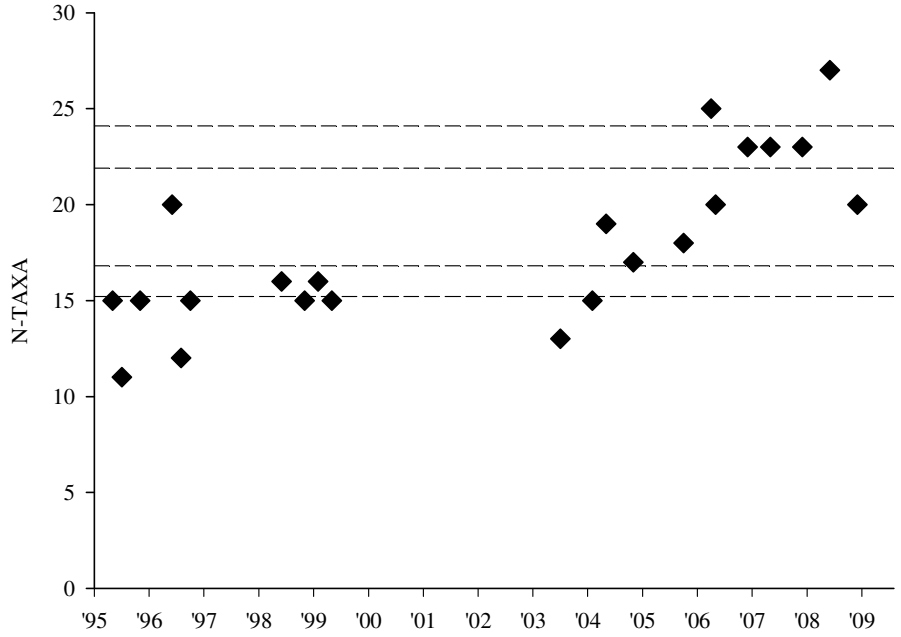
Key: Upper lines: West Rivulet Mean BMWP 1989-1995 (±SE), Lower lines: East Rivulet Mean BMWP 1989-1995 (±SE)

Figure 5.3-g East Rivulet BMWP Scores (1995-2009)



Key: Upper lines:West Rivulet Mean ASPT 1989-1995 (±SE), Lower lines: East Rivulet Mean ASPT 1989-1995 (±SE)

Figure 5.3-h East Rivulet ASPT Scores (1995-2009)



Key: Upper lines:West Rivulet Mean Ntaxa 1989-1995 (±SE), Lower lines: East Rivulet Mean Ntaxa 1989-1995 (±SE)

Figure 5.3-i East Rivulet Ntaxa (1995-2009)

The surveys carried by ENVIRON UK Ltd, although not included in the analyses, supported the Environment Agency findings. An improvement in the biological quality of the East Rivulet (BMWP, 166; ASPT, 5.12; Ntaxa, 32) and overall improvements in water quality (as measured by BMWP, ASPT and Ntaxa were found compared to surveys carried out in 2007, at sites below the confluence of the two arms of the Bourne Rivulet (Murdock, 2008a). The macroinvertebrate survey carried out in 2009 similarly reported good biological quality (East Rivulet BMWP,150; ASPT, 5.17; Ntaxa, 29) (Murdock, 2009).

Gammaridae counts for samples from the East Rivulet channel, The Island and Ironbridge are presented in Figure 5.3-j. The numbers found in the East Rivulet channel in the last four surveys (2007-2009) show a marked and consistent increase compared with previous results. The numbers of *Gammaridae* found also appear to be more consistent with other locations downstream of the watercress farm.

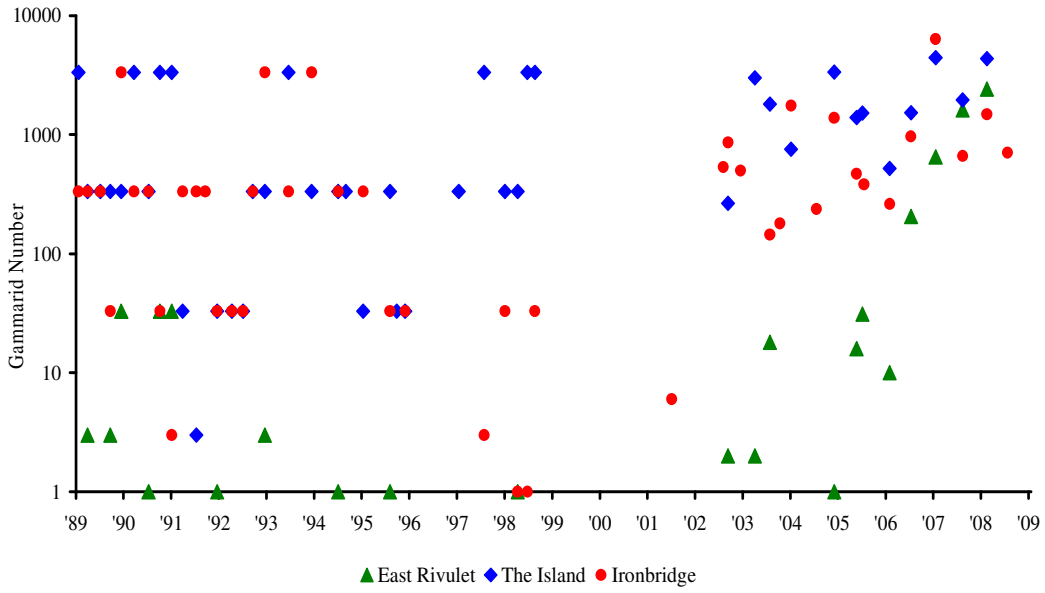


Figure 5.3-j Long Term *Gammaridae* Counts (1989-2009)

Counts (log scale) are shown for three sites downstream of Lower Link Farm; East Rivulet (green triangle), The Island (blue diamond), Ironbridge (red circle). Note: some sites were surveyed more than once per year. Pre-2000 counts were recorded as abundance category.

5.3.3 Macroinvertebrate Abundance

The macroinvertebrate abundance class of six families is shown in relation to pollution sensitivity for East Rivulet and West Rivulet samples in Figure 5.3-k and Figure 5.3-l respectively. A comparison of East and West Rivulet abundance class data showed a similar and consistent long term pattern in the pollution sensitive families with little change in the long term trend. There was however, an increase in the abundance class of each of the pollution sensitive families analysed from the most recent samples from the East Rivulet. In the most recent surveys the abundance of *Ephemerellidae* and *Gammaridae* (Spring 2008 and 2009) and *Limnephillidae* (Spring 2008 survey) were increased by 2 classes and were consistent with observations made from samples of the West Rivulet.

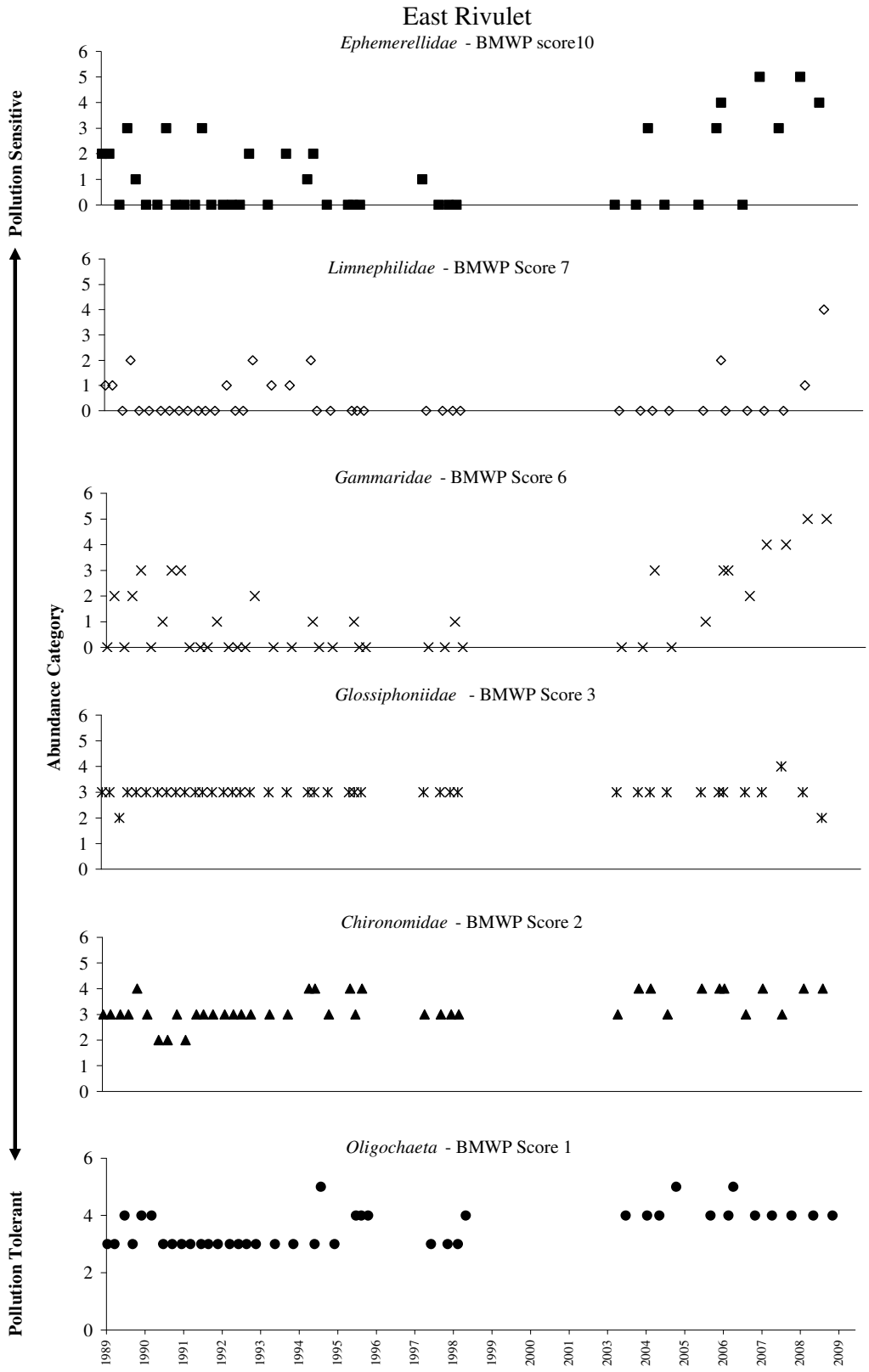


Figure 5.3-k East Rivulet (1989-2009) Macroinvertebrate Abundance

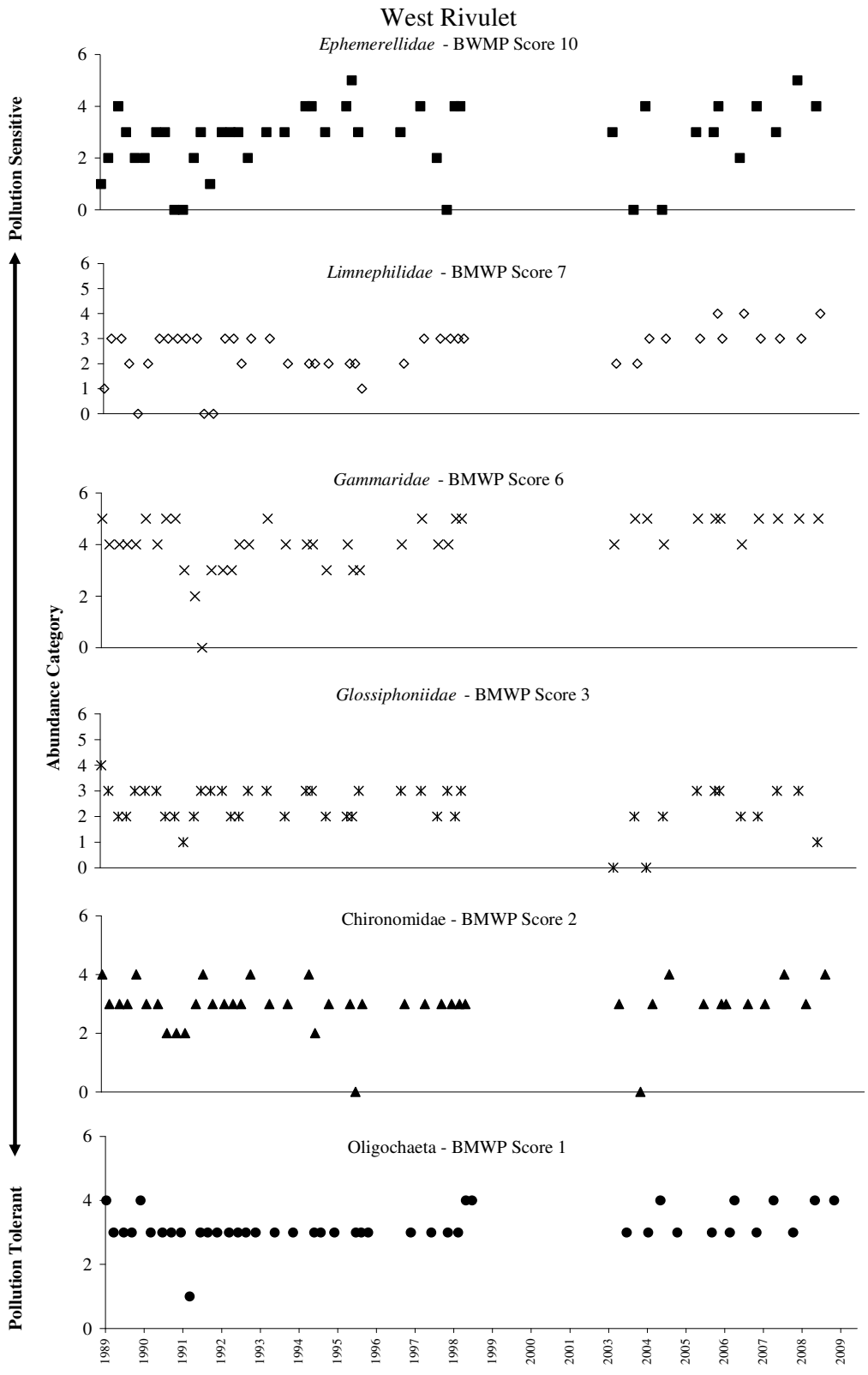


Figure 5.3-l West Rivulet (1989-2009) Macroinvertebrate Abundance

Ephemerellidae in samples from the West Rivulet showed seasonal variation due to larval development and spring emergence of many species, with Spring and Summer surveys in Class 4 and 5 and Winter surveys in Class 1 and 2. The lower class data in 1992 may be related to drought conditions and low flows experienced over the previous years. Archive hydrological data for the nearest monitoring station, the River Test at Broadlands (Centre for Ecology & Hydrology, 2009) showed an annual total flow which was 70-85% of previous records for the years 1989 to1992. In East Rivulet samples, the *Ephemerellidae* low class scores were not seasonal.

The use of abundance classes may result in boundary effects being seen. This was illustrated by the variation exhibited for *Glossiphonidae* from samples from the West Rivulet. Scrutiny of the original data was not possible for data prior to the year 2000 as records were only held in abundance class format. However, after 2000 the data showed the numbers of individuals fell very close to the cut off point between Class 2 and 3. The number of *Glossiphonidae* present from in samples from the East Rivulet surveys were consistently higher placed within Class 3 and thus a boundary effect was not seen.

5.4 Discussion

5.4.1 Assessment Methodology

The availability of the Environment Agency data set presented a unique opportunity to investigate the long term pattern of change to the macroinvertebrate community of the Bourne Rivulet. Macroinvertebrate samples had been taken annually and sometimes biannually at four sites on the Bourne Rivulet below Lower Link Watercress Farm (except for a two to four year gap, depending on site, from 2000). Through analysis it was possible to see whether changes to farm management practice, which had been put into place with the aim of improving discharge water quality, reflected in a change in the macroinvertebrate populations downstream of the watercress farm.

Due to the absence of information describing what the ideal or preferred biological communities specifically relating to chalk stream headwaters are, it was difficult to quantify improvement; there is no widely accepted benchmark or reference condition for chalk stream headwater macroinvertebrate populations to measure against. The River Invertebrate Prediction and Classification System (RIVPACS) (Wright *et al.*, 2000) which has been adopted widely within the UK to assess the biological quality of rivers and streams is not suitable. RIVPACS makes an assessment of biological quality based on a comparison with a reference condition for a particular reach. Sear *et al*. (1999) found that sites on the upper reaches of chalk streams fell into different groups according to the TWINSPAN classification used in RIVPACS used to make this comparison. Difficulties arise due to the intermittent hydraulic regime and inherent variability in community structure in headwaters, making identification of a reference condition difficult.

Instead, a temporal comparison was made, initially defined by the available Environment Agency data set (1989-2009), but also a notional before/after, impact/control approach, based on the dates when improvements were made to the discharge quality at Lower Link Farm (Table 5.3-a). The use of biotic scores provided a method of assessing differences in biological quality between-sites based

on benthic macroinvertebrates present, but not their abundance. The ASPT and BMWP being a commonly used tool to summarise the river water quality in terms of freshwater invertebrate families present (Medgett, 2008).

5.4.2 Chalk Stream Headwater Macroinvertebrate Communities

In this study, assessment of biological quality was made using family level identification and the BMWP and ASPT biotic scores. Clarke *et al.* (2008) however, found that family level identification may drastically underestimate the true diversity in headwater streams. For example, families with high levels of within-family and within-genus diversity (e.g. chironomid midges) may not be well represented in studies using low taxonomic resolution.

With reference to the typical and likely invertebrates to be found in chalk streams (Appendix F), not all the species listed by Mainstone (1999) were identified from samples taken from the Bourne Rivulet (all sample sites). It should be noted that the species list drawn up by Mainstone (1999) only refers to perennial reaches, a limited number of families and used a data set drawn from seasonal samples collected from the Test, Itchen, Frome and Hampshire Avon. The West Rivulet sample site was located at the extreme upstream reach of the perennial section of the Bourne Rivulet. Even so, only half of the species likely to occur in the upper reaches were identified and if family level identification was included this was only increased to three quarters of those anticipated by Mainstone (1999).

The taxon richness for both the East Rivulet and West Rivulet has shown an increasing trend over the twenty year monitoring period, although the overall increase in number of taxa recorded from each reach may be a result of different influences. For example, an increase in Ntaxa at the East Rivulet site may have been more influenced by removal of toxicants present in the factory wash water discharge, whereas West Rivulet populations would have only been influenced by removal of toxicants applied to the cropping beds. West Rivulet communities may have been more influenced by low flows during drought years.

The aim however, in this case, was not primarily to measure or assess biological diversity, but to provide a measure by which the West and East Rivulet sites could be compared in an assessment of their change with respect to change in farm management practice.

5.4.3 Influences on Macroinvertebrate Community

Pollutants within an ecosystem cause species to be affected in several different ways depending on their tolerance and response to the pollutant. Numbers of a particular species may decline sharply and even disappear, as seen with the Gammaridae downstream of the Lower Link Farm outfall. The species may decline but persist in lower numbers or it may increase in numbers to take advantage of the change in community dynamics. Following the cessation of input of a pollutant, the population may recover and return to its previous size and reach the equilibrium state it existed in prior to pollution (Walker, 2006). Alternatively, where physical change has taken place the population may increase to the carrying capacity of a reach. Where dredging, along with habitat creation (Cain Bio-Engineering Ltd, 2009) has taken place within the East Rivulet channel downstream of the Lower Link Farm, it is possible that the carrying capacity of this habitat would extend the numbers of some species or even present colonisation opportunities for additional taxa. The increasing taxon richness seen in the most recent samples from the East Rivulet (Figure 5.3-i) supports this. Mild pollution produces subtle changes in fauna which show differences over short distances and these can persist for some time after pollution has ceased (Hynes, 1970).

In the case of Lower Link Farm, there was a number of different pollutants which were removed from the system by changes in farm management practice throughout the twenty year monitoring period. Early improvements to the discharge quality reduced sediment input by the installation of a settlement tank. In a comparison of the macroinvertebrate counts for the East Rivulet site before and after improvement to discharge quality, samples taken soon after improvements began can be identified as most dissimilar to those at other sites (Figure 5.3-b). The East Rivulet channel is cut off from the main Bourne Rivulet channel and receives no flow from upstream; therefore there is no potential for downstream drift of recolonising species. Also, the

channel only receives flow from the watercress farm and previous accumulations of silt and the unsuitable habitat it provides for many species may have been or even continue to be removed gradually.

This also shows that the later improvements had a greater effect on macroinvertebrate abundance at this site. These improvements included removal of inputs of chlorine, ammoniacal nitrogen and a potentially significant proportion of PEITC (§ 4.4.1) from the site discharge, as well as the reduction of coarse (plant) debris. For the East Rivulet site, the potential reduction of PEITC from the discharge (by recirculation of salad wash water back through the cropping beds) was the improvement measure following which there was a clear change in the presence or absence of macroinvertebrates (Figure 5.3-c). Other improvements to the discharge quality were demonstrated by analysis of samples taken from the West Rivulet, which receives no discharge from the watercress and salad wash process. The elimination of ammoniacal fertiliser use at the farm was independent of this process. Following this, a clustering of macroinvertebrate samples (Figure 5.3-f) dissimilar to those before this event was evident. The increasing taxon richness throughout the monitoring period may also be attributable to this.

Chalk stream macroinvertebrate communities are also influenced by drought and periods of low flow. The prolonged groundwater drought experienced between 1989 and 1992 (Department of Environment, 1993) had a severe effect on macroinvertebrate assemblages in many English chalk streams, although recovery in the three years following the end of the drought was swift with recolonisation from perennial sections (Boulton, 2003). Long term predictions with reference to climate change (Department for Environment Food and Rural Affairs, 2009c) indicate that heavier winter rainfall events will occur and lower average summer rainfalls, although annual precipitation for the south east of England is predicted to remain similar (748 mm to 749 mm per annum) over the next 50 years. The maintenance of winter rainfall would ensure aquifer recharge, although this may be counteracted by increased abstraction rates from the catchment and thus greater aquifer depletion during the drier summer months. Abstraction is a primary concern for the health of the Test and Itchen catchment (Environment Agency, 2008). A decrease in summer rainfall could cause earlier or more widespread drying of winterbourne stream

sections with the associated loss of aquatic habitat. Low flows would cause a reduction in stream velocity with associated changes to the channel substrate and clean gravel beds would give way to an accumulation of silt.

The consistency of macroinvertebrate assemblages may also be influenced by mesohabitat (Armitage and Pardo, 1995). The similarity in taxonomic composition between sites on a temporal and spatial basis (using cluster analysis, ANOVA compared to RIVPACS) was assessed. The mesohabitat relationship with species assemblage was stronger than the site relationship. However, North (2009) found this not to be the case for the Bourne Rivulet. Four types of mesohabitat were recorded at sites sampled in the vicinity of Lower Link Farm; in-stream vegetation, gravel, marginal vegetation and silt. There was a lack of distinction in mesohabitat macroinvertebrate community at the sites sampled downstream of Lower Link Farm. This was chiefly attributed to its status as a headwater, where the community experiences disturbance in the form of seasonal low flows or drought and faunal compositions of habitats are considered to be more alike (Cannan 1999 in North, 2009). In fact, at unaffected sites on the Bourne Rivulet, where a significant difference in the macroinvertebrate communities of different mesohabitats would be expected (e.g. West Rivulet) there was found to be most similarity. A between-site difference in the taxon richness of mesohabitats downstream of Lower Link Farm was however evident. The highest values were reported for in-stream vegetation and followed by gravel, marginal vegetation and silt respectively.

Arbuthnott (2001) explored the temporal recolonisation dynamics of chalk stream macroinvertebrate communities and found that factors such as substrate size and availability, feeding strategy and ability to exploit available food materials, intra- and interspecific competition for space, mobility and drift from upstream and the progression of predator-prey relationships all contributed to patterns observed. *Gammarus* spp., in particular are very mobile species (Hynes, 1955) and rapid colonisers as the exponential increase in their numbers found in the East Rivulet (see Figure 5.3-j) has illustrated. The presence of increased numbers of Gammaridae, as shredders, would also benefit other species downstream by making nutrients available as they break down coarse organic particulate matter.

5.4.4 Watercress Farm Management

The situation is complex and unusual at the Lower Link Farm site, as, in addition to the watercress beds, there is a large salad processing and packing plant which discharges wash water to the East Rivulet channel. Biological surveys of the Bourne Rivulet (Marsden, 2005, 2006, White and Medgett, 2006, Medgett, 2008, Everall and Bennett, 2007, Murdock, 2007, 2008a, 2009) showed that there has been a community response to inputs to the watercourse from the watercress farm discharge, although there was a gradient of improvement in biological quality to approximately 2 km downstream.. Samples from the East Rivulet in particular, downstream of the farm discharge, showed a notable reduction or absence of Gammaridae in many cases, as well as low biotic scores and taxon richness. Elmidae and Gammaridae were absent from samples taken downstream of the watercress farm and outfall in Spring, 2004, Autumn 2005 and Spring 2006 and there were comparatively higher numbers of Asellidae, Oligochaeta and Planariidae than at other sites on the Bourne Rivulet (White and Medgett, 2006). The Test and Itchen Catchment Management Strategy (Environment Agency, 2008) also noted a continued measurable effect on macroinvertebrate communities below the watercress farm, although found an improvement in numbers of *G. pulex* and other pollution sensitive groups in samples taken from the East Rivulet, which were attributed to changes made to the farm process and practice at Lower Link Farm.

It is likely that the changes in farm management practice to make improvements to the watercress farm discharge have all resulted in changes to the macroinvertebrate fauna of the receiving water. Certainly there is documented evidence of the impact of zinc on *G. pulex* (Roddie *et al.*, 1992), the sublethal effect of pesticides on macroinvertebrates (Beketov and Liess, 2008) and sublethal stress exhibited by *Gammarus* spp. due to ammonia (Maltby *et al.*, 1990a, Prenter *et al.*, 2004).

Macroinvertebrate populations in the chalk receiving waters below watercress farms are likely to be exposed to a variety of different stressors or subject to habitat change due to the nature of the farm discharges. In the light of this study, consideration of potential effect on macroinvertebrates would need to be made on a case by case basis. Consideration of the current farm management practice, status of the

receiving water and the dilution it offers for the farm discharge. The use of macroinvertebrate monitoring did reveal that of all the improvements made to the most impacted site below Lower Link watercress farm; the East Rivulet channel, it was the removal or reduction of PEITC in the discharge which was followed by a marked improvement in macroinvertebrate abundance and diversity.

5.5 Conclusions

Chapter 3 demonstrated the lethal and sublethal effects of PEITC and watercress wash water on *G. pulex* under controlled environmental conditions. Chapter 4 has demonstrated how mitigation of ecotoxicological effects on adult *G. pulex* was measurable and possible by recirculating the farm discharge to allow it to flow back through a series of watercress cropping beds. This Chapter has explored the changes to the macroinvertebrate community of the receiving water below the Lower Link Farm discharge which have taken place in the receiving water.

As well as a marked increase in the numbers of Gammaridae present at the formerly depleted East Rivulet site, there has been an increase in abundance of other pollution sensitive families such as Limnephilidae and Ephemerellidae. Biological data have been used as a tool to confirm that changes to discharge quality are reflected by an increase in macroinvertebrate abundance and diversity.

Chalk stream macroinvertebrate communities are influenced by a complexity of anthropogenic and non-anthropogenic factors and these must be considered alongside any alterations in farm management practice. It has however, been possible to illustrate that the removal or reduction in PEITC from discharge to the East Rivulet site has had the greatest effect on the macroinvertebrate community at this site.

It is anticipated that monitoring of the macroinvertebrate populations of the Bourne Rivulet will continue, at least in the short term. It will be interesting to see whether the macroinvertebrate populations continue to change and how they change. It must be noted that the future of the macroinvertebrate communities of the Bourne Rivulet is not solely dependent on the quality of the discharge from Lower Link Farm. Water abstraction by the watercress industry is the primary resource use in the Bourne Rivulet catchment (Environment Agency, 2008) and impacts due to over-abstraction are recognised as a key issue.

6 DISCUSSION

6.1 Introduction

6.1.1 The Nature of the Problem

A link was proposed between the activities and outputs of the watercress farm and the unusual macroinvertebrate community found in the Bourne Rivulet (§ 1.4.1). This final Chapter considers what has been established by the findings of the experimental work carried out in the Chapters 2, 3 and 4 in helping to understand the nature of the problem. The ecology of the stream was thought to be affected by the naturally produced isothiocyanate PEITC. This was released at artificially elevated, levels due to the manipulation of the watercress crop and its washing and processing along with other cruciferous and non-cruciferous salads.

The present Chapter also explores the way in which change has taken place on a temporal basis. Reference is made to both the long term and short term changes to the status and management of the receiving water at the watercress farm, The Bourne Rivulet, and to the more recent changes in management of wash water at the farm. The implications for the watercress industry are examined and the potential application by other watercress farms is described.

6.1.2 Evolution of Chalk Stream Management

It is management practice which largely determines the form and function of chalk rivers in England today. They no longer exist in their 'natural' state, only as watercourses which are artificially maintained to comply with the numerous anthropogenic pressures exerted upon them (Berrie, 1992). This was applicable even a century ago when Bradley (1909) describes the fishery of the River Wiley [*sic*], a Wiltshire chalk stream. His description tells how it was possible to look into the crystal waters and watch trout or grayling above the clear gravelly bottom "an interesting spectacle only possible in the chalk streams, and, one might almost add, only in those that modern fish-culture and science have been busy with." He recognised that the river and its habitat existed in the observed state only because it was a cultivated fishery.

Ladle and Westlake (1976) describe how chalk streams unaltered by human intervention would probably have existed as a series of ill-defined channels surrounded by alder (*Alnus glutinosa*) carr and willow (*Salix* spp.) fen. However, during the late 17th and 18th Centuries most of the wet woodland was cleared, drained and the flow controlled for use as water meadows or to operate mills (Mainstone, 1999). There are currently numerous additional pressures exerted on chalk streams and rivers including those associated with effluent discharge, development and recreation (see Table 5.1-a). With reference to chalk stream headwaters in particular, the rate of abstraction and its effect on river flows is of key concern (Environment Agency in Vitacress Conservation Trust, 2009, Environment Agency, 2008). Stressors due to the effect of climate change, such as change in rainfall and therefore future flow patterns will also need to be considered.

6.1.3 Management of the Bourne Rivulet

In assessing the condition of the Bourne Rivulet prior to the start of commercial watercress farming, a useful benchmark is provided by historical maps and accounts. In maps prepared in the early 19th Century (Ordnance Survey, 1817), The Bourne Rivulet is shown as a series of small channels, drains, flood plain and marshy ground. In a series of journals from his travels around southern England, Cobbett (1830) describes the intermittent nature of the headwaters of the Bourne Rivulet at Hurstbourne Tarrant (upstream of St. Mary Bourne), which "has, in general, no water at all in it from August to March". Similarly, Stephens (1888) describes the Bourne Rivulet at St Mary Bourne (then known as the Upper Test) as an intermittent stream, but only above St. Mary Bourne, "as at about a mile and a half lower down there is some water always present." This would be in the locality of the watercress farm.

It was interesting to note that this stream was seasonally choked by vegetation. Stephens (1888) refers to summer flooding due to the prolific aquatic plant growth in the channel "which chokes up the course, causing some stagnation, and, rendering the stream more swollen than it would be from the actual supply it receives from the springs." The current management of the stream for salmonid fisheries and flood prevention now ensures summer weed clearance to maintain a fast flow and

stagnation is rarely seen. Stephens (1888) also describes the practice of "penning back of water where the brook flows through water-meadows, in order to force the herbage." The once common practice of using water meadows to provide pasture, in particular good quality early spring grazing, has long since ceased.

There was clearly no mention of watercress cultivation in these accounts and prior to the establishment of the watercress farm the Bourne Rivulet in these environs was intermittent. The Bourne Rivulet is today managed by landowners, in particular for salmonid fisheries and at St. Mary Bourne for watercress farming. The watercress farm has an effect on flow characteristics particularly during the summer months when the headwater is intermittently dry. Flow above the watercress farm discharge to the West Rivulet is intermittent at these times although the pumped borehole supply to the watercress beds sustains the Bourne Rivulet below this point. The East Rivulet flows year round, maintained in dry months by the farm discharge at its head (Figure 1.2-c).

6.2 The Source and Fate of PEITC

6.2.1 Temporal Variability

Watercress farms are a source of PEITC, both from the watercress grown and harvested from the cropping beds and that produced during any washing process on-site. There are many references relating to the production of isothiocyanates from glucosinolates by watercress, with the greatest proportion being contributed by PEITC. Gil and Macleod (1980) found that 91% of the glucosinolate degradation products from watercress leaves were contributed by PEITC.

In the late 1800s and early 1900s, anecdotal reports (The Watercress Alliance, 2009) suggest that the largest watercress grower (James & Son, which was later to be taken over by Vitacress Salads Limited) was handling in the order of 50 tonnes of watercress in a weekend harvested from beds in Surrey and Hampshire. This figure would have included watercress harvested from Lower Link Farm. Over the past half century, Lower Link Farm has been developed and has increased in size from less than half a hectare of watercress beds in 1951 to the current farm size of 18 hectares. During the course of its development, the farm has not only increased in size in terms of the number of cropping beds, but also the rate of crop production due to an increase in the intensity of cultivation. The intensive cultivation techniques used now mean that harvesting throughout the year is possible rather than just during the winter months. Therefore, the potential for year-round PEITC production by the farm has also emerged. During a two day period in July 2007, for example, 15 tonnes of watercress (31 tonnes in total of PEITC producing crops) were washed and processed at Lower Link Farm alone. This figure includes watercress harvested from all the Vitacress Salads Limited farms in Southern England which is transported to Lower Link Farm for processing, concentrating the PEITC produced during the washing process into a single location. In the UK, a total in the order of 2,000 tonnes of watercress is produced per year (Department for Environment Food and Rural Affairs, 2009a).

Temporal variability in PEITC production also exists over different timescales. The watercress (and other salad) wash schedule is operated on a daily basis, therefore, PEITC from this source will cease overnight, once the process plant completes its daily schedule. There is also an annual variation with peak watercress production in the summer months between May and July. As well as watercress, other cruciferous salad crops are also processed, for example, black cabbage, kale, mizuna, wild rocket and tatsoi (see §1.2.6). There is likely to be some compositional variability with the production of isothiocyanates other than PEITC from other cruciferous crops included in the salad mixes. During an eight week period between May and July 2008, for example, the ratio of isothiocyanate producing crops to the total weight of crops washed each day did not remain constant (CV = 26%).

6.2.2 PEITC Reaching the Bourne Rivulet

Prior to the recirculation of salad wash water back through the watercress beds before its discharge to the Bourne Rivulet, enough PEITC reached the stream to cause significant changes to the macroinvertebrate community downstream of the watercress farm. The most notable effect of the watercress farm on the Bourne Rivulet was a profound reduction in or absence of the Gammaridae population (Medgett, 1998), along with an increase in pollution tolerant macroinvertebrate taxa.

Following its production during the harvesting, manipulation and washing of the watercress crop, PEITC may be transported to the receiving water in two ways. First, even though water flow though the watercress bed is temporarily blocked during harvesting, it is possible that PEITC is subsequently washed from the remaining stubble on resumption of flow. Flow from 13 beds at Lower Link Farm (and thus also any PEITC produced) discharges directly to the West Rivulet. Flow from the remainder of the beds discharges to the East Rivulet. Secondly, PEITC produced during the factory process may be re-circulated within the factory, as water is recycled within the wash process, increasing the PEITC concentration of the wash water. Before mitigation by recirculation was put in place, the factory salad wash was discharged to the Bourne Rivulet via a parabolic screen and sedimentation tank. The change in the transport process resulted in the salad wash water being transported through watercress cropping beds prior to discharge to the East Rivulet.

During this period, the transformation, decomposition and volatile loss of PEITC could potentially occur.

Evidence from the *in situ* ecotoxicological tests carried out (Chapter 4) showed that after passing through watercress beds as a surrogate wetland system the effect of wash water on *G. pulex* was lowered. Macroinvertebrate surveys carried out at locations downstream of the watercress farm following the installation of the recirculation system showed much increased numbers of Gammaridae, in particular at the site on the East Rivulet immediately downstream of the outfall (§ 5.3.2). When these two pieces of evidence are considered in tandem we can conclude that levels of the PEITC in the watercress farm outfall are now lower than effective on *Gammarus* spp. We can also therefore infer that it is the loss of PEITC during its transport between the wash water source and the target community in the Bourne Rivulet which makes the difference, although not actually demonstrated by chemical analysis.

6.2.3 Recirculation as a Surrogate Wetland

Chapter 4 demonstrated that recirculation of the salad wash water from the processing factory via a series of watercress cropping beds prior to its discharge to the East Bourne Rivulet was an effective measure to reduce acute impact on *G. pulex*. The recirculation of wash water through the watercress beds effectively acts as a surrogate wetland, allowing dissipation of PEITC as the water flows through them. Recirculation extends the distance and time between the release of PEITC and the point at which it reaches sensitive macroinvertebrate communities in the receiving water. Additional dilution would also be provided where the factory wash water supply is supplemented by pumped borehole water.

The scale, success and role of constructed wetland treatment systems has been examined in numerous studies (Vymazal, 2005, Thullen *et al.*, 2005, Wetzel, 2001, Kassenga *et al.*, 2003, Ahn and Mitsch, 2002, Price and Probert, 1997). Reviews of the design and management of constructed wetlands (Nuttall *et al.*, 1997) and operation guidelines (Cooper, 1990) are available. Thullen (2005) describes how the treatment capabilities of the wetland are greatly affected by the water quality,

hydraulics, water temperature, soil chemistry, available oxygen, microbial communities, macroinvertebrates, and vegetation.

The most widely used concept of constructed wetlands in Europe is that with horizontal sub-surface flow (Vymazal, 2005) and this has a number of similar features as the watercress cropping bed. For example, both have an impermeable liner, a filtration medium (gravel, crushed rock), vegetation and a maintained water level in the bed. The gravel watercress bed substrate ensures high hydraulic conductivity and the watercress plants provide oxygenation. Any suspended solids would also be removed by settlement and filtration through the gravels at the top end of the bed. Loss of PEITC by its adsorption to the deposited sediments could occur. Sorption of methyl isothiocyanate to soil increases with increasing organic matter content (Smelt and Leistra, 1974 cited in Brown and Morra, 2005). Rather than clogging the gravels, these sediments are flushed out regularly as part of the routine clearing and re-planting of the watercress beds and are collected in the sedimentation tank on site.

Ahn and Mitch (2002) found an increase between the inlet and outlet temperature of large wetland features. During the summer months ambient temperatures would also increase the temperature of the salad wash water as it flows through the watercress bed. The potential increase could be from approximately 7ºC at the end of the wash process to greater than 20ºC at the bottom end of the watercress bed depending on the level of crop cover, the flow rate through the bed and the prevailing weather conditions. An increase in water temperature would act to decrease the stability of PEITC and therefore increase its rate of degradation. Ji *et al*.(2005) found that at pH 7.4, the stability of PEITC was significantly greater at 4ºC than that at room temperature (half life; 108 h and 56 h respectively). The pH of carrier water recorded *in situ* at Lower Link Farm (§ 4.3.1) was between pH 7 and pH 8 (mean value pH 7.6). Ambient winter temperatures could conversely be lower than salad wash water, although dilution with borehole water at the top of the watercress beds (a constant 11ºC), would maintain temperatures above ambient. PEITC may also be degraded by photolysis and this may be an important factor especially during summer months when peak crop and therefore most PEITC production occurs.

The *in situ* study described in Chapter 4 found that a degree of dilution of the salad wash water with pumped borehole water was evident during its progress through the watercress beds. The amount of dilution to each particular watercress bed was not consistent, depending mainly on the location of the salad wash water outlet pipe, the source of the pumped borehole water and the resultant mixing zones. Any dilution with borehole water would contribute to the decrease in effectiveness of PEITC. It should also be noted that the water flow within the beds is maintained each night, when the salad wash process is not operational, by pumped borehole supply.

A sump level controlled pump provides an even flow of salad wash water discharge to the watercress cropping beds. However, high levels of turbulence and aeration are created by the salad wash supply as it enters the above bed water carriers and the additional aeration would potentially also act to decompose PEITC. PEITC is very sensitive to oxidation and the pure standard is stored under nitrogen to prevent oxidation (Sigma-Aldrich, 2009).

Re-use of spring water within the wash lines of the wash factory, although contributing to reduction of abstracted flow, is however minimal compared to the total volume of water pumped and flowing through the beds. Approximately 5,000 gallons water per acre per hour are used for mature watercress beds and Lower Link Farm uses 40,000 gallons of water per hour for newly seeded and mature beds (Natural England, 2009). The recirculation of salad wash water would also decrease the overall demand for borehole water to be pumped to the watercress beds, albeit by a small amount. The benefits of the re-use of water are reflected primary in the improvement of health of the receiving water, rather than in a significant reduction in the abstraction rate; i.e. an improvement in the quality of the water rather than the quantity used. In general, the installation of the recirculation system; a relatively small change to the way water management took place on site, had a large impact on the reach of the Bourne Rivulet which had been affected.

The time taken for PEITC, produced by crops being washed in the factory at Lower Link farm, to reach the outfall to the East Rivulet is in the order of two to three hours (Vitacress Salads Ltd, 2010). During this time degradation of PEITC will occur. Water is abstracted and pumped to the wash lines and throughput time is

approximately 15 minutes. The wash water is then pumped from the wash lines to the parabolic screen, over the settlement beds and then to the top of the watercress beds and this takes approximately 5 minutes. The water flows through the watercress beds in an estimated 2 hours (this time will vary primarily depending on crop age with younger crops receiving slower flow). It is then carried from the beds to the outfall in approximately 15 minutes.

6.3 Impact of Watercress-derived PEITC

6.3.1 Measured Impact on *Gammarus pulex*

The studies described in Chapter 3 have established the impact of watercress wash water at lethal and sublethal levels. It is well established that glucosinolates present in cruciferous plants are the precursors of isothiocyanates (Bones and Rossiter, 1996, Fahey *et al.*, 2001, Rosa *et al.*, 1997). Benn (1977) refers to the biological properties associated with the catabolites and reminds us that "The importance of the glucosinolates resides in their disappearance [*sic*]". PEITC is the primary catabolite released from the glucosinolate gluconasturtiin in watercress (Fenwick *et al.*, 1982, Gil and MacLeod, 1980). The study described in Chapter 2 establishes that PEITC is measurable in watercress wash water.

The amounts of PEITC released into wash water, (artificially prepared using frozen watercress, although realistic based on the leaf quantities and water volumes used in the factory wash process), were enough to elicit a reaction from *G. pulex* under controlled lab conditions. This reaction could be provoked both in the adult reproductive and juvenile state. Additionally, a response to watercress leaf has been shown in feeding adults (Newman *et al.*, 1996) and Worgan (2005) suggested an avoidance response to watercress wash water. However, laboratory tests do not characterise a causal relationship which happens under complex natural regimes (Cormier *et al.*, 2008), for this purpose the use of an *in situ* approach was appropriate.

The *in situ* study described in Chapter 4 established that there was a measurable acute effect on *G. pulex* placed in salad wash water. Therefore, it was possible to see a response both with the specifically defined controlled conditions of the *ex situ* ecotoxicological tests (described in Chapter 3) and in tests taking place under 'actual' environmental conditions (described in Chapter 4). In order to extrapolate between laboratory tests and *in situ* tests, there are a number of factors should be considered but which may not be possible to quantify. For example, the route of exposure, exposure to complex mixtures, biotransformation (enhanced or decreased

toxicity), change in environmental exposure (chemical binding to solid phase), the nutritional and physiological status of the organism, multi-stress situations, variation of exposure intensity over time, indirect effects *in situ* not present in the laboratory and physiological or genetic adaption. Furthermore, a direct correspondence of the results could not easily be made as the laboratory acute tests were carried out using juvenile *G. pulex* with a 48 hour endpoint and adults were used for the *in situ* tests and a seven day endpoint. However, the experimental set-up on site at the watercress farm meant that not only were we able to expose *G. pulex in situ,* but also to salad wash water in isolation from the receiving water. It is reasonable therefore to link the results from controlled testing with PEITC and watercress wash water with those carried out in the salad wash water discharge to infer that PEITC was the causal agent in both cases.

6.3.2 Impact on the Macroinvertebrate Community

In addition to assessing the impact by ecotoxicological testing *in situ* and *ex situ*, a third means of assessment was used. The macroinvertebrate community in the receiving stream, the Bourne Rivulet, was considered. In fact, this was the starting point for concerns relating to the effect of discharging salad wash water from Lower Link Farm. There are however, uncertainties with respect to the expected reference conditions for chalk stream headwaters. Although an improvement in the macroinvertebrate community of the Bourne Rivulet has recently been recorded, it is difficult to assess completely due to the lack of benchmarking criteria available for this purpose. The River Invertebrates Prediction and Classification Scheme (RIVPACS) was developed as a tool to predict expected macroinvertebrate communities in running waters (Wright *et al.*, 1993). RIVPACS uses a large database to provide a standard against which assessment of the macroinvertebrate fauna of new sites can be made, as well as evaluation of their status within a national context. Sear *et al.* (1999) examined the position of sites along the length of chalk streams within the TWINSPAN classification used in RIVPACS to test the hypothesis that groundwater dominated rivers possess distinct faunal communities. They found that although the upper reaches of northern chalk streams mostly fell within a single TWINSPAN category, there was no consistency for southern chalk streams. There is also a lack of characteristic macroinvertebrate fauna for

headwaters described in the Chalk Rivers Biodiversity Action Plan (Environment Agency, 2004a).

In selecting a particular state for an ecosystem to be considered healthy moral, value and ethical judgements about the system are often made (Fisher 1998 in Den Besten and Munawar, 2005). Section 6.1.3 describes the change in the use management of the Bourne Rivulet over the past Century. Chalk streams, and in particular their headwaters, are now managed often with requirements from a number of different stakeholders to be met rather than to meet a set of rigorously defined reference conditions. The need for industrial water use for abstraction, agriculture, milling, private fisheries and recreational uses has to be balanced with the importance of preserving the diversity of chalk stream habitat. This may include fen meadow, wet grassland, wet woodland, as well as historical features of chalk streams such as water meadows. Unlike the downstream reaches of many larger chalk streams and rivers, their headwaters are very variable habitats, generally not protected by designation such as SSSI's and do not have rigorously defined criteria for expected macroinvertebrate community, although benchmarks are drawn for plants, fish and birds (Environment Agency, 2004a).

6.3.3 Use of Biological Assessment and Ecotoxicology

The depletion of populations of Gammaridae in the reaches of the Bourne Rivulet below the watercress farm highlights a limitation of the biological survey when used as a pollution identification tool. The symptom of a problem is shown, but not the causal factor. The use of ecotoxicological tools can fill in the important details as to the specific pollutant, their target and their mode of action. Ecotoxicological tests can be used to identify the response of a test organism to a whole effluent or a single chemical. The results from such tests can be applied to the effluent discharge and the dilution it receives by the receiving environment to estimate a safe concentration. The studies described in Chapters 3 and 4 use ecotoxicological tests to assess the effect that the farm discharge may have upon the receiving environment. Ultimately, however, an understanding of the target environment through biological assessment informs us of the biota that overall the habitat has the capacity to support, whereas

the results of effluent testing may be used to represent the overall condition of the effluent (Diamond *et al.*, 2008).

In interpreting results of toxicity tests there are a number of factors which will influence the relationship observed with biological assessment. These may include the statistical endpoint used (e.g. NOEC or EC_{25}), the quality criteria used to design the testing regime or the representative effluent dilution rate used (as opposed to the actual low flow dilution in stream). The use of the EC_{50} does not indicate environmental safety, but indicates a measure of toxicity that should be employed in a relative context. It is dependent on the conditions surrounding the toxic response (time, concentration, temperature etc.) i.e. 'under test conditions'. *In situ* and laboratory tests measure the toxicity in the water column, whereas the effects on the macroinvertebrate assemblage in the receiving water may occur because of other effluent-related causes (La Point and Waller, 2000). There may be site specific water quality effects or other indirect effects of pollutants, for example change in the intensity of trophic dynamics and functional feeding groups (Cotter, 2005). A direct evaluation of the health of the receiving water community using biological assessment techniques is needed to evaluate fully systems affected by waste water discharge.

Girling *et al.* (2000) were able to use laboratory tests to identify concentrations that were chronically toxic in similar and/or related species in mesocosms. They found that the lowest NOEC, or EC_x values were comparable with the lowest values obtained in the mesocosms. However, it can be difficult to interpret accurately the results of effluent toxicity testing. Diamond *et al.* (2008), found a lack of relationship between whole effluent toxicity and biological assessment results (possibly because frequency of effluent testing was not great enough to provide representation of the toxicity potential of the effluent).

The use of biological assessment, describing the condition and status of a chalk stream, is most suited to use as an indicator of any alteration in health of a biological community. It may also be used as an indicator of the success of any mitigation measure applied to reduce the impact of a discharge source on the chalk stream receiving water. Ecotoxicological tests can then be used to further inform, i.e. in how

the nature, presence and extent of for example, a PEITC problem arising at a watercress farm, can be established.

6.3.4 Ecotoxicological Approach

The case study of the Bourne Rivulet and Lower Link Farm was a specific problem, with a unique and complex set of variables to consider. It required the adaption of existing ecotoxicological methods to help provide information to address the questions asked. The data from each Chapter were used to supplement and support the others, i.e they were integrated and/or complementary. Existing biological data described the status of the receiving environment, supplemented by the *in situ* ecotoxicological study which, more specifically, showed that the 'untreated' factory wash water had a lethal effect on *G. pulex*. The *ex situ* study complemented the *in situ* work by describing more specifically the effect of watercress wash water on *G. pulex* juveniles and adult reproductive behaviour and showing that the same effect resulted from exposure to PEITC solution.

Burton *et al.* (2002) used different lines of evidence as part of a Weight of Evidence Approach (WOE). Several different approaches build up a more complete picture or assessment. Another example of this was the Triad approach applied by Van de Guchte (1992, in Den Besten and Munawar, 2005) which used surface water monitoring, chemical analysis and ecological survey to make a complete assessment. This approach may be more suited to situations where large amounts of data are already or readily available, although may be prohibitively costly otherwise.

A chemical approach was not suitable in this case as PEITC could not be quantified easily in aquatic samples. Furthermore, mixture effects could not be considered, there were missing or incomplete data on its environmental characteristics and its degradation products were unaccounted for (Tonkes and Balthus (1997) in Den Besten and Munawar, 2005). Chemically orientated tests could however, be used to focus on the mode of action of PEITC. In studies relating to the use of PEITC as an anticarcinogen, which have investigated and quantified the uptake of isothiocyanates, rapid cellular uptake has been demonstrated (Zhang, 2001, Chung *et al.*, 1992, Chiao *et al.*, 2004). This would concur with the sublethal response by

reproductive *G. pulex*, seen within 2 hours and their subsequent recovery. We can therefore speculate that the mode of toxic action of PEITC on *Gammarus* spp. is probably initially at a cellular level. PEITC may additionally acts separately via ingestion (Newman *et al.*, 1992) with long term exposure (exposure possibly via several pathways) leading to mortality.

It may be possible to use metabolomics to identify the mode of toxic action of PEITC on *Gammarus* spp. Metabolomics is the study of the entire composition of small molecule biochemicals (metabolites) in a given cell, tissue, biofluid or whole organism. Changes in the concentration of these metabolites can be induced by environmental changes or by environmental pollutants. It is possible to analyse a large proportion of the metabolome at once in an untargeted approach using a high resolution nuclear magnetic resonance (NMR) technique.

6.3.5 Other Sources of Environmental Impact of Watercress Farming

There is a prevalent 'belief' of more widespread impact to chalk streams due to watercress farming, based on those that have been best recorded in the Bourne Rivulet below Lower Link Farm, St. Mary Bourne (Natural England, 2009). Impacts to macroinvertebrate populations downstream of watercress farms have been noted at sites on the Pilhill Brook, River Ebble (tributary of the River Avon), Bere Stream (tributary of the River Piddle) and the River Frome and its tributaries. The role of PEITC as a causative factor for depletion of macroinvertebrate populations in watercress farm discharge streams is also highlighted (Natural England, 2009). Beside PEITC, there are a number of other potential sources of impact on chalk streams due to watercress farming. However, many of these are subject to strict control or regulation to mitigate their effects on the environment.

Regulation relating to the application of pesticides to watercress crops and use of pest and disease control measures applies to all producers of watercress. The use of any pesticide is subject to statutory regulation by DEFRA and any release to receiving waters is controlled within discharge consents set by the Environment Agency. Since water used in watercress production is discharged to rivers, few pesticides are used in its production. There are only two insecticides approved for

use on watercress cropping beds (Assured Produce, 2006), although there are additionally a number of off-label approvals for use on watercress. Off-label approvals provide for the product use in situations other than those included on the product label and are undertaken at the users risk entirely. Insecticides are approved for the control of plant damage by Chironomid midge larvae. Propamocarb hydrochloride is licensed for application to peat/compost, prior to seedling emergence, to prevent fungal attack by *Pythium* spp. and *Phytophthora* spp. Low concentrations of zinc are approved for application to the water inlets above beds to control crook root (*Spongospora subterranea f. sp. nasturtii*). There are no herbicides approved for use with watercress; weed removal by hand is the only method available.

Changes to the chemical composition and water quality of chalk streams downstream of watercress farms was first documented by Casey (1981). Nutrient enrichment is of concern, although primarily in relation to phosphates. The high levels of nitrates present in chalk aquifer water mean that its addition to crops is unnecessary, although phosphate rich fertilisers are more commonly used. The amount of nutrients added to the crop ('topping up') varies with the nutrient content and flow rate of the water. Discharges from watercress beds have been shown to cause significantly elevated phosphate (as biologically available soluble reactive phosphate) loading in the headwaters of chalk streams (Natural England, 2009) which may have undesirable consequences for growth of algal communities. Chlorinated water may be used on-site at watercress farms to wash the product, although discharges from such operations are required to be made to foul sewer or treated to neutralise the chlorine before discharge. At Lower Link Farm the use of chlorination ceased in 2006.

Low concentrations of zinc, conforming to Environment Agency requirements, may be added into the inlet water above the beds. The application of zinc is permitted to control for crook root disease. Prior to the employment of Environment Agency control measures, Casey (1994) reported that high concentrations of zinc were found in sediments and plants downstream of watercress farms where zinc applications to the crop had been made and, although not directly toxic to *G. pulex*, such sediments caused reduced feeding rates and behavioural avoidance responses. In addition to

guidance on the application of zinc, Assured Produce (2006) advises that "crop removal and bed preparation must be conducted so as to minimise suspended solid discharge to watercourses in accordance to the procedures agreed with the Environment Agency for intensive or traditional farms". Traditional farming techniques resulted in the release of large quantities of suspended sediment during bed cleaning operations, but only once a year for a relatively small period of time. With the increasing employment of intensive cultivation, more frequent bed clearing operations increased the discharge of suspended solids to the receiving water. Watercress growers are required to meet suspended solid consent conditions specified by the Environment Agency and this may require the installation of settlement facilities.

Abstraction of large quantities of chalk aquifer water remains a concern with respect to the maintenance of flows in chalk streams and headwaters in particular. The Environment Agency plan water resource management annually via a Catchment Abstraction Management Strategy (CAMS). The Bourne Rivulet is described in the Test & Itchen CAMS (Environment Agency, 2008) as being at risk of over-abstraction and twelve percent of the total licensed abstraction for the Test and Itchen catchment is for watercress cultivation. The Test & Itchen catchment is subdivided into water resource management units and abstraction due to watercress farming is 80% of the total for the unit in which the Bourne Rivulet is located. The assessment for additional abstraction licence purposes in this unit gives its status as 'no water available' to protect the over-abstracted reaches of the River Test downstream, but also to allow investigation of the causes of observed ecological stress on some reaches, for example the Bourne Rivulet (Environment Agency, 2008).

6.4 Applications to the Watercress Industry

6.4.1 Diagnosis of Problems Due to PEITC

The extent of effect on macroinvertebrate communities in chalk streams below watercress farms due to the release of PEITC is not formally known. This thesis has described the impact recorded in the Bourne Rivulet below the large scale and intensively cultivated watercress beds at Lower Link Farm. In particular, the elevated levels of PEITC released due to the presence on site of a salad washing and packaging factory have been implicated in the deterioration of the macroinvertebrate populations of the Bourne Rivulet downstream of its discharge. The mitigation of impact has been successfully achieved in this case.

In the case of smaller scale or traditionally farmed watercress cultivation operations, it is unclear whether efficiencies of scale or size are occurring. The amount of PEITC released may be lower than effective to macroinvertebrate populations in the case of small scale farming operations. Alternatively, PEITC release may be great enough to cause an effect, but this is not 'seen' as the discharge is already receiving adequate dilution by the receiving water or is treated prior to discharge. Finally, 'real' impacts may occur but are unmeasured or unreported.

An *in situ* test, using the methodology described in Chapter 4, could be used as a relatively straightforward method of assessing whether a perceived impact, due to PEITC release, on the macroinvertebrates community downstream of a watercress farm exists. A series of cages containing *G. pulex*, placed upstream and downstream of the watercress farm discharge would identify any reduction in the survival rate due to the discharge and demonstrate that release of PEITC was of cause for concern.

6.4.2 Application of Methodology

Where an effect on the survival of *G. pulex* was identified by using an *in situ* test, a case-specific assessment would be required to propose and implement a solution.

Although the National Farmers Union and the Watercress Association provide guidance protocol (Assured Produce, 2006) which their members are recommended to sign up to and abide by, there remains variability throughout the industry. Production techniques (e.g. application of fertilisers/disease control and bed clearing) vary at each farm with the prevailing conditions and the geographic location. In addition the water source may vary, for example, some growers divert part of the chalk river through the cropping beds before discharging to the main channel downstream of the farm, rather than use springs or pump borehole supply. The methods of crop washing may also differ from the unusual situation at Lower Link Farm where crops are washed in borehole water in a washing and processing factory. Harvested crops may simply be submersed briefly in a chlorine solution which is then either de-chlorinated prior to discharge to the receiving water or discharged to sewer.

The construction of an additional wetland, balancing pond, settlement lagoon or tank may not always be possible due to the additional space/land requirement and associated construction and maintenance costs. The use of an existing watercress cropping bed to recirculate discharge would require no additional land. Furthermore, eutrophication problems arising in balancing ponds or settlement lagoons do not arise when existing cropping beds are used as nutrients are used by the growing plants.

The use of recirculation as a surrogate wetland 'treatment' measure for reducing the levels of PEITC reaching the receiving water and the macroinvertebrate community would also address problems of high suspended solid levels in discharge which cause impact on macroinvertebrate communities and reduce suitable fish spawning grounds by smothering the gravels in chalk stream beds. It is also possible that the sediments act as a sink for isothiocyanates. At Lower Link Farm, silt present in re-circulated watercress wash water is deposited in the cropping beds as it flows through them and is cleared when the bed is cleared prior to replanting. Silt washed from imported crops and from watercress grown elsewhere within southern UK is also therefore prevented from reaching the receiving water. This minimises the input of silt from geologically differing regions to the local chalk stream and the deleterious effect of silt on the coarse gravels of the chalk stream bed.

Consideration of the potential increase in the rate of production of PEITC should also be made where a watercress grower proposes to change from traditional cropping techniques to intensive cultivation. Expansion of farm size, i.e. an increase in the number or area of watercress cropping beds could also result in an increase in the rate of PEITC production.

6.5 Suggestions for Further Work

6.5.1 Analysis of PEITC in Aqueous Samples

Although Chapter 2 described the identification and measurement of PEITC from watercress wash water by GC-MS techniques, there are other methods reported which could be used. For example, Section 2.2.3 describes the alternative use of High Performance Liquid Chromatography (HPLC) techniques. A cyclocondensation method which coverts volatile isothiocyanate into non-volatile dithiocarbamate to effectively measure organic isothiocyanates by proxy has also been reported (Zhang *et al.*, 1996, Zhang *et al.*, 1992). Since PEITC contributes over 80% of the isothiocyanates present in watercress (Cole, 1976), the use of this method may be a more straightforward way to monitor PEITC concentrations in watercress wash water and could be explored further. The concentration of ascorbate in watercress was found to linearly increase with plant age, similarly to PEITC (Palaniswamy *et al.*, 2003) and it is also possible that ascorbate could be used as a proxy for PEITC. Analysis of ascorbic acid is reported as relatively straightforward, using the 2,6-dichlorophenol indophenol visual titration method (Association of Official Analytical Chemists, 1995 in Palaniswamy *et al.*, 2003).

Section 6.4.1 indicates that the level of PEITC produced from watercress grown, harvested and washed at other farms is not known. It may be useful to carry out comparative analysis of wash water samples from different farms which could be used to further characterise PEITC release at watercress farms and the dilution it receives in the receiving water.

6.5.2 Biological Assessment

The assessment of two decades of biological sampling at selected sites on the Bourne Rivulet provided the opportunity to investigate how changes in management practices at the watercress farm have influenced the chalk stream macroinvertebrate communities. The continuation of biological sampling would confirm the continued

improvement of populations which have been seen following the use of watercress cropping beds to 'treat' the farm discharge.

An assessment of the availability of suitable macroinvertebrate habitat in the receiving water would also provide evidence to show that the recovery of *G. pulex* and the macroinvertebrate community can be supported. The relationship between mesohabitat and species assemblage has been shown to be stronger than that between site and species assemblage (Armitage and Cannan, 2000). However, below the Lower Link farm, North (2009) described how the macroinvertebrate community structure was influenced more strongly by site rather than by mesohabitat. Of the four mesohabitats sampled (in-stream vegetation, marginal vegetation, silt, gravel/pebble), none were specifically affected by the watercress farm discharges. Tickner (2000) also concluded that the rehabilitation of impoverished reaches should aim to improve mesohabitat diversity. North (2009) also suggested that the concreted and sedimented nature of the substrate at sites on the Bourne Rivulet immediately below Lower Link Farm may negatively affect the macroinvertebrate diversity and richness. Management to clean gravels and break up the substrate would provide additional and more diverse habitat for macroinvertebrates. In particular, this would allow species which rely on the habitat provided by gravel interstices to thrive. A low suspended solid content would ensure clear water with high light penetration to allow algal and macrophyte growth.

The habitat creation project at the head of the East Rivulet, where Lower Link Farm discharges to the Bourne Rivulet East channel, has been successful in providing additional habitat for typical chalk stream macroinvertebrate communities and native fish populations. Anecdotal reports have described native brown trout caught immediately downstream of the watercress farm outfall (Cain Bio-Engineering Ltd, 2009). Other than local angling club catch statistics and an Environment Agency fish survey following dredging of the East Rivulet (Gent, 2006), few data are available to assess fish populations of the Bourne Rivulet. Future monitoring of fish stocks would address the lack of information.

6.5.3 Phosphates

Phosphate is supplied as a supplementary nutrient to watercress crops as it is not present in high enough quantities in groundwater to produce marketable crops. Impact on macroinvertebrate populations due to phosphates is unknown and future studies to address this would provide valuable information. Evaluation of fertiliser regimes and advice on the sustainable use of phosphate fertilisers is available to watercress growers, (Agriculture and Horticulture Development Board, 2009). This, for example reports that discharge levels of total reactive phosphate into watercourses are high at bed clearing and after fertiliser application although they return to normal within 24 hours.

6.6 Concluding Remarks

Using the resources supplied by the chalk geology of southern England the watercress industry has flourished over the past two Centuries. The industry has also relatively recently (within the past two decades) diversified in terms of individual farm size, output and approach to cultivation. With continued agronomic development this is likely to develop further. This work has considered a complex issue, arising in part due to the changes taking place within the industry, with an approach comprising several different layers of investigation.

Poor macroinvertebrate communities were recorded downstream of the largest watercress farm in Europe. The circumstances at the farm were further complicated by the operation of the salad wash and processing factory on site, which also discharged to the receiving water. This work has collectively used a long term biological data set available for sites downstream of the farm, ecotoxicological testing both *in situ* and under controlled laboratory conditions and the chemical analyses for PEITC in wash water to examine a series of research hypotheses.

The hypothesis that it was possible to identify and quantify levels of PEITC from water in which watercress had been washed was examined in Chapter 2. Despite this work showing that it is possible to measure PEITC in watercress wash water, the development of a straightforward means of monitoring PEITC from samples taken from watercress farm outfalls still remains a future challenge. In the absence of this, it would be appropriate to use an *in situ* test with *Gammarus* spp. as an indicator of whether watercress bed or wash water discharge was potentially harmful with respect to PEITC.

Chapter 4 examined the hypothesis that mitigation measures, in place at the watercress farm to reduce the impact of water used in the production and processing of watercress on the receiving water, are successful. An *in situ* test at the farm with caged *G. pulex* showed that the use of watercress beds as a 'treatment' system, to

allow dissipation and dilution of isothiocyanates from watercress and other salads washed on-site, was a successful mitigation measure.

The hypotheses that isothiocyanates produced by the watercress crop have a detrimental effect on *G. pulex* and that macroinvertebrates other than *G. pulex* have been affected in the downstream community of the Bourne Rivulet were also examined. Ecotoxicological testing (Chapter 3) showed that juvenile *G. pulex* were acutely affected by watercress washwater. The EC_{50} was shown to be in the order of 2 g frozen watercress washed per litre of water. Adult reproductive pairs were also shown to have their precopular behaviour disrupted by watercress wash water (prepared using frozen watercress at a ratio of approximately 1 g leaf per litre water) during a two hour exposure. Repeated exposure to watercress wash water indicated that a sustainable population would not be possible under these conditions. The use of PEITC standards showed that the response was analogous to that of watercress wash water. Chapter 5 showed that, in addition to significant changes in Gammaridae abundance, a community response was also evident in the receiving water at sites below the watercress farm. Analysis of a long term macroinvertebrate dataset also showed community changes which reflected modifications in farm management practice.

The chalk streams and rivers of southern England are an important resource and are recognised and protected as diverse habitats. The nutritional and anti-carcinogenic benefits we gain from our consumption of watercress should be achieved without harm to the environment within which it is produced. This work has shown that this is possible and that farm management techniques sensitive to PEITC production by watercress crops can be successful with respect to this.

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Appendices

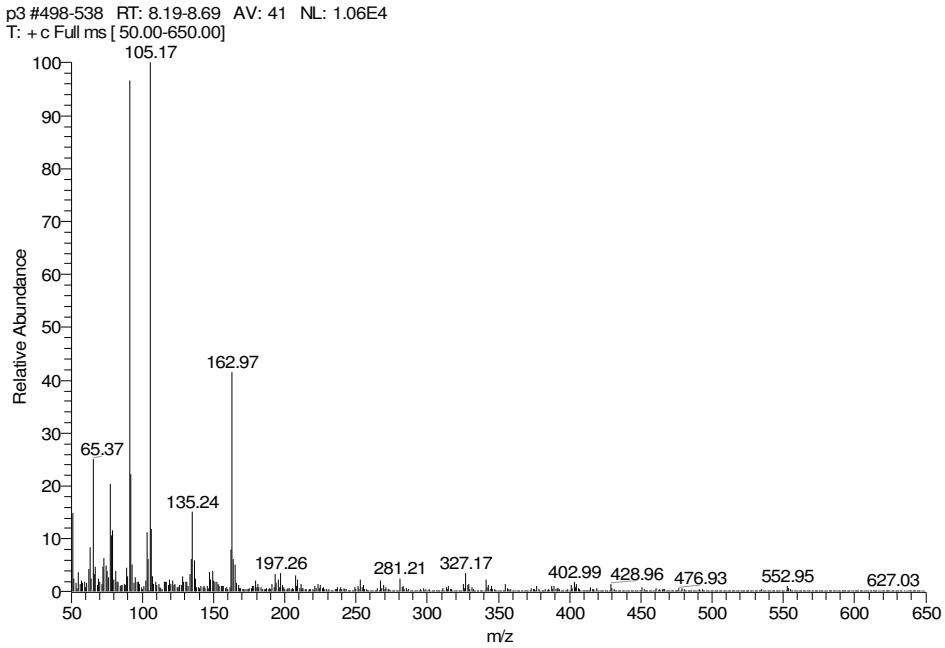
Appendix A Watercress Beds on Chalk Rivers in Southern England

Watercourse	Catchment	Watercress Bed	Grid Ref	Size(ha)
Tadnoll Brook	River Frome	Warmwell Mill	SY749873	1.31
Tadnoll Brook	River Frome	Warmwell Mill	SY749873	2.10
River Frome	River Frome	Tincleton (East)	SY767917	0.42
River Frome	River Frome	Tincleton (West)	SY766917	1.15
River Itchen	River Frome	Ilsington	SY756916	0.81
River Itchen	River Frome	Brockhill	SY837929	1.00
River Itchen	River Frome	Waddock Cross	SY795909	2.47
River Crane	Moors River	Holwell Watercress	SU074124	3.24
Bere Stream	Bere Stream	Doddings	SY852938	2.86
Bere Stream	Bere Stream	Manor Farm	SY847946	0.84
Bere Stream	Bere Stream	Holly Bush	SY839956	2.01
River Loddon	River Loddon	Black Dam, Basingstoke	SU653520	?
River Lyde	River Loddon	Huish Farm, Mapledurwell	SU672515	?
River Lyde	River Loddon	Andwell Mapledurwell	SU689522	?
River Nadder	River Avon	Ludwell Watercress	ST907225	2.02
River Ebble	River Avon	Chalke Valley Watercress	SU031252	1.62
River Wyle	River Avon	Stonewold Watercress	ST869405	3.24
River Test	River Test	Home Beds/Crane's Beds	SU444447/ SU439422	0.81
Bourne Rivulet	River Test	St. Mary Bourne	SU430489	6.88
Pilhill Brook	River Test	Abbotts Farm	SU327438	3.24
River Dever	River Test	Bullington	SU463413	0.57
River Dever	River Test	Norton	SU466409	0.42
River Arle	River Itchen	Manor Farm	SU585335	2.43
River Arle	River Itchen	Drayton	SU549333	3.77
River Arle	River Itchen	Maxwells	SU591334	1.21
River Arle	River Itchen	Bishop Sutton	SU604323	1.43
Headbourne Worthy Stream	River Itchen	Springwell	SU486322	1.58
Candover Brook	River Itchen	Fobdown	SU570338	2.43
River Arle	River Itchen	Pinglestone	SU581330	1.64

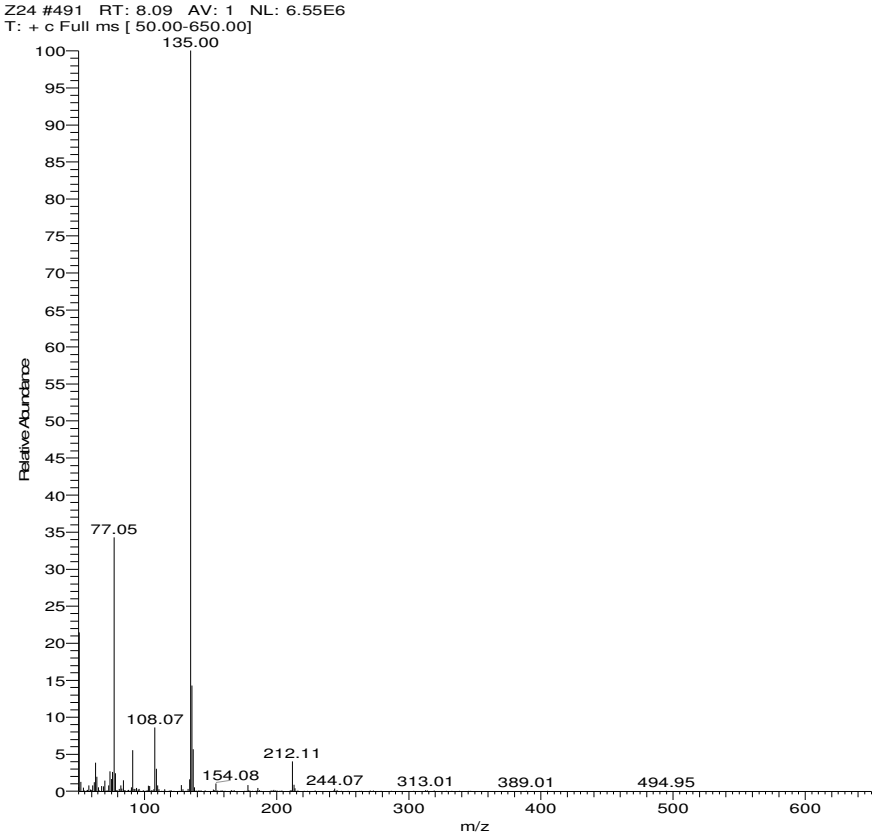
Watercourse	Catchment	Watercress Bed	Grid Ref	Size(ha)
River Itchen	River Itchen	Spring Gardens	SU577317	2.43
		Borough Farm	SU569324	
		Weir	SU587333	
		Itchen Stoke	SU554324	
River Allen	River Stour	Winbourne St Giles	SU024126	0.81
River Stour	River Stour	Spetsbury	ST908300	2.05
River Meon	River Meon	Warnford	SU621230	1.23
Sherfield Stream	R. Blackwater	Sunbeam Watercress	SU292226	?
Tilling Bourne	River Wey	Kingfisher Watercress	TQ097473	0.50?
Ham Brook	Chichester Harbour	Hairspring Watercress	SU780059	4.00

Taken from Natural England (2009)

Appendix B Example Chromatograms



Example Spectral chromatogram for PEITC



Example Spectral Chromatogram for PITC

Appendix C Trial Comparison of PEITC extracted in Methanol from Fresh and Frozen Watercress

It would be expected that the level of PEITC from fresh watercress leaves, which had not undergone complete cell lysis due to the freezing process, would be significantly less than that from frozen leaves. An attempt was made to compare the PEITC levels in fresh and frozen watercress in order that this could be related to discharge from the industrial washing process of freshly harvested watercress.

Due to the requirement for fresh leaves, a different batch of watercress was used for this study. Half of a pack of supermarket bought product (John Hurd's Traditionally Bunched Organic Watercress Class 1, source UK, unwashed) was frozen overnight and half was stored refrigerated until the following day. Samples of both the fresh and frozen leaf were weighed and 'washed' in methanol (0.5g leaf in 50 ml methanol). The leaf was washed in methanol rather than water as this procedure was quicker; the SPE phase was not required and the samples could be analysed directly using GC-MS. The watercress tissue thawed quickly during the weighing process and it was assumed that as the freezing process would have broken down the cell walls, hydrolysis had taken place (i.e. glucosinolates hydrolysed to PEITC) on thawing due to the water present within the plant cells.

The same method was employed as for leaves washed in water (i.e. the wash water was stirred once, then filtered to remove any plant matter using a 250 µm mesh). Aliquots were spiked with 5µl of the internal PITC standard (at a concentration of 0.113 g/L) and analysed for PEITC.

The mean concentration of PEITC from frozen leaf was 0.00053 g/L (n=2) and PEITC from fresh leaf was 0.00008 g/L (n=2). Therefore the concentration of PEITC washed from fresh leaves was found to be 15% of that from frozen leaves. As a direct comparison of frozen tissue extracted from water with frozen tissue extracted into methanol was not carried out this is a comparative rather than absolute measure of PEITC.

This data can however be used to relate levels of PEITC (by weight of leaf washed from frozen plant tissues) measured in this study (§ 2.5.3), to those potentially released in the factory wash water from freshly harvested plants. PEITC between 397 and 696 µg/g leaf was measured from frozen watercress leaf/stem tissues washed in water. It can therefore be estimated that 15%, i.e. 60-104 µg/g leaf would be washed from fresh plant. As watercress is washed in the factory at an approximate ratio of 10 g leaf per litre of water, it can be estimated that factory wash water will contain approximately 600-1040 µg/L PEITC.

Appendix D Summary of Mean Proportion *G. pulex* Precopular Pairs Separated during Sublethal Tests

	Proportion of total separated %								
Time (mins)	**0**	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**
Control WW 1	0	5	5	5	5	5	5	5	5
WW 1	0	5	10	20	25	43	60	75	90
Control WW 2	0	0	0	0	7	7	7	14	21
WW 2	0	0	0	3	3	15	33	50	70
Control WW 3 & WW2R	0	0	0	10	10	10	10	10	10
WW2R	0	0	15	20	30	50	60	70	75
WW 3	0	0	10	20	29	49	54	63	71
Control WW5	0	14	14	14	14	14	14	14	14
WW5	0	16	11	21	32	42	3	79	95
Control PEITC 1	0	0	8	15	23	23	23	31	31
Solvent Control PEITC 1	0	0	0	0	0	10	10	10	10
PEITC 1	0	6	25	44	81	88	94	94	100
Control PEITC 2	0	0	6	6	6	6	13	13	13
Solvent Control PEITC 2	0	0	0	0	0	7	7	7	0
PEITC 2	0	3	9	9	12	18	39	39	55
Control PEITC 3	0	8	8	8	17	33	33	33	33
Solvent Control PEITC 3	0	7	21	21	29	36	29	29	29
PEITC 3	0	3	9	12	21	50	71	79	88
Control PEITC 5	0	13	17	21	25	25	25	29	29
PEITC 5	0	27	36	50	55	64	68	77	82
Control WW5R	0	0	0	11	11	11	11	22	22
WW5R	0	9	45	55	82	82	91	100	100
Control PEITC5R	0	0	6	11	11	17	22	28	28
PEITC 5R	0	29	50	64	71	71	79	86	86
Mean Control (SE) (n=8)	0	5(2)	7(3)	9(3)	11(4)	14(4)	13(3)	15(3)	15(4)
Mean Wash water (SE) (n=4)	0	5(4)	8(3)	16(4)	22(7)	37(8)	52(7)	67(7)	81(6)
Mean PEITC (SE) (n=4)	0	10(6)	20(7)	29(11)	42(16)	55(14)	68(11)	72(12)	81(10)
WW – wash water, R – re-exposure									

Appendix E *In Situ G. pulex* Deployments - Organisms Immobile after 7 days

	Number of individuals per cage immobile after 7 days			
Test No.	**Control u/s**	**Control d/s**	**Wash water u/s**	**Wash water d/s**
Test 1(25 Jun 07)	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	2	0
	0	0	1	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
Test 2 (13 Jul 07)	0	0	2	0
	1	1	0	1
	0	0	3	0
	0	1	0	0
	0	0	3	0
	0	0	3	0
	1	0	0	0
	1	0	0	2
Test 3 (20 Jul 07)	1	0	2	0
	0	0	0	0
	2	1	0	0
	1	0	0	0
	1	0	0	0
	0	0	1	0
	0	0	3	0
	1	1	0	0
Test 4 (14 May 08)	1	0	1	0
	1	0	0	0
	0	0	1	0
	0	0	1	0
	0	0	0	0
	0	0	2	0
	0	0	1	0
	0	0	0	0
	0	0	1	0
	0	0	2	0
	0	0	0	0

	Number of individuals per cage immobile after 7 days			
Test No.	**Control u/s**	**Control d/s**	**Wash water u/s**	**Wash water d/s**
Test 4 cont…	0	0	1	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
Test 5 (4 Jun 08)	0	0	0	1
	0	1	0	0
	0	0	0	0
	0	0	0	0
	0	0	1	0
	0	0	0	0
	0	0	0	0
	0	0	0	1
	0	0	1	0
	0	0	0	*
Test 6 (11 Jun 08)	0	*	0	0
	0	0	0	0
	0	0	0	0
	0	1	0	0
	1	0	1	0
	0	1	0	0
	0	0	0	0
	0	0	0	1
	0	0	0	0
	0	0	0	1
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	ND	ND	0	ND
	ND	ND	0	ND
Test 7 (18 Jun 08)	0	0	0	0
	0	0	0	0
	0	0	0	0

	Number of individuals per cage immobile after 7 days			
Test No.	**Control u/s**	**Control d/s**	**Wash water u/s**	**Wash water d/s**
Test 7 cont…	0	0	0	0
	0	0	0	0
	0	0	1	0
	0	0	0	0
	0	1	0	0
	0	0	0	0
	0	0	1	0
	0	0	0	0
	0	0	1	0
	0	1	0	0
	0	0	1	1
	0	0	0	0
	0	0	1	0
Test 8 (25 Jun 08)	0	0	1	0
	0	0	1	0
	0	0	1	0
	0	0	2	0
	0	0	0	0
	0	0	0	1
	1	0	2	0
	0	0	1	0
	1	0	0	0
	0	0	1	0
	0	0	2	0
	1	0	1	0
	0	1	0	0
	0	0	1	1
	0	0	1	0
	0	0	2	0
Test 9 (2 Jul 08)	0	0	0	0
	1	0	1	0
	0	0	0	1
	0	0	1	0
	0	0	0	1
	1	0	0	0
	1	0	0	0

	Number of individuals per cage immobile after 7 days			
Test No.	**Control u/s**	**Control d/s**	**Wash water u/s**	**Wash water d/s**
Test 9 cont…	0	0	1	0
	0	0	0	0
	0	0	1	0
	0	1	0	0
	0	0	0	1
	1	0	0	0
	*	0	1	0
	0	0	0	0
	0	0	0	0

ND No data; cages not deployed

* * All organisms escaped through hole in mesh

Note: Three *Gammarus pulex* were deployed in each cage at the start of each test.

Appendix F Typical and likely chalk stream invertebrate species (Mainstone, 1999)

	upper, middle & lower reaches		**upper & middle reaches**	**upper reaches only**
Ancylus fluviatilus	*Baetis vernus*	*Hydropsyche siltalai*	*Pisidium milium*	*Pisidium personatum*
Anisus vortex	*Caenis luctuosa*	*Hydroptila sp.*	*Caenis rivulorum*	*Lymnaea stagnalis*
Lymnaea peregra	*Centroptilum luteolum*	*Lepidostoma hirtum*	*Oreodytes sanmarkii*	*Ecdyonurus sp.*
Physa fontinalis	*Ephemera danica*	*Limnephilus lunatus*	*Brychius elevatus*	*Rithrogena semicolorata*
Potamopyrgus jenkinsi	*Ephemerella ignata*	*Polycentropus flavomaculatus*	*Silo nigricornus*	*Habrophlebia fusca*
Pisidium nitidum	*Heptogenia sulphurea*	*Potamophylax* spp.	*Silo pallipes*	*Nemoura cambrica*
Pisidium subtruncatum	*Paraleptophlebia sumarginata*	*Psychomyia pusilla*	*Limnephius rhombicus*	*Leuctra hippopus*
Sphaerium corneum	*Isoperla grammatica*	*Phyacophila dorsalis*	*Melampophylax mucoreus*	*Leuctra nigra*
Erpobdella octoculata	*Leuctra fusca*	*Sericostoma personatum*		*Agabus sp.*
Glossiphonia complanata	*Sigara sp.*	*Simulium aureum*		*Anacaena limbata*
Helobdella stagnalis	*Elmis aenea*	*Simulium angustitarse*		*Elodes sp.*
Piscicola geometra	*Limnius volckmari*	*Simulium ornatum*		*Riolus cupreus*
Assellus aquaticus	*Orectochilus villosus*			*Plectrocnaemia geniculata*
Gammarus pulex	*Platambus maculatus*			*Hydropsyche angustipennis*
Baetis muticus	*Agapetus sp.*			*Oxyethira sp.*
Baetis niger	*Athripsodes albifrons*			*Drusus annulatus*
Baetis rhodani	*Halesus sp.*			*Simulium costatum*
Baetis scambus	*Hydropsyche pellucidula*			