# Materials and Methods

## Test System

### Study Site

### The study was carried out at the Syngenta Jealott’s Hill Research Station. For a diagram of the study site showing microcosm layout see Figure 2.1

Figure 2.1: Study site and microcosm layout (not to scale)



### Microcosm Construction

Each individual microcosm (Figure 2.2) consisted of a rectangular fibreglass tank (1.0 m in width, 4.5 m long) divided into 3 discrete sections of different depth and length. Microcosm depth and length of the ‘shallow,’ ‘medium,’ and ‘deep,’ sections were approximately 0.3 m x 2 m, 0.5 m x 1.5 m and 0.9 m x 1 m respectively. A total of 24 microcosms were available. The microcosms were sunk into the ground with the top edge at ground level, in order to buffer and stabilise the temperature in the microcosms. Turf was laid in between the microcosms. The microcosms were uniquely labelled M01 – M24 as shown in Figure 2.1.

Figure 2.2: Diagram showing microcosm dimensions and depths of water and sediment (scale approximate)

### Preparation of Test Systems

####  Initial Water and Sediment Addition

Sediment was added to a depth of approximately 10 cm to each section and water was added to give depths of approximately 10 cm (shallow section), 30 cm (medium section) and 70 cm (deep section). The length of these sections was 2 m, 1.5 m and 1 m, respectively, although the 2 m shallow section also incorporated a mud bank, which sloped from the edge to approximately half the length of the shallow section (see Figure 2.2). The approximate water volume of the shallow, medium and deep sections was 117, 450 and 700 litres respectively, giving an overall microcosm capacity of approximately 1267 litres.

Mixing and addition of sediment and water was carried out prior to sampling. Sediment was transferred by wheelbarrow (~80L capacity) from source pond 5 (See Figure 2.1) and distributed between the microcosms to give a depth of approximately 10 cm in each section. The sediment was levelled with a spade and initially sufficient water (taken from source ponds 4 and 1 (see Figure 2.1) was added to cover the sediment to protect the microcosms from frost damage. The ponds were later topped up with water, from the same source, to the required depth.

Mud banks were incorporated into the shallow sections of the microcosms, to form a gradual transition of depth. Two wheelbarrow loads (approximately 50L per load) of sediment was added to the shallow region of each microcosm, and then smoothed over with a spade to form a slope. A double layer of mesh (approximately 1 cm2 mesh size, purchased from “World of Water Aquatic Centre”, Shinfield, Reading, Berkshire) was placed on top of the slope to help hold it in place, followed by another one and a half wheelbarrow loads of sediment to finish forming the slope. When completed, the bank extended to approximately 1 m into the shallow region from the top of the microcosm edge (see Figure 2.2). Each microcosm was prepared in a similar manner to promote uniformity of experimental units.

Samples of water and sediment were sent to Natural Research Management Ltd (NRM) for analysis of parameters including selected nutrients, metals and organic compounds. These data confirmed that the sediment and water tested were suitable for use in the study.

#### Biological Establishment and Enhancement

 Much of the biota was added along with the naturalised sediment and water (described in Section 2.1.3.1). Further enhancement of the phytoplankton or periphyton communities was not considered necessary as the initial addition via the sediment and water and rate of natural colonisation was considered to provide a sufficient introduction. However, due to the short-term nature of the study, abundances of macrophytes, macroinvertebrates and zooplankton were enhanced via planting and addition. This was carried out prior to any pre-treatment sampling. Additions were made to each microcosm in a similar manner in order to promote uniformity of experimental units. After commencing biological sampling, no further additions of biota were made to the microcosms.

### Test System Monitoring and Maintenance

After completion of the microcosm set-up phase (the addition of sediment, water and biota), the systems were monitored for water level and macrophyte cover using methods described below.

#### Water level assessments

#### The water levels in the microcosms were monitored at least fortnightly throughout the study period. A piece of tape was placed on the microcosm lip, 1 m down from the bank end of the shallow section of each microcosm to mark the positioning for water depth measurements. Water depth, to the nearest centimetre, was measured at this position in the microcosms using a ruler placed in front of the tape marker.

#### Water level adjustments were made when fluctuations were +/- 5cm from the desired level, or when considered necessary. Microcosm water additions were made to the deep section using water pumped from source pond 4 (see figure 2.1), filtered through a 100 µm mesh filter. Where water needed to be removed, the water was pumped out via a 100 µm mesh filter and poured down the drain.

#### Macrophyte Assessments

#### The microcosms were set up to include a natural shallow zone providing a good cover of marginal vegetation, in addition to the deeper areas supporting lower numbers of macrophytes. The different sections aimed to provide a range of habitats and enhance the diversity of flora and fauna in the systems. Microcosms were prepared as similarly as possible.

#### A list of species present in the microcosms was prepared and the percentage cover (when viewed from above) of submerged/floating/emergent plants, open water and bare-ground was also visually assessed on these days. Assessments were made in 5% increments with any group present at <5% being scored as <5%.

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2. 1.
	2.
	3.
	4.

## Biological and Physico-Chemical Determinations

All samples were taken in a fully randomised manner. The only exception to this was the Hydrolab (used for physic-chemical determinations) where post-application measurements were done in ascending treatment order to prevent cross-contamination.

### Water Sampling for Physico-Chemical and Biological Determinations

Water samples were taken for various biological and chemical measurements. On each sampling occasion, the deep section of each microcosm was sampled using a mechanical depth integrating, trap door water column sampler (as illustrated in Figure 2.3). The sampler was lowered vertically through the water column, with the entrance flap open, until it was approximately 10 cm above the sediment. The sampler was then sealed, by pulling the lever-handle, and the entire water column then lifted vertically from the microcosm. If a large amount of filamentous algae was present, the sample was discarded and another sample taken. The sampler contents were then poured into a bucket. This was repeated until approximately 12 litres were collected in the bucket.

The combined water sample was mixed vigorously with a wooden stirrer and sub-samples were taken for the following determinations :

1. Alkalinity/Hardness and Chlorophyll *a* analysis. See Sections 2.4.2.2 and 2.4.3 respectively.
2. Phytoplankton. See Section 2.4.4.
3. Zooplankton. See Section 2.4.5.

Excess water was then returned carefully to the deep section of the sampled microcosm.

**Figure 2.3: Diagram of Water Column Sampler**



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### Physico-Chemical Measurements of the Water

#### Temperature, Dissolved Oxygen, pH, Turbidity and Conductivity

Measurements of water temperature, dissolved oxygen (DO), pH, turbidity and conductivity were carried out *in situ* on all of the study microcosms, using a “Hydrolab” H20 or YSI6820 multiparameter instrument. The probe was placed at approximately mid-depth in the deep section of the microcosm and a record of each parameter was taken, once the readings had stabilised.

#### Alkalinity and Hardness

The alkalinity and total hardness of water from each microcosm were measured. A 500 mL labelled bottle was immersed in the combined water column sample (see Section 2.4.1), and the bottle was filled and then capped. The sample was taken to the laboratory for processing and analysis, as soon as possible after sampling.

In the laboratory, 100 mL of the sample was removed and alkalinity was determined by titration with a standard hydrochloric acid (HCl) solution in the presence of bromocresol green/methyl red indicator. A further 50 mL was removed and total hardness was determined by titration against ethylenediaminetetra-acetic acid (EDTA) disodium salt in the presence of Erichrome Black indicator.

The calculations used were as follows: -

Alkalinity (as mg L-1 CaCO3) = Volume of HCl titre (mL) x Molarity of HCl x 500.5

Total hardness (as mg L-1 CaCO3) = Volume of EDTA disodium titre (mL) x 1000 Volume of water sample

### Chlorophyll *a* Analysis

Water column sampling for the analysis of chlorophyll-a was carried out. A 500 mL bottle was immersed in the combined water column sample (see Section 2.4.1), and the bottle was filled and then capped. The sample was taken to the laboratory to be processed as soon as possible for alkalinity, hardness and chlorophyll-a determinations (see Section 2.4.2 for details of alkalinity and hardness measurements). Chlorophyll-a content was determined by fluorometry.

#### Fluorometric Method

Chlorophyll *a* concentration in the microcosm water samples was determined using a bbe Algae Analyser Cuvette Fluorometer. The measurements were performed as follows:

Background adjustment – a subsample (> 25 mL) of the microcosm water sample was filtered under vacuum through a 0.45 μm filter membrane. The filtered sample was transferred to a glass cuvette, placed in the fluorometer for analysis in order to provide a background reading.

Measurement of the test sample – a subsample (> 25 mL) of well-mixed, unfiltered microcosm water was poured into a cuvette via a coarse mesh filter (approximately 0.5 mm) to eliminate large particles of e.g. filamentous algae. The sample was analysed by fluorometery, taking into account the background adjustment described above, to give total chlorophyll-a in μg/L.

### Phytoplankton

Phytoplankton samples were taken using the water column sampler, as described in Section . A 250 mL bottle was immersed in the combined water column sample (see Section 2.4.1) filled and then capped. The sample was taken to the laboratory to be processed and preserved as soon as possible by adding approximately 6 mL of Lugol’s-iodine solution to the sample bottle.

Samples were transferred to Institut fuer Gewaesserschutz or Technische Universität München for analysis. A summary of the methods used to identify and count the phytoplankton samples are as follows.

Samples were prepared for identification and enumeration according to the following method. The phytoplankton sample was shaken thoroughly and the tubular chamber was then filled with the sample water. When the settling volumes deviated from 50 ml, the desired volume was pipetted into the tubular chamber, and the remaining volume was made up with water. For sedimentation the samples were allowed to stand for at least 24 hours. The supernatant water was removed by inserting a glass disc between the tube and the base chamber (containing the sample to be counted) and excess water was taken up with cellulose.

The samples were evaluated using an inverted microscope at a magnification of 400x. A strip of the counting chamber (width 100 µm) along its diameter (2.6 cm) was counted with the aid of a counting grid. In cases where the individual count of a species (or other taxonomic level being evaluated) consistently exceeded 15 in an area measuring 100 x 100 µm, the whole strip was not counted - instead 10 quadrants measuring 100 x 100 µm were counted for this particular species/category. Care was taken to ensure that the 10 quadrants were uniformly distributed over the whole counting strip. Where this was carried out it was recorded on the data sheet. The count from these 10 quadrants was multiplied by 26 to account for the total area of the strip. Following identification and counting, the number of individuals per mL of each taxon was determined.

### Zooplankton

Zooplankton samples were taken using the water column sampler, as described in Section . A 12 L subsample of the microcosm water was removed from the composite water column sample using a plastic measuring jug, and poured through a 100 μm mesh size conical plankton net with detachable end sieve. The inside of the plankton net was washed into the end sieve with tap water from a low-pressure hose. The end sieve was then detached from the net and the contents washed into a 125 mL sample bottle with tap water from a washbottle. The amount of water used was kept to a minimum, so that the bottle was only approximately one quarter to one third full. The sample was taken to the laboratory to be processed and preserved as soon as possible by topping up the volume with industrial methylated spirit (IMS) to give approximately a 70% aqueous IMS solution. Two drops of 0.4% rose bengal stain were then added.

Samples were transferred to Institut fuer Gewaesserschutz or Technische Universität München for analysis. A summary of the methods used to identify and enumerate the zooplankton samples are as follows.

The zooplankton sample was filtered through a gauze of 63 µm mesh size. Organisms remaining on the gauze were rinsed off with water into a counting chamber along with any organisms remaining in the sample bottle. The samples were evaluated under a stereomicroscope with transmitted light illumination. The following groups were identified and counted in the zooplankton samples: Rotatoria, Cladocera, Copepoda, Insecta (Chaoboridae) and Ostracoda. All adult Crustacea (Cladocera, Copepoda and Ostracoda) were identified to species level if this was possible without extensive .preparation of the organisms. No size classifications or egg counts were made, with the exception of the Copepoda for which counts of nauplii and copepodites were included. Following identification and enumeration, the number of individuals per litre of each taxon was determined taking into consideration the sampling volume, the counted area and the total area of the counting chamber.

### Macroinvertebrates

Macroinvertebrate sampling was carried out using 3 techniques:

1. Enhanced Surface Area Substrate samplers (subsequently referred to as ‘ESAS’) – artificial colonising substrates to sample benthic and epiphytic dwellers.
2. Sweep-netting (‘NETS’) – to sample swimming and epiphytic organisms.
3. Emergence Traps (‘ET’) – to sample emergent adult insects.

During the study conducted in 2000 only techniques 1 and 2 were implemented.

Due to the small size of the test system and the resultant potential for destructive sampling of the populations, the organisms collected by ESAS and NETS were identified and enumerated live. The organisms were then returned to the originating microcosm.

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#### ESAS Sampling

Each ESAS comprised of a plastic colonising area (constructed from 14 polypropylene pall rings); weights (sealed plastic bottles containing sand); retaining mesh (1 mm) at the base (to avoid loss of macroinvertebrates from the sampler when raised through the water column), and a floating marker attached by a cord (see Figure 2.4).

Two sets of ESAS (labelled A and B), were employed during the sampling period. Each set consisted of two samplers in the shallow section and one in each of the medium and deep sections. The samplers were labelled in order to identify the microcosm number, position (shallow, medium or deep) and set (A or B) in each microcosm. They were placed in the microcosms so that the mesh was in contact with the sediment surface, away from each other and from plants where possible. Each set of samplers was left to colonise for two weeks before sampling, and was returned to the microcosms for recolonisation on completion of sample processing. ESAS sets A and B were alternated to ensure the two-week colonisation was maintained.



**Figure 2.4: Enhanced Surface Area Substrate sampler (ESAS)**

On each sampling occasion, the ESAS positioned in the medium and deep sections of the microcosms were removed and processed as a combined sample (MD). When these samples had been processed, the two samplers in the shallow (S) section were removed. The sampling procedure is detailed below:

1. The ESAS were carefully removed from the microcosm by pulling on the attached cord. They were placed in a bucket labelled with microcosm number and section (‘S’ or ‘MD’) and containing a small amount of water from the originating microcosm.
2. The organisms were then gently washed from the samplers and bucket using a low pressure hose into a large tray. Any excess plant material or debris was removed from the tray using forceps, making sure no organisms were removed from the sample. The samplers were also checked to ensure that all organisms were removed; however, where an organism could not be dislodged it was identified and recorded on the sample datasheet. The sample was concentrated by passing it through a 250-425 μm mesh sieve. It was then transferred from the sieve to a counting tray, labelled with the microcosm number and section (‘S’ or ‘MD’), by inverting the sieve and washing with water from a low-pressure hose.
3. Macroinvertebrates were identified and counted live, by transferring individuals carefully using either a plastic pipette (the end of the plastic pipette had been cut to prevent damaging the organisms) or forceps into a separate tray. Individuals left in the originating tray were counted systematically from top to bottom, left to right. Where necessary a magnifying lens or microscope was used to aid identification. Organisms were identified to species level when possible or genus/family level where organisms were too small, immature or identification of live specimens to a lower level was not practically feasible.
4. Following identification and enumeration, and after all ESAS had been sampled, organisms were returned to their microcosm and section depth. The samplers were also returned to their original positions in the microcosms after completion of sample processing.
5. Counting results were captured on hard copy data sheets and were then checked and transferred from these sheets into an electronic database.

#### Sweep Net Sampling

A single macroinvertebrate sample was taken from the shallow (S) section and a combined sample from the medium and deep (MD) section of each microcosm, using a 1 mm mesh sweep net supported by a 15 cm x 15 cm frame. For the shallow sample, the water was swept for a 30 second period incorporating as much of the section as possible, tapping the sediment and plants. The medium and deep combined sampling was split into two 15-second periods, one for each section. Netting in the medium and deep sections was carried out in continuous top to bottom sweeps, incorporating as much of the water depth and width as possible. The netting procedure was timed using a stopwatch.

The samples were transferred to sample trays (labelled with microcosm number and section) containing a little water from the appropriate microcosm. Each sample was transferred by inverting the net over the sample tray, and the water in the tray used to gently rinse the net of all organisms.

The organisms were then gently washed from the trays using a low pressure hose into a large tray. Any excess plant material or debris was removed from the tray using forceps, making sure no organisms were removed from the sample. The sample was concentrated by passing it through a 425 μm mesh sieve. Following this clean-up process, the sample was transferred from the sieve to a counting tray, labelled with the microcosm number and section (‘S’ or ‘MD’), by inverting the sieve and washing with water from a low-pressure hose.

Macroinvertebrates were identified and counted live as described for the ESAS sampling (see above) and data recorded in the same way. Following sample processing, and after completion of all net sampling, organisms were returned to their originating microcosms and depth section.

#### Emergence Trap Sampling

Emergent adult insects were sampled using emergence traps placed over the shallow section of each microcosm (see Figure 2.5 for a diagram showing the trap design).

Each trap consisted of three panels of white mesh (104 x 26 mesh per inch), and a front panel of clear, non-toxic PVC plastic with one sleeve opening for access to the interior. The bottom of the trap was open with four corner loops, which were attached to a plastic frame (145 x 60 cm) made from PVC tubing (diameter: 27mm) using cable ties.

**Figure 2.5: Emergence trap design (illustrating the main part of the trap ‘A’ and plastic frame design ‘B’)**

**‘A’ – main part of trap ‘B’ – plastic frame design**

 

The traps were positioned over the shallow section of a microcosm starting from the 1 m tape mark to 60 cm towards the shallow/medium section divide (the emergence trap frame being 60cm wide). Pegs were placed in the ground next to the microcosm to attach the frame securely, and to prevent the trap blowing away. When not in use the traps were placed away from the study microcosms.

The traps were placed over the microcosms to collect emergent insects for a minimum of one night every two weeks during the study period. The frequency of sampling depended on weather conditions and sample size. Traps were positioned late afternoon for collection the following morning. In the morning the emergence trap of a microcosm would be carefully lifted and emergent insects collected using a battery-powered pooter to carefully suck up the adult insects. The fly sample was then carefully transferred into a labelled bag partially filled with air to avoid sample damage. Large organisms, such as Anisoptera and Zygoptera, were identified live at the study site and then allowed to fly away. These taxa counts were recorded on the activity record for that day.

All the sample bags were taken to the laboratory for transfer to glass vials. Transfer was aided by placing the samples in the freezer for approximately 30 minutes to temporarily stun the flies. After this time the bags were removed from the freezer and the fly sample transferred, by pooter, from the plastic bag into a pre-labelled vial containing 70% IMS.

Identification of these samples was carried out to family with further identification of selected samples to produce a species list.