Assessment of the Efficacy of BTI Larvicide, VectoBac 12AS® (BTI AM65-52) Against "Nuisance" Flies at Water Treatment Plants in North West England.

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Abbreviations and keywords

Biocide: a chemical designed to kill, deter or render harmless or induce a controlling effect on harmful organisms.

Biological/percolating/sprinkling/trickling filter: Interchangeably used to refer to bacteria beds used for secondary/biological treatment of sewage.

Biotoxin: A poisonous substance of biological origin.

BT: *Bacillus thuringiensis*

BTI: *Bacillus thuringiensis* var. *israelensis*

EA: Environmental Agency

Entomopathogen: an organism that is parasitic/infectious or toxic to insects

IGR: Insect growth regulator.

JHA: Juvenile hormone analogue.

Larvicide: also referred to as larvacide, is a larvae-targeting insecticide.

NTOs: Non-target organisms.

VectoBac 12AS® strain AM65-52 (BTI AM65-52): The commercial name of the biocidal product or BTI-derived larvicide formulation under investigation.

WWTW: Waste Water Treatment Works

Abstract

In treating wastewater using percolating filters, nature employs diverse biota, which includes nematoceran larvae, to sustain filter function. This study sought to generate evidence on the efficacy of a *Bacillus thuringiensis* var. *israelensis*-derived larvicide widely used in England for the control of adult flies that emerge. This was in part-fulfilment of the European Union's new biocidal products licencing requirements.

Adult and larval fly counts of four species of "nuisance flies", *Limnophyes minimus, Metriocnemus eurynotus, Psychoda alternata* and *Sylvicola fenestralis* were monitored at two wastewater treatment works (WWTW) in North West of England. At selected points relative to peak fly presence, selected filters were treated with the larvicide at concentrations of 80mg/L (low dose), 160 mg/L (medium dose) or 230 mg/L (high dose). Fly reduction and effectiveness of different doses were determined based on pre- and post-treatment counts.

A single treatment against *S. fenestralis* using the medium (recommended) dose achieved 100% larval and adult fly reductions within two and fourteen days respectively. Two treatments against *P. alternata* at the low (recommended) dose achieved 93% and 95% larval and adult reductions overall, 48 hours and 23 days after the second treatment respectively. Forty-eight hours and one month after second treatment, larval and adult *L. minimus* respectively showed 100% and 30% overall reductions at low dose, 100% and 91% at medium (recommended) dose and, 100% and 90% at high dose. At similar post-treatment points, larval and adult *M. eurynotus* showed 100% and 94% overall reductions respectively at low dose, 97% and 99% at medium (recommended) dose and 100% across both larvae and adults at high dose.

The biolarvicide showed no effect against non-target organisms but was harmful and effective against target-species. Reduced and increased treatment strengths trialled against chironomid species showed slightly reduced efficacy and no added benefit respectively.

Chapter 1. Introduction

1.1 General overview

Sewage or wastewater is a combination of water (99.9%) and solid material (0.1%) (Gray, 2005) in terms of volume. The solid components, "faeces, food particles, grease, oils, soap, salts, detergents, plastics, salts, metals, sand and grit" (Gray, 2005) are made up of 70% organic material like nutrients. Although wastewater covers a wide spectrum of polluted water, the major cause of concern to environmental authorities is organic waste originating from domestic, commercial, agricultural and industrial processes (Gray, 2005).

Wastewater treatment primarily aims to reduce organic components of sewage into manageable sludge (Gray 2005). Through regulatory compliance (Gray, 2010), wastewater treatment achieves separate final sludge and water with qualities of no adverse effects of pollution on ecology and public health including nuisance or offence.

Wastewater treatment comes with undesirable elements like odour (Rudolfs *et al*. 1950); Ofwat, 2016) and fly nuisance and, to a much lesser extent in the UK, risk of sewer rat-transmitted leptospirosis or Weil's disease (Chan *et al*., 1987) to WWTW staff. The control of nuisance flies using *Bacillus thuringiensis* israelensis (BTI)-derived larvicide, VectoBac 12AS® strain AM 65-62 (BTI AM65-52) is at the core of this study. Throughout the text, this product is interchangeably referred to by its full name or simply as VectoBac®. The management/regulation, history, rationale and method of wastewater treatment are also explored.

1.2 Water industry: companies and regulators

There are 17 licenced water supply companies in England and Wales (Office of Water Services, 2016). Ten of these manage both drinking water and wastewater with nine of them operating in England alone (Environment Agency, 2016). The major water companies in North of England are United Utilities, Yorkshire Water and Northumbrian Water servicing North East, North West and Northumbria regions respectively. These water companies are accountable to a number of regulatory bodies to protect the environment and the public.

The government department in charge of water policy and regulations in England and Wales is the Department for Environment Food and Rural Affairs (Defra) (2017). Based on UK and EU laws, they set quality of drinking water and environmental standards to be met by water and sewerage firms (Defra, 2017). The Environment Agency (EA), a public body accountable to government through Defra is one of the regulators of the water industry. Its main focus is on minimising the negative impact of water companies' operations on the environment (EA, 2016).

The UK Government's Office of Water Services (Ofwat) is the 'The Water Services Regulation Authority' (Ofwat, 2016). It is the water supply licensing authority and the industry economic regulator. Over and above setting various regulations protecting consumers on water rates and access to information, Ofwat also offer "financial and reputational incentives" to water and sewerage firms who do well in regulatory compliance through their "service incentive mechanism" (SIM) (Ofwat, 2015).

Another arm of Government involved in water industry regulation in England and Wales is the Drinking Water Inspectorate (2017) who enforce drinking water quality and standards. Consumer interests like tariffs, service and value for money are taken care of through their membership in The Consumer Council for Water (2017). There is also Water UK (2017) composed of all licensed water and sewerage services providers in the UK working together on industry policy, for example, on markets and legislation. This also serves as a platform for exchanging notes on best practice and research on areas of common interest.

The synergy of all these organisations and regulatory bodies provide "an integrated approach" (EA, 2016) in the running of the industry for an outcome beneficial to all parties

1.3 Advent of wastewater treatment: environmental and public health

Thomson (1921) provided one of the earliest historical accounts of sewage disposal and wastewater treatment. It is explained that practices gradually shifted from natural sewage disposal methods of applying raw sewage into the soil (for consumption and breakdown by animals and plants). This was followed by sewage "disposal … by dilution" which involved discharge into streams and cesspools "with and … without preliminary treatment" (Hommon *et al.,* 1920). In fact, chemical treatment of sewage before disposal into streams came when rising urban populations made earlier practices environmentally unsustainable (Fig 1). The effect of turbidity and bacteria load of the improved effluent soon gave rise to the need and development of effluent filtering (Thomson, 1921). With sewage filtering setting in, wastewater treatment evolved and keeps improving.

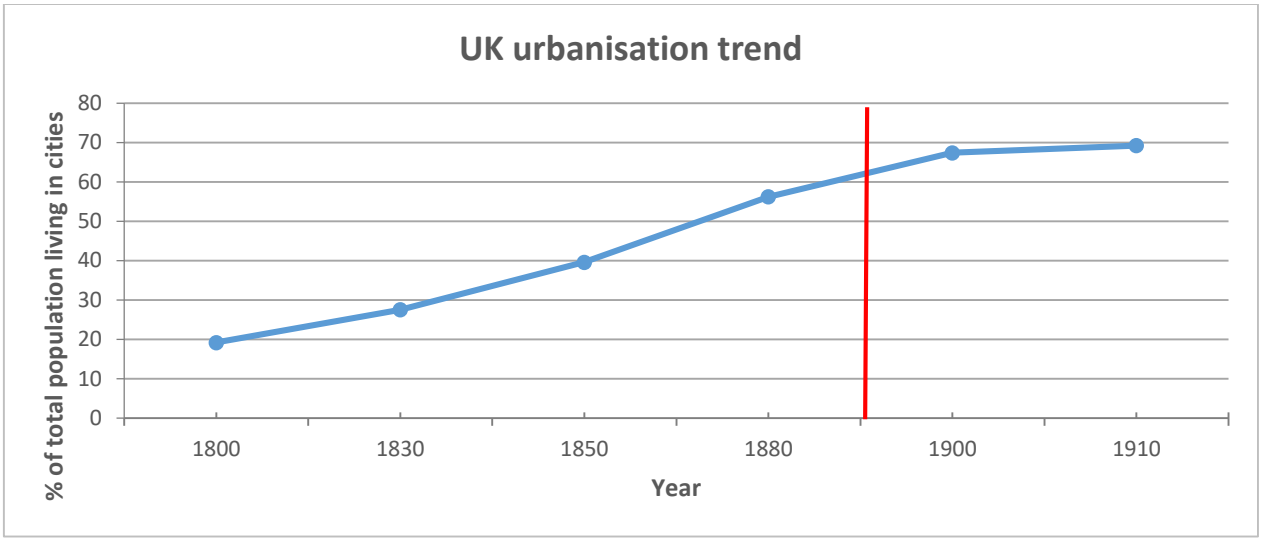


Fig. 1: UK level of urbanisation at the beginnings of the 19th and 20th centuries based on historical data from Bairoch and Goertz (1986). Red line gives a rough indication of the beginning of centralised sewage sprinkling filters.

A quick glimpse through history shows that water and sanitation were recognised as important aspects of public health since the Roman times. However, wastewater treatment was adopted just over one and half centuries ago in Britain as urbanisation (Bairoch and Goertz, 1986) brought challenges that included diseases linked to the working and living conditions of factory workers. A good example was the cholera epidemic of 1831 and 1832 (Fee and Brown, 2005) which made the UK government recognise the link between disease burden and working/living conditions of people in industrialised cities. At this time and over the next one and half decades, the Poor Laws were revisited and reviewed several times to alleviate poverty and suffering whilst also safeguarding the prosperity of the rich (Report of the House of Commons Select Committee on the Health of towns, 1840, cited in Fee and Brown, 2005).

As another epidemic of cholera swept westwards across Europe in 1848 (Fee and Brown, 2005), clean water supply and sewage disposal were tackled through the introduction of the Public Health

Act of 1848 (UK Parliament, 2016). This made the state guarantor of health and environmental standards (Fee and Brown, 2005) by setting up proactive structures to achieve set standards. The Act was amended in 1875 (Fee and Brown, 2005) to set up and bring uniformity across rural and urban sanitary districts. This separation of sewage treatment into two stages seems to have first been suggested by an English sanitary engineer, Scott-Moncrieff when, in 1891, he built a closed tank where anaerobic putrefaction was to take place with a series of trays containing coke for the second stage of nitrification (Metcalf and Eddy, 1916). Fly control through safe sewage disposal was shown to significantly reduce diarrheal diseases in both moderate (Lindsay *et al.,* 1953) and high (Watt and Lindsay, 1948) morbidity areas. Wastewater treatment and clean water supply also directly reduce transmission of faecal-oral pathogens like**,** *Escherichia coli, Campylobacter, Salmonella, Shigella,* Norwalk virus, hepatitis A virus, protozoa e.g. *Cryptosporidium and Giardia* (Rose *et al.,* 2000*).*

With environmental health concerned with "health problems relating to man's need for and use of air, water, food, and shelter" (Hollis, 1951), ecosystem and human health laws primarily ensure that mankind have a safe environment to work, live and play in their different settings. Improving and sustaining public health practices was and/or is always done with "complementary ecological focus" (O'Connor, 2016) to minimise anthropogenic pressures (Lencioni *et al.,* 2012) on the environment. Over and above, reducing diseases (McCabe and Haines, 1957) in humans, organised sewage disposal and wastewater treatment also provides for sustainable conservation of "endangered biota" (Marina *et al.,* 2014) including marine life. The high biological oxygen demand (Gu *et al.,* 2013) in sewage inevitably depletes vital dissolved oxygen profile (Fan and Wan, 2008) of rivers and their aquatic life (Manahan, 2000, cited in Simoes *et al.,* 2008).

This would explain why companies licenced to provide water and sewerage services in the country operate under a multifaceted regulatory regime outlined earlier.

1.4 General outline of wastewater treatment

Biological treatment of sewage is done using fixed film reactors, activated sludge and natural systems (Gray, 2005). In England, use of fixed film reactors in the form of percolating sewage filters (Hawkes, 1963) has lasted the longest (Van Poppelen, 1998). These are also referred to as trickling or sprinkling filters (Hommon *et al.,* 1920) or simply, bacteria beds. A bacteria bed for municipal wastewater treatment is a tank of different sizes and shapes which is usually filled with inorganic material, normally small pieces of rock or clinker. When fully operational, the filter bed becomes an artificial semiaquatic habitat of diverse and vital micro- and macro-biota (Gray, 2005). Therefore, nature provides and employs different biota for these facilities to fulfil their purpose.

Sewage or wastewater treatment process (Fig. 2) is a combination of physical and biological phenomena (Agersborg and Hatfield, 1929) involving screening, sedimentation, coagulation, reduction and oxidation (Dighe *et al.,* 2015) of organic and inorganic matter. The process was originally restricted to pathogen removal and discharging effluent with acceptable oxygen level (Akhtar and Ghaffar, 1986). The process now has 5 basic functional stages – preliminary, primary, secondary, tertiary and sludge treatment stages (Gray, 2010) with sedimentation mostly achieved at the second stage but continues downstream of the process.

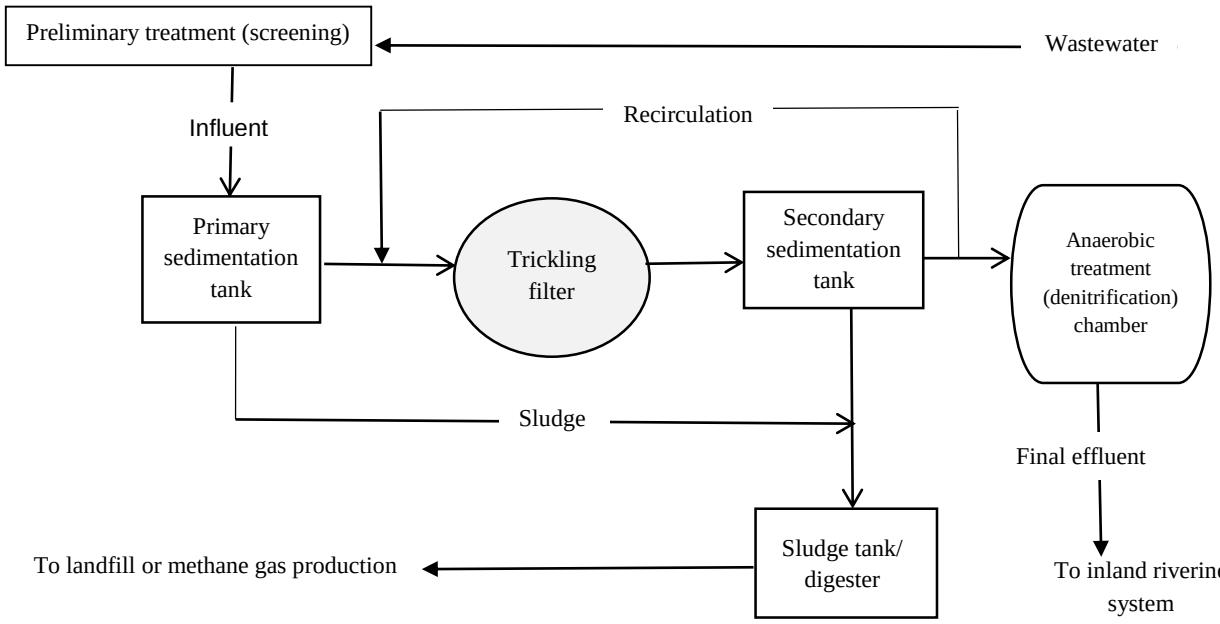


Fig. 2: General layout of a municipal WWTW that uses a fixed film reactor in the form of sewage trickling/percolating filter based on descriptions by Gray (2005, 2010) and on-site observations.

Preliminary sewage treatment involves removal of gross solids from incoming sewage (influent) by means of coarse and fine screening, grit separation and if present, removal of oil grease (Gray, 2010). This is followed by primary/sedimentation treatment stage in primary settlement tanks in which, as the name implies, solids settle (Gray, 2010) at the bottom effluent as sludge. Predominantly through gravity, the sludge drains to sludge tanks whilst effluent drains to distribution chambers in direct transit to bacteria beds for secondary treatment (Gray, 2010).

At the secondary treatment stage, effluent is automatically and intermittently (Hommon *et al.*, 1920) drizzled onto the bacteria bed by rotary effluent dispensers (Gray, 2004) (Fig. 3). As effluent trickles through the filter media to the bottom, it gets in contact with the "active purifying element" (Hommon *et al.*, 1920), the pollutant-degrading biological film (Cooke, 1959). This biological aspect of wastewater treatment is summarised in subsection 1.5.1.

Fig. 3: Influent being dispensed onto the filter bed. Shown is a portion of a filter bed and part of the four-arm rotary distributor covered with bird droppings. The green colour is filter flora - part of the biological film. (Picture by: S. Wenjere).

Biological filters drain into secondary sedimentation or humus tanks where the microbial biomass and invertebrate and organic debris (Gray, 2010) from the percolating filter settle as humus or filter sludge (Cooke and Hirsch, 1958). The humus is either recirculated to the beginning of sewage treatment to support microbial activity upstream of the process (Cooke and Hirsch, 1958) or channelled to sludge treatment stage (Gray, 2010) for thickening and stabilisation. The treated sludge is disposed of in different ways including use in landfill or methane gas production (Gray, 2004). On the other hand, the effluent is pumped to the final/tertiary treatment (Gray, 2010) stage in denitrification reactors. At this stage, by-products of the biological treatment process like nitrates (Gray, 2010) are removed or reduced to acceptable levels in the final effluent by the activity of anaerobic bacteria, *Nitrosomonas* and *Nitrobacte*r (Holtje, 1943; Skadsen, 1993). Through regulatory compliance, wastewater treatment must achieve an acceptable water quality index (Egborge and Benka-Coker, 1986; Simoes *et al.*, 2008) in the final water discharged into the inland riverine system.

One of the most important aspects of biological treatment of wastewater through percolating filters is the diverse micro- and macro-biota at the secondary treatment stage - in biological sewage filter beds.

1.5 Ecology of bacteria beds

Temperature (Learner, 1975) aside, filter ecology is dependent on the material make-up of its filter media (Terry, 1956), methods (Hawkes, 1959) and frequency (Coombs, 1997) of sewage dosing/application. The latter also has an effect on the seasonal population fluctuations (Hawkes and Shephard, 1972) of the filter flora and fauna.

The percolating sewage filter is helped to fulfil its purpose by a complex ecosystem of micro- and macro-biota that include binding and free-living organisms (Cooke, 1956). These include "bacteria, fungi, algae, protozoa, nematoda, rotatoria, chaetopoda, crustacea, arachnida and insecta" (Holtje (1943). The following are part of the diverse filter biota and therefore non-exhaustive.

1.5.1 Bacteria

The fact that the percolating sewage filter is also referred to as a bacteria bed suggests that, as shown in section 1.4, bacteria are at the core of biological treatment of wastewater. They sit at the fixed film reactor food chain's basic trophic level (Gray, 2010). There is wide spectrum of heterotrophic microorganisms (Gray, 2010) at different levels of the filter bed with specific roles in sewage treatment.

Organic matter is broken down by biological oxidation/respiration of aerobic bacteria to give soluble end-products and through biosynthesis whereby suspended particles and soluble organic matter is converted to new cellular biomass (Gray 2010). A good example of aerobic oxidation is when carbohydrates are oxidised into carbon dioxide and water by aerobic bacteria species (Gray, 2004). A lot of anaerobic faecal bacteria are also abundant in the filter beds where they are useful at the low-oxygen bottom layers and downstream of the process (Gray, 2004).

There are numerous other bacterial associations responsible for stabilising sulphur compounds, assimilation of iron and manganese products and involved in breaking down different compounds including cellulose (Holtje, 1943). The entomopathogenic bacteria (Learner, 2000), *B.*

thuringiensis has been found to exist in bacteria beds with their insecticidal activity (Mizuki *et al.,* 2001) helping with natural control of insects. In fact, wastewater sludge is a viable raw material in the production of *B. thuringiensis*-based biopesticides (Brar *et al.*, 2006).

1.5.2 Algae, Fungi and protozoa

Cooke and Hirsch (1958) listed numerous fungi including various *Fusarium*, *Aspergillus, Penicillium* species and algal species, *Stigeoclonium nanum*, *Ulothrix tenuissima*, *Phormidium uncinatum, Amphithrix janthina* etc. whose associations assist in the maintenance of the biological film. In one test, *A. flavus* was found to be most efficient at reducing ammoniacal nitrogen (NH3-N) (Akhtar and Ghaffar, 1986) in wastewater. Furthermore, the association between branched mycelia of fungi Holtje (1943) and filamentous algae (Cooke and Hirsch, 1958) holds together the biofilm at the upper levels of the filter bed to withstand vagaries of weather (Peng e*t al.,* 1992) and constant flow of effluent Holtje (1943).

Protozoa species are another member of filter invertebrate community (Hawkes, 1963, Learner, 1975; Gray, 2004) with important functions. Sewage percolating filters in the UK were found to be inhabited by more than 50 protozoan ciliate species (from four subclasses) including *Opercularia microdiscum, Chilodonella uncinata,* and *Cinetochilum margaritaceum* (Curds and Cockburn, 1970). Species like *Epistylis* and *Opercularia* provide competition to fungi over and above increasing oxidation Holtje (1943) in biological sewage treatment. The bacteria-ingesting protozoa help sustain aerobic oxidation by causing continuous bacterial replication to replenish the constant population decline (Cooke, 1959). Effluent clarification and mechanical agitation of filter slime (Rudolfs, 1950) are some of their functions in these filter beds.

1.5.3 Annelid worms

Annelid worms are abundant (Reynoldson, 1939) in the upper levels of bacteria beds. They include, *Lumbricillus rivalis, Enchytraeus coronatus* (Solbe *et al.,* 1974), *L. lineatus* (Lloyd *et al.,* 1940*;* Reynoldson, 1948), *Aelosoma hemprichi, Pristina spp., Limnodrilus spp.,* and *Tubifex spp.* (Cooke, 1959), *Eiseniella tetraedra* and *Dendrobaena subrubicuda* (Solbe *et al.,* 1967). These worms also provide important natural control of flora and fauna of bacteria beds with their wriggling movement (Reynoldson, 1939) helping to loosen the biofilm and keep surface growth in check. They feed on algae, fungi, bacteria, and break down organic debris to promote sloughing (Holtje, 1943) one of the mechanism by which solids are removed from the filer-bed (Williams and Taylor, 1968). A lab-based experiment (Hyvonen et al., 1994) showed that Lumbricid and Enchytraeid worms, *Dendrobaena octaedra* and *Cognettia sphagnetorum* controlled nematode populations by competing for food in the form of microorganisms. In another experiment (Williams and Taylor, 1968), it was proved that, once adequate population levels were present, annelid worms achieved high sewage treatment efficiency even in the absence of dipteran larvae. Therefore, these annelid worms help maintain the ecological balance of bacteria beds and promote efficiency in sewage treatment.

1.5.4 Nematode worms

Several nematode species from about dozen families are listed (Petersen, 1982) as filter bed inhabitants. Common species in England include *Diplogaster strictus,* and *Rhabdites spp* (Cooke, 1959) among others. These parasitise and kill or stunt development of a wide range of insect Orders

including Diptera (Petersen, 1982). Nematodes are also known to be biological control agents of Molluscan grazers through direct parasitism or by transmitting lethal bacteria (Grewal *et al*., 2003).

1.5.5 Molluscs – snails and slugs

There is little information on molluscan activity in the artificial habitats of WWTWs. However, in natural habitats, snails and slugs play an important ecological role (Penha-Lopes *et al.*, 2010) by feeding on organic matter and regulating algal growth by crawling and feeding on them. Cleworth, (2006) reported *Lymnae peregra,* now called *Radix peregra* (Bargues *et al.*, 2001) as one of the prominent aquatic snail species at WWTWs in the North West of England.

1.5.6 Nematoceran diptera

Different insects also persist in biological filters namely Collembola, Coleoptera, Hymenoptera and Diptera (Learner, 1975). However, most relevant to this study are the semiaquatic Nematoceran flies (Salmela, 2011) namely, *Sylvicola fenestralis, Metriocnemus eurynotus, Limnophyes minimus* and *Psychoda alternata* (Coombs *et al*., 1996; Van Poppelen, 1998; Learner, 2000; Cleworth, 2006).

The larval stages of these dipteran insects are beneficial to the maintenance of the biological film (Holtje, 1943) and therefore the efficiency of the filter beds. The larvae feed on living and decaying material including other members of the filter community (Holtje, 1943). This stabilises the organic matter in the filter media and facilitates the sloughing process (Holtje, 1943) over and above improving ventilation (Cooke, 1959) in the filter bed. Filter bed ventilation is improved as larvae

eliminate organic debris and fungal accumulation by perforating and loosening the biofilm (Reynoldson, 1948) during feeding. *P. alternata* larvae were also found to feed on eggs, miracidia and cercaria (El Bardicy *et al.*, 2009) of different snail species. Improved ventilation and, as explained in section 1.5.2, constant bacterial population depletion and replenishment promote efficiency of sewage treatment.

Clearly, one of the problems arising from biological treatment of wastewater by percolating sewage filters arises from their vital macroinvertebrate community - emergence of adult nuisance flies.

1.6 Common sewage-associated fly species

The nuisance fly families Anisopodidae (*S. fenstralis)*, Chironomidae (*M. eurynotus* and *L. minimus*) and Psychodidae (*P. alternata, P. albipennis* (= *P.severini) and P. cinerea)* are common throughout England (Tomlinson and Stride, 1945; Woods *et al*., 1978; Van Poppelen, 1998; Learner, 2000). These insect families are also listed as targets of the biological control product being investigated in this study. With dipteran larval insecticidal susceptibility not uniform across instars (Coombs *et al.,* 1997) and with no feeding occurring in preparation for moulting (Dhadialla *et al.,* 1998), studying and understanding the flies' morphology, lifecycles and feeding habits is important for controlling them.

In general, Nematocera, the sub-order of the nuisance flies has a life-history over five phases – adult mating, female maturation, egg incubation, larval growth and pupation (Murray, 1939) periods. Diptera eggs always hatch into legless larvae whose life is completely different from adult forms (Freeman, 1950). The sub-order also has four larval instars (Solbe and Tozer, 1971).

Micrographs of the immature and adult stages of the four fly species monitored in this study and described in this text (1.6.1 – 1.6.4) are shown chapter 3, table 5.

1.6.1 *Sylvicola fenestralis*

S. fenestralis is one of the 120 species belonging to seven genera of the Anisopodidae insect family (Amorim *et al.*, 2016). The adult species' shade-seeking tendency (Hawkes, 1952) makes the fly a nuisance to WWTW workers and local residents. The adults normally seek refuge on windows or tree trunks which is why they are also referred to as window or wood gnats (Amorim and Tozoni, 1994). In fact, their tendency to collect at windows makes their presence conspicuous and annoying (Learner, 2000) in any numbers. Robinson (2005) provided the most comprehensive description of the life history and morphology of the species.

Grey masses containing 150 eggs are laid on filter substrate. After hatching, the larva goes through a series of moults with the final instar relocating to a drier place (Robinson, 2005) for pupation in the same habitat with no cocoon formation (Freeman, 1950). After maturation, pupae move to the filter bed surface in preparation for adult emergence (Freeman, 1950). The complete temperature-dependant life-cycle takes between 50 and 88 days (Robinson, 2005) or 39 -121 days (Hawkes, 1951) (table 1).

Table 1: Summary of *S. fenestralis* life-history showing duration of developmental stages at different temperatures based on findings of cited workers.

	Duration of developmental stages (days)				Full duration (days)	Reference
Temperature	Egg	Larva	Pupa	Adult		
~20^{0}C	4	20	8	7	~35	Robinson (2005).
~10.5^{0}C	-	50	-	7	~88	Robinson (2005).
9^{0}C	-	-	-	-	121	Hawkes (1951).
13^{0}C	-	-	-	-	73	Hawkes (1951).
18.5^{0}C	-	-	-	-	50	Hawkes (1951).
21^{0}C	-	-	-	-	39	Hawkes (1951).

Of the four nuisance flies found in the North of England and monitored in this study, *S. fenestralis* is the most physically distinct. Their nearly cylindrical larvae are yellowish-white progressing to yellowish-brown (or mottled orange) at the fully-grown stages which can measure up to 15mm in length (Robinson, 2005). Larval body segments are separated by narrow constrictions to form enlarged rings at anterior ends although the 10th/last segment ends in 5 tubercles (Robinson, 2005). Based on head capsule and body dimensions, Coombs *et al.* (1997) determined that *S. fenestralis* had four larval instars (table 2).

Table 2: Morphometric characters of *S. fenestralis* larval instars as determined by Coombs *et al.* (1997).

	Head capsule (mm)		Body length (mm)
	Width	Length	
Instar I	< 0.175	< 0.215	2.5
Instar II	0.175-0.270	0.215-0.320	2.5-5.5
Instar III	0.270-0.420	0.320-0.430	5.5-9.0
Instar IV	> 0.420	> 0.430	> 9.0

Adult *S. fenestralis*, at 6mm in length, (Robinson, 2005) is the biggest and most conspicuous (Learner, 2000) of the nuisance flies. The species adult is distinguishable with three blackish stripes on the thorax (Edwards, 1923), has antennae longer than the head and dark brown wings with scattered pale spots (Robinson, 2005) and about 5 – 7.5mm in length (Freeman, 1950).

1.6.2 *Metriocnemus eurynotus*

M. eurynotus (formerly *M. hygropetricus* (Kieffer, 1911) or *M. longitarsus* (Gortghebuer, 1921) is a non-biting midge (Raunio *et al.*, 2011) (family: Chironomidae, subfamily: Orthocladiinae) (Cranston and Reiss, 1983) common in British aquatic ecosystems including sewage filter beds. Despite them comprising much of the global biodiversity and playing important roles in different habitats, not much information is available at genus- or species-levels (Raunio *et al.*, 2011).

However, adult species of this family are structurally similar but not the immature stages (Oliver, 1971). They are grey to black, bear midline furrow on thorax, measure 1-10mm in length and are

mosquito-like with shortened or no mouthparts (Robinson, 2005). Although adult *M. eurynotus* can also mate in confined spaces (Lloys *et al*., 1943), when conditions suit them, they form compact mating swarms (Terry, 1956) above or around a conspicuous feature (Robinson, 2005) like a tree, footpath etc. Further to this fly abundance causing annoyance (and in some cases, hypersensitivity), it also brings a safety hazard on motorways and roads (Robinson, 2005).

The ephemeral Chironomid adults complete their reproductive cycle (mating, egg maturation and oviposition (Oliver, 1971) in 2-3 days (Robinson, 2005). *Metriocnemus* eggs are laid in gelatinous masses onto the substrate but the period they take to hatch at different temperatures does not appear to be covered (Oliver, 1971) in citeable literature. The larvae feed on algae, fungi and can tolerate low-oxygen conditions. Chironomids can complete development between 0^0C and 32^0C.

Pupation happens inside the case of the final larval stage. The pupal stage is short, ranging from hours to few days and adult emergence is rapid - from few seconds to several minutes followed by immediate flight (Oliver, 1971). However, in his lab-based investigations, Lloyd (1937) found that *M. eurynotus'* complete life cycle takes as much as 100 days at 7 ^{o}C and 33 days at 18^{o}C.

1.6.3 *Limnophyes minimus*

Limnophyes [=*Hydrobaenus*] *minimus* (Learner, 2000) (formely *Spaniotoma minima*) (Lloyd, 1937) is another non-biting midge (family: Chironomidae) (Raunio *et al.*, 2011) and one of the species that causes most sewage-associated nuisance (Painter, 1980, cited in Learner, 2000) in England. The presence and prominence of these two Chironomid species in the North, or England at large, is corroborated by nearly each of the many workers who have been involved in the ecology

of wastewater treatment in the past including Lloyd 1937; Lloyd, 1943; Tomlinson and Stride (1945); Terry (1956); Houston *et al.* (1989[a]); Coombs (1997); Learner (2000) and Cleworth (2006). However, there also appears to be limited citeable literature on its biology at genus- or species-levels.

L. minimus mating swarms make them a nuisance (Gibson, 1942). A lab-based study by Lloyd (1937) gave a limited insight into their life-history. Most mated adults oviposit at 10^{o}C followed by a good hatching success and few that manage oviposition at 5^{o}C yield nothing. The eggs are deposited under the top filter stones in mucus batches of 200 eggs. Larval development favours filter bed temperatures between 8 and 21^{o}C, maturing in 38.3 days at 13.5^{o}C and in 25.5 days at 18.5^{o}C. Larva creates a mouse dropping-like cocoon from debris to pupate in or around its food source within the filter bed. The larva also helps pupal hatching by breaking the cocoons. They display carnivorous tendencies – eating *Psychoda* eggs, and are able to break *Metriocnemus* cocoons and eat *the* pupae and also feed on *Lumbricillus* cocoons (Lloyd, 1943).

Delettre (1977) provided head capsule dimensions of the 4 larval instars of this species as follows: instar 1: 50-90 µm, instar 2: 90-140 µm, instar 3: 140-2l0 µm and instar 4: 210-310 µm. The distinguishing features of *L. minimus* is its much smaller adult size and larval appearance which is similar to the early stages of *M. eurynotus* but with distinct purple patches on the body.

1.6.4 *Psychoda spp.*

Psychoda spp. belong to sub-family Psychodinae of family Psychodidae with 84 (16 genera) of the 1200 global species (Smith, 1989) found in Britain. Species of this genera found in sewage beds include moth fly-like *P. cinerea* and *P. alternata* (Fair, 1934). Psychoda is commonly referred to as owl midge (Van Poppelen, 1998) or "trickling filter fly" (Quatte, 1955, cited in Redborg *et al.*, 1983). Their 4-stage lifecycle (Holtje, 1943; Redborg *et al.*, 1983) takes about 21-27 days (El Bardicy *et al.*, 2009) to complete.

The adult lays transparent irregular masses each containing 15-40 (Fair, 1934) and sometimes more (Holtje, 1943; Van Poppelen, 1998) eggs measuring between 0.2 and 1mm in length (Fair, 1934) on the biofilm. At around 21^0C, (Fair, 1934) eggs can hatch in approximately two days or less (Fair, 1934; Holtje, 1943). The emergent whitish (*P. alternata)* or blackish (*P. cinerea*) larvae move into the filter media where they feed and grow from 0.75 – 9mm (Fair, 1934) over 4 larval instars (Redborg *et al*., 1983). Larval stages take 9-15 days (Solber and Tozer, 1971) at 21^{o}C, 24 days at 15.5^{o}C and only 8 days above 29^{o}C (Fair, 1934) to pupate for 20-40 hours (Satchell, 1947). Emerging adults quickly move to dry spots like the under-surface of filter stones, unfold wings, rest, copulate, lay eggs and/or escape from the filter (Fair, 1934). Some adults are reported to rest in the lower levels of the filter bed (Zuelzer, 1909, cited in Fair, 1934) where they die.

Psychoda larvae are legless wrigglers. Their whitish (*P. alternata*) (Fair, 1934) and black (*P. cinerea),* cylindrical, dorsoventrally flattened bodies which taper slightly (Satchell, 1947) at the anterior and posterior ends. The larva is amphipneustic (Satchell, 1947), its 11-segment body ends in a tubular siphon with posterior spiracles at its apex and fan-like brushes (Smith, 1989). Larval

mouth opens on the underside but the complex nature of fully developed mouthparts means the breathing siphon stands out as the best diagnostic character (Satchell, 1947).

The non-feeding pupa is shorter and thicker than larva at 6mm and 3.5mm respectively and their breathing horns protruding from thorax (Fair, 1934) are their best diagnostic features. Adult *P. cinerea* is darker (nearly-black) (Fair, 1934) than *P. alternata* which is dark grey (El Bardicy *et al.*, 2009) in colour. These species also have hairy body and wings (Fair, 1934) (Fig. 4) giving them a moth-like appearance with body size ranging from 3 to 5mm (Van Poppelen, 1998) or smaller. In this study and of the two *Psychoda spp.*, focus was placed on the more abundant *P. alternata.*

Fig. 4: Female *P. alternata* wing (Tokunaga, 1953) showing the typical hairy characters.

1.7 The fly nuisance and public health problems

As shown earlier, presence of the immature stages of the above dipteran species in sewage filter beds is beneficial (Holtje, 1943). However, although the adult flies that emerge are non-biting, enough sanitary evidence was found to justify concerns about their "potential menace to health as well as … aerial nuisance" (Turner, 1923, cited in (Fair, 1934).

P. alternata was found to accidentally cause myiasis in Japan (Tokunaga, 1953) whilst, together with Chironomid species, it was also found to cause asthma attacks in people (Van Poppelen, 1998; Failla *et al.,* 2015). On the other hand, Chironomid habitats include both sewage and drinking water facilities, making mechanical transmission of pathogens like *Salmonella* and *Vibrio cholerae* (Failla *et al.,* 2015) a plausible concern. With the help of wind, the flies can drift away up to about a mile of WWTWs (Fair, 1934) and often reaching enough aerial densities to cause annoyance to sewage plant operators and the public (Woods *et al*., 1978, Coombs *et al.* 1997; Learner, 2000; Cleworth, 2006). Disease concerns aside, it is understandable for members of the public to be uncomfortable and annoyed with these flies because of their association with sewage.

For these reasons, sewage-associated flies are classified as a statutory nuisance under the Environmental Protection Act 1990. The law in-part defines insect statutory nuisance as "any insects emanating from relevant industrial, trade or business premises and being prejudicial to health or a nuisance". Therefore, when a business' operation gives rise to nuisance flies, controlling them (flies) becomes a matter of regulatory compliance in England and Wales. However, giving guidance on Neighbourhoods and Environment Act 2005, (Defra, (2006) reiterated that "it should not be assumed that killing insects is necessarily the most appropriate way to cease or abate a nuisance." This suggests that larviciding is encouraged as a last resort if/when adult fly egression is predicted at levels high enough to cause a nuisance.

1.8 Nuisance fly control – history and methods

There is a long history of nuisance fly control through biological, chemical, physical (Fair, 1934) and nutritional (Learner, 1975) interventions since percolating filters came into use at the end of

the 19th century (Van Poppelen,1998). Whilst all control methods would be expected to be economical, they must be target-specific and not upset the biological integrity of the filter beds, ecosystem of the receiving waters or persist in the food chain. Therefore, ever since the advent of biological treating wastewater through percolating filters, different fly control methods have been employed. For different reasons, some methods fell away and the more reliable ones are continually improved on.

1.8.1 Physical Control

Filter bed flooding (Kamei *et al.*, 1993) for 24 to 36 hours ((Fair, 1934; Holtje, 1943), drying (Van Poppelen, 1998) and fly burning with gasoline torches (Fair, 1934) were used against *Psychoda* and *Sylvicola spp*. with varying degrees of success and challenges. The same species have been successfully controlled by covering the filter bed with a layer of fine media (Tomlinson and Stride, 1945; Learner, 1975) but it was not feasible across all seasons. Total enclosure of percolating filters by surface barriers including glass covers, tight walls (Learner, 1975) and fine-meshed nets (Van Poppelen, 1998) was practiced but came with operational challenges and high costs. In South Africa, enclosing filters resulted in reduced heat and cold extremes (Murray, 1939) which led to abnormally high fly emergence and exit through false floors.

Filter bed mechanical media in the form of smooth pebble gravel (Rachesky and Petty, 1968) was also found to curtail excessive fly breeding. However, it was found that media size alone could not achieve desirable results (Terry, 1956) if other factors were overlooked.

Another form of physical control was observed at the WWTWs worked on in this study which were all surrounded by thickets of trees and shrubs. These must serve as physical barriers against the drifting away of large swarms of nuisance flies into the community.

1.8.2 Nutritional control

As already covered in section 1.5, it would be better for the less troublesome Annelid worms to dominate Dipteran species in the filter bed trophic pyramid (Learner, 1975). However, more understanding of the complex filter-fauna community would need to be fully established first. Upsetting the filter ecological balance promotes biofilm accumulation (Hawkes, 1963) and therefore higher breeding success by the flies.

Increasing sewage dose to filter beds was found not to have any fly-drowning effect in South Africa but actually promoted their breeding success (Murray, 1939). In fact, constantly reducing the food source, i.e. biological film, was found to promote filter-fauna diversity (Learner, 1975) at the expense of the flies. However, decrease of sewage loading balances fly populations by inherently allowing for competition for limited food resources (Woods et al., 1978). This is accompanied by a drop in *Psychoda* populations and an increase in chironomids, *L. minimus* and *M. eurynotus* (Tomlinson and Stride, 1945). Resting filters as much as possible in winter was used against *Psychoda* and *Metriocnemus* but it was/is operationally infeasible to give rest for long periods (Lloyd, 1937; Woods *et al.*, 1978) at some WWTWs.

1.8.3 Chemical control

All fly control methods have disadvantages but the immediacy with which insecticides provide relief, has always made them the most attractive option. Before the onset of organochlorine (Learner, 1975) and organophosphorus (Bruce-Chwatt, 1971; Kamei *et al.,* 1993) pesticides, "repellents and contact insecticides" (Fair, 1934) like lead arsenate (Magalhaes, 2002) were used in fly control. Repellents showed noticeable impact on their targets and insecticides like arsenates, benzene, chlorine and more substances also worked (Fair, 1934). However, these were mostly expensive, not target-specific and deleterious to the filter biota. "Environmental accumulation of arsenical residues or their mobilization into water supplies" (Magalhaes, 2002) led to a gradual departure from arsenate compounds.

Creosote oil, paraffin, chloride lime or chlorine gas (Murray, 1939) and bleaching powder (Tomlinson, 1945; Tomlinson and Stride, 1945) were also some of the early chemical interventions used. Furthermore, ordinary housefly chemical sprays (Murray, 1939) were found to kill *P. alternata.* Of these early methods, spraying filter beds with a mixture "of creosote and crude oil" (Scouller and Goldthorpe, 1932) and acidification of sewage (i.e. dropping sewage pH by adding sulphuric acid) (Fair, 1934) proved to be the most satisfactory interventions.

There were other problems with chemical control. It was expensive due to repeated applications necessitated by the tendency of fly populations to quickly re-establish (Hawkes, 1963) after treatment. Repeated treatments create altering filter ecological makeup (Hawkes, 1955) and upsetting microbial activity thereby causing ponding (or filter bed clogging). Most importantly, the long-term impact of repeated treatments on utility of the sewage works and in receiving waters had

to be fully studied (Rachesky and Petty, 1968). One of the most important challenges of chemical fly control was the non-selective nature of the insecticides (Tomlinson, 1945).

Following the disadvantages of earlier insecticides, development of organic insecticides was accelerated after the second world war (Woods *et al*., 1978). Gammexane, one of the isomers of Benzenehexachloride (BHC) and dichloro-diphenyl-trichloroethane (DDT) achieved extremely high fly reduction levels (Brother, 1946; Jenkins, 1949). BHC was found to be very effective against *S. fenestralis* (Hawkes, 1951). Different workers found gammexane as the most effective and economical biocidal choice of the post-war era (Jenkins *et al.,* 1949; Rudolfs *et al.,* 1950). Unfortunately, these organochlorides contained some level of toxicity to trout and filter bed "scouring population" (Tomlinson and Muirden, 1948) which led to ponding at one of the studied sewage works. Because DDT targets the nervous system, it was not very effective against larvae but lethal against a wide spectrum of insects (Brothers 1946). Furthermore, the organochlorine pesticides persisted in the food chain and their mode of action caused insects to develop resistance (Bruce-Chwatt, 1971) to them and other insecticides.

DDT use in many countries ended around the 1970s to be replaced by organophosphates (Oberemok *et al*., 2015). Organophosphorus insecticide, Pirimiphos-Methyl achieved high fly-reduction success although it was later found to reach the receiving waters (Harbott and Penny, 1983) meaning, the product lacked rapid degradation. Recently, Borras *et al.* (2017) went further and discovered that the same product also generates organic aerosols which "may pose further health and environmental hazards because of higher toxicity."

Williams (1967) summed up the chemical control journey, in his time, as being made up of first generation (arsenates), second generation (DDT) and third generation (insect growth hormones) pesticides.

1.8.4 Biological control

The entomological definition of this control method is given as “the reduction of pest populations by their natural enemies” (Sawyer, 1990). A good example of biological/natural control of insects is when they are predated upon birds, other fly species, spiders, mites and/or get infected by certain parasites. In nature, populations of different insect orders are under constant check from entomopathogens or insect killing bacteria, viruses, fungi and nematodes (Lacey *et al.*, 2002). However, known parasites of insects in biological filters were found not to target three of the four nuisance flies (Learner, 1975) monitored in this this study.

Predators and entomopathogens aside, in the artificial habitats of WWTW, biological or natural fly control is also provided through feeding competition by snails, leeches and worms (Fair, 1934). Therefore, biological/natural control means the flies are controlled to some extent with or without man’s knowledge.

In sewage filter beds, some *Limnophyes* and *Metriocnemus spp*. control other dipteran flies through direct attack (Lloyd, 1937, 1941; Learner, 1975) or competition for food (Reynoldson, 1948, cited in Rachesky and Petty, 1968). Although this would appear to make them even more successful, there are other members of the filter community which are predaceous on them. In fact, the Chironomid species have been found to be self-limiting by virtue of their complex microbiome

(Halpern and Senderovich, 2015) whose other members are deleterious to their eggs. On the other hand, *Sylvicola* struggle to compete with *Psychoda* where the latter is abundant (Tomlinson and Stride, 1945). Known parasites of insects in filters were found not to target three of the four nuisance flies (Learner, 1975) monitored in this this study. Literature review on filter ecology (section 1.5) also touched on how (or which) other members of the filter flora and fauna help in controlling each other's populations.

Entomopathogenic nematode, *Steinernema feltiae* trading as Nemasys® was successfully tested (Cleworth, 2006) against sewage filters in North West of England. In this case, insecticidal activity arose from symbiotic entomopathogen, *Xenorhabdidus nematophilus* (Thomas and Poinar, cited in Cleworth, 2006) transmitted into insect larva. Therefore, control methods requiring human intervention, are secondary to the aforementioned natural phenomena. The need for human intervention only arises after natural controls have already played a part.

No citeable literature was found pointing to commercial use of natural insect enemies against sewage filter flies in England. However, biological control also includes commercial microbial- and hormone-based pest control agents. The manipulation of different invertebrate biological phenomena in the production and use of synthetic components or analogues means the original use of the term "biological control" (Rajendran and Singh (2016) has been interfered with. In that regard, biological control ceases to be synonymous with natural control.

Two biological control methods involving commercial exploitation of different biological phenomena are looked at separately as part of the current control methods below. In fact, one of them is at the centre of this study.

1.9 Current control methods

With all their shortcomings, chemical control was the most viable option of those discussed above. However, target-specific, biodegradable and resistance-proof pesticides were needed. The continuous search for sustainable methods/products that meet or exceed these standards was, and remains, largely driven by human quest to control human, animal and plant insect pests (Williams, 1967).

As opposed to the predominantly neurotoxic chemicals (Miyamoto, 1993), the discovery and development of insect hormone mimics and bacteria-derived larvicides pushed insect pest control frontiers to new levels. These targeted a very specific biological pathway in given species, meaning their chance of harming non-target organisms was always going to be highly unlikely.

1.9.1 Insect growth regulators (IGR)

Insect growth regulators (IGR) or, as now proposed, insect growth disruptors (IGD) (Subramanian and Shankarganesh, 2016) came into use after about 50 years of research by earlier workers between 1913 and 1918 (Gilbert *et al*., 2000) and built upon by others including Kopec (1922), Wigglesworth (1934). Studying and understanding of arthropod endocrinology (Spindler-Barth, 1992) made it possible to manipulate insects' own hormones for their destruction.

Hormone-based pesticides have two main classes (Beckage, 2000). Juvenile hormone analogues (JHAs) or synthetic juvenoids (Staal, 1975; Miyamoto *et al., 1993*) disrupt oviposition, hatching and larval maturation (Spindler-Barth, 1992). Among other aberrations, stunted pupation often

leads to early pupation and adult dwarfs (Graf, 1993). The other class belongs to moulting hormones (or ecdysteroids) which, as the name suggests, are chitin synthesis inhibitors (Graf, 1993; Miyamoto, 1993; Subramanian and Shankarganesh, 2016) which disrupt ecdysis. Application of analogues or antagonists of these hormones at "inappropriate" developmental stages is deleterious to the insects (Subramanian and Shankarganesh, 2016).

Diminiln®, diflubenzuron is one chitin synthesis inhibiting IGR which was experimentally shown to control filter flies (Painter, 1980, cited in Coombs *et al.*, 1996) at high treatment strengths (Ali and Kok-Yokomi, 1990). Altocid®, a methoprene, (Ali and Kok-Yokomi, 1990; Kamei *et al.*, 1993), cyromazine, (Follas and Patterson, 1994) and pyriproxyfen (or S-31183) (Coombs *et al.*, 1996; Schaefer and Miura, 1990, Cleworth, 2006) are all JHAs that are effective against different nuisance flies but harmless to non-target organisms. However, methoprene is most effective against mosquitoes (Ali and Kok-Yokomi, 1990; Kamei *et al.*, 1993) and shows negligible bioaccumulation in fish and no irreversible persistence in the receiving waters (Schaefer *et al.*, 1988).

One of the important advantages of IGR is that the biological site they target and act on in insects is non-existent in mammals (Tunaz and Uygun, 2004). Conversely, the most notable disadvantage of IGRs is their action on embryonic, larval and pupal development. This mode of action means their effect is not as immediate as conventional pesticides and often has to be supplemented with adulticides (Graf, 1993). Fortunately, the discovery of entomopathogenic bacteria-derived larvicides followed.

1.9.2 Bacteria-derived larvicides

Of all bacterial agents, it is entomopathogenic bacteria (Learner, 2000; Mizuki, 2001), *B. thuringiensis* that is the most important (Sanchis, 2011, cited in Oberemok *et al*., 2015). In fact, it "accounts for greater than 90% of all marketed Bioinsecticides" (Nicholson, 2002). The species was first isolated and described in Germany in 1915 (Angelo *et al.*, 2010). First attempt to exploit the bacterial species in control of Lepidopteran insects came around the end 1920s (Oberemok *et al*., 2015) with a commercial breakthrough occurring in France in 1938. However, it later turned out that in 1902 a Japanese researcher had described a bacterium that has now been linked to an important *B. thuringiensis* subspecies.

In view of the disadvantages of different pesticides against Dipteran larvae covered in this section, the discovery of *B. thuringiensis* subspecies *israelensis* (BTI) in 1976 (Goldberg and Margalit, 1977) in Israel was ground-breaking. The Gram-positive, spore-forming (Fig. 5) bacteria produce proteinaceous parasporal inclusions (Mizuki, 2001) that have larvicidal activity against Dipterans, and in particular, the suborder Nematocera. Going by the reasoning of Williams (1967), BTI-derived larvicides could be viewed as 4^{th} generation pesticides.

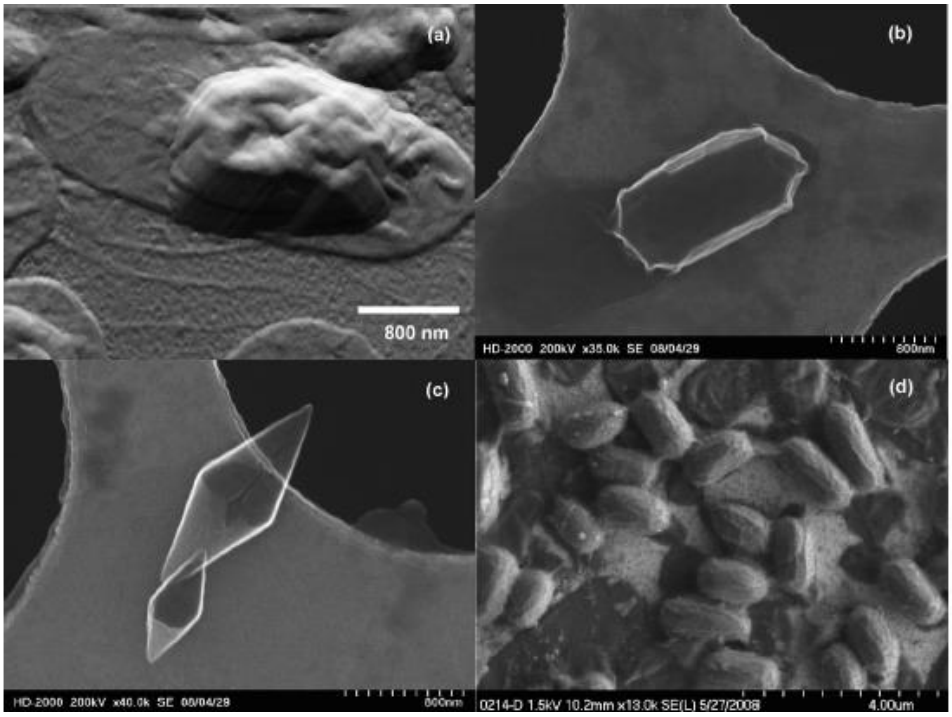


Fig. 5: BT spores (a and b), protein crystals (c), and both (d) from Chung *et al.* (2010) using different capability microscopes.

Different companies produce different BT-based invertebrate pest control products (Brar *et al.*, 2006). In fact, there are more than 20 licenced BT formulations of which eight (Inci, 2014) are derived from the serovariety *israelensis.* The biocidal formulations include Aquabee®, Bactimos®, Gnatrol®, LarvX®, Mosquito Attack®, Skeetal®, Teknar®, Vectobac® (Houston, 1989[b]; Sulaiman *et al.*, 1990; Thiery *et al.*, 1996; Lima *et al.*, 2005; Inci *et al.*, 2014). It is one specific strain of the latter (produced by Valent Bioscience Corporation) that concerns this study - VectoBac-24® strain AM65-52 (BTI AM65-52). Throughout this text, the biolarvicide is referred to by its full name or simply as VectoBac®.

1.9.2.1 VectoBac 12AS® strain AM65-52 (BTI AM65-62)

VectoBac 12AS® is one of the BTI-derived larvicides (subsection 1.9.2). In this study, the product was tested against its listed target nematoceran families, Anisopodidae, Chironomidae and

Psychodidae (section 1.5.6) which happen to persist in biological filters at WWTWs in North West of England.

1.9.2.2 Biological and toxicological characteristics of VectoBac®

There does not appear to be a detailed biology of BTI in terms of its multiplication, sporulation and crystal production (Angelo *et al.*, 2010) but this is obviously well studied and guarded in commercial secrets. However, evidence was found showing that during sporulation some toxin crystals "insert into the spore coat where they are displayed for binding to specific receptors in the insect midgut" (Du and Nickerson, 1996, cited in Nicholson, 2002). This facilitates attachment and germination of spores within the host. It is also known that during sporulation, the bacteria produce proteinaceous "Crystal (Cry) and Cytolitic (Cyt) toxins" (Bravo *et al.*, 2007). The highly target-specific Cry and Cyt proteins have toxic and hemolytic effects (Bravo *et al.*, 2007) on target organisms respectively. There are three different toxin classes and sizes from these two.

In their technical bulletin, Valent (2017), manufacturer of VectoBac®, revealed that it is the synergy of four endotoxins, Cyt1A (27kDa), Cry4A (134kDa), Cry4b (128 kDa) and Cry11A (66 kDa) that has enabled BTI larvicide, VectoBac® to remain effective over the years. This suggests that resistance potential exists if only these toxins acted alone. The existence and mode of action of these toxins was substantiated in various peer-reviewed literature including Phytoparasitica (2003); Bravo *et al.* (2007); Stalinski *et al.*, (2014) and Gwal *et al.*, (2015).

1.9.2.3 Product mode of action and host interaction

Specificity of BTI toxins is dependent on its configuration and host physiology (Lacey and Siegel, 2000). Because the toxins' mode of action (Fig. 6) is based on specific membrane conformations and binding receptors, they are harmless to vertebrates and plant life (Valent, 2017).

After ingestion, the Cry toxin crystal complex, is solubilised to protoxins in midgut pH environment and cleaved by enzymes (Bravo *et al.*, 2007) there. Active form of toxins is released and bind to specific receptors in the midgut leading to pore-formation (Angelo *et al.*, 2010) in cell membrane to create osmotic imbalance (Chilcott & Ellar, 1988, cited in Gwal *et al*., 2015).

Although this has not been studied across all target species, the toxin binding or Cry mode of action was also explained in terms of signal transduction (Angelo *et al.*, 2010). In this case, receptor binding induces intracellular reactions involving G-protein and adenylate cyclase which lead to elevated levels "of cyclic adenosine monophosphate (cAMP)" (Angelo *et al.*, 2010) and protein kinase activation.

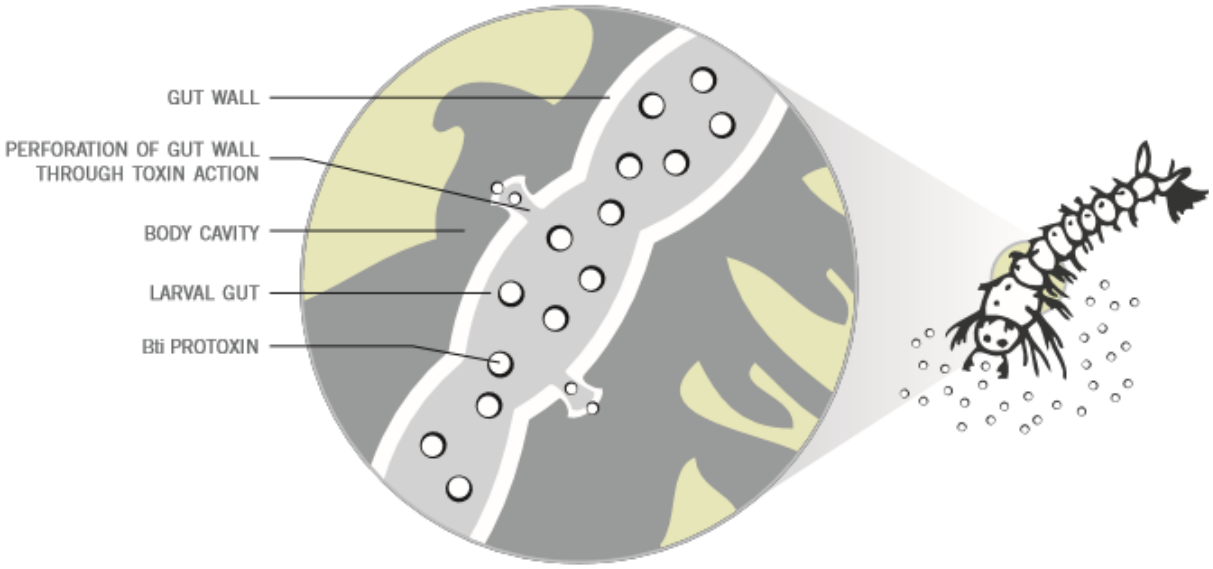


Fig. 6: Basic illustration of BTI larvicide mode of action in insect midgut (Valent, 2017).

Both modes of action tend to destabilise cellular osmotic balance leading to cell lysis (Bravo *et al.*, 2007; Gwal *et al.*, 2015) and larval death due to poisoning, subsequent lack of feeding and paralysis (Angelo *et al.*, 2010).

On the other hand, Cyt proteins are broken down, have their C-terminal and N-terminal portions (Angelo *et al.*, 2010) cleaved. The protoxin is converted to the active form to bind to membrane lipids inducing pore-formation, which disrupts the cell membrane lipid bilayer (Angelo *et al.*, 2010).

The synergy of the Cry and Cyt proteins is credited with be the major product toxicity.

1.9.2.4 Advantages, disadvantages and efficacy

The main advantages of the VectoBac® include its target specificity (Gwal *et al.*, 2015),

harmlessness to humans, other non-target organisms and the environment. There is also very low likelihood of host resistance, compatibility with other pesticides and ease of genetic modification (Inci, *et al*., 2014). The existence of multiple toxins (subsection 1.9.2.2) working together makes the product more toxic (Gwal *et al.*, 2015) whilst also making it difficult for target species to develop resistance.

Notable disadvantages of the product include its "high host specificity and problems of shelf-life" (Inci, *et al*., 2014)

According to Brar, *et al.* (2006) the product efficacy is also dependent on a few environmental factors. UV-B (280–310 nm) and UV-A (320–400 nm) portions of the radiation (UV) spectrum are deleterious to product toxicity. About 3 cm of rainfall cuts BTI efficacy by 20%, optimal pH is narrower than pH 3-10, temperatures lower than 10^0C and higher than 30^oC is not ideal for optimum performance of product and lastly, foliage (e.g. leaves) are not only a physical barrier to the product but could also have natural chemicals that degrade BTI spores.

Although there is no evidence of the product affecting non-target organisms, the deleterious effects of repeated applications on other organisms at higher trophic levels or on the structure of the ecosystem community (Lacey and Siegel, 2000) cannot be dismissed out of hand.

In the current study, the product was tested against species of its target nematoceran families, Anisopodidae, Chironomidae and Psychodidae (section 1.6) which persist in biological filters at WWTW in North West of England.

1.10 Rationale of study

This study was done in part-fulfilment of European Union's (EU) new insecticide licensing criteria necessitated by new findings on the unintended effects of pesticides in the environment. For example, pharmaceutical bioaccumulation together with damage to beneficial and/or non-target species by pesticides is concerning environmental authorities.

A recent study by Ruhi *et al*. (2016) on effluent-receiving inland riverine system found that riverine food web macroinvertebrates are capable of amplifying the bioaccumulation of different compounds including endocrine disruptors. Another recent study on a Mediterranean riverine system by Ccanccapa *et al.* (2016) revealed that more than four dozen pesticides used in crop husbandry showed worrying concentrations in the receiving watercourses causing some ecotoxicological harm at different trophic levels in that immediate food web. On the other hand, there is also the topical issue of "global bee apocalypse" (EU Times, 2013). This was brought about by the wide use of reproduction- and behaviour-altering nicotinoids (Laurino *et al*., 2011; Stanley and Raine, 2016) in crop farming.

There have been protracted studies and discussions on the use and licensing of such pesticides in China (Copping, 2008[a]) and Europe (Copping, 2008[b]; 2008[c]; 2009; 2013[a]; 2013[b]; 2013[c,] 2016). These have provided the EU with increased evidence that some pesticides harm beneficial insects, persist in the environment and at different trophic levels (Gross, 2014; Hallman *et al*., 2014). Therefore, the risk of multiple ecological stressors (Schuhmacher *et al.,* 2016*)* has been brought into focus and wide-ranging restrictions and bans have already been effected. In fact, Ruhi *et al*. (2016) indicated that emerging knowledge has seen the EU putting more compounds on their watch

list. Therefore, the current study was necessary for the licensing and continued use of the biocidal product, VectoBac® in nuisance fly control at WWTWs in UK and other EU countries.

1.11 Research objectives

It is not unusual for authorities to want to know more about products that are finding their way into the environment. According to the studentship sponsorship letter, this study was *"*requested and authorised under the Chemicals Regulation Directive (CRD) of the Health and Safety Executive (HSE) due to the introduction of the EU Biocides Regulation 528/2012 (EU BPR) – Authorization NO: UK-2015-0947" (Cleworth, 2016). Therefore, with VectoBac being used for nuisance fly control at some WWTWs in North of England, this study sought to achieve the following objectives:

1.11.1 Evaluate the efficacy of VectoBac® in the control of sewage filter flies by comparing pre- and post-treatment data.

1.11.2 Determine the effective dose against the hard-to-control Chironomid species.

1.11.3 Assess the need for larviciding repeats against sewage filter flies.

1.11.4 Investigate the effects of the larvicide on non-target organisms (NTOs).

1.11.5 Find gaps in the body of knowledge on the biology and ecology of wastewater treatment.

2.1 Research sites

The research was conducted on two United Utilities wastewater treatment sites in Cheshire at Helsby (53.9328° N, 2.0086° W) and Macclesfield (53.3009° N, 2.1544° W) (Fig. 7).

Fig. 7: United Utilities WWTW and sites of this research in Cheshire East and West. The area demarcated in red, on the bottom map, was the section of the sewage works researched on.

2.1 Experimental design

The experimental design was in line with earlier studies by Coombs *et al*. (1996) and Cleworth (2006). Only abundant and easy-to-enumerate NTOs, nematodes, aquatic earthworms and molluscs were monitored alongside the target dipteran species. Atmospheric and filter bed temperatures were also monitored throughout. Initially, monitoring was done on two percolating sewage filters per site before being increased to four. The preliminary stage served to test and fully establish sample handling techniques whilst getting an indication of availability of target species.

At each site, fly populations were monitored for several months in advance of treatment to establish the relative populations of the common fly species and to predict their population peaks. Before starting treatment, differences of all filter-population counts were compared for statistical significance.

The recommended concentration of the larvicide under investigation, VectoBac 12AS® strain AM65-52 (BTI AM65-52) is 160mg/L for all species except *P. alternata* at 80mg/L. However, in this study, lower (80mg/L) and higher (230mg/L) concentrations were also trialled against Chironomid species, *L. minimus* and *M. eurynotus.*

The standard field practice in fly control with this biocidal product is that each treatment cycle is comprised of two product applications seven to twenty-one days apart depending on the targeted species. Further doses are applied where necessary according to the product label. In this study, second treatments were done within seven to fourteen days of the first.

Four filters were monitored on each site and two fly species were targeted for treatment at each site. This experimental design is summarised by way of flow charts (Figs. 8 and 9). The execution of monitoring and treatment plan outlined here is detailed in sections 2.3 to 2.4.

Helsby experimental design

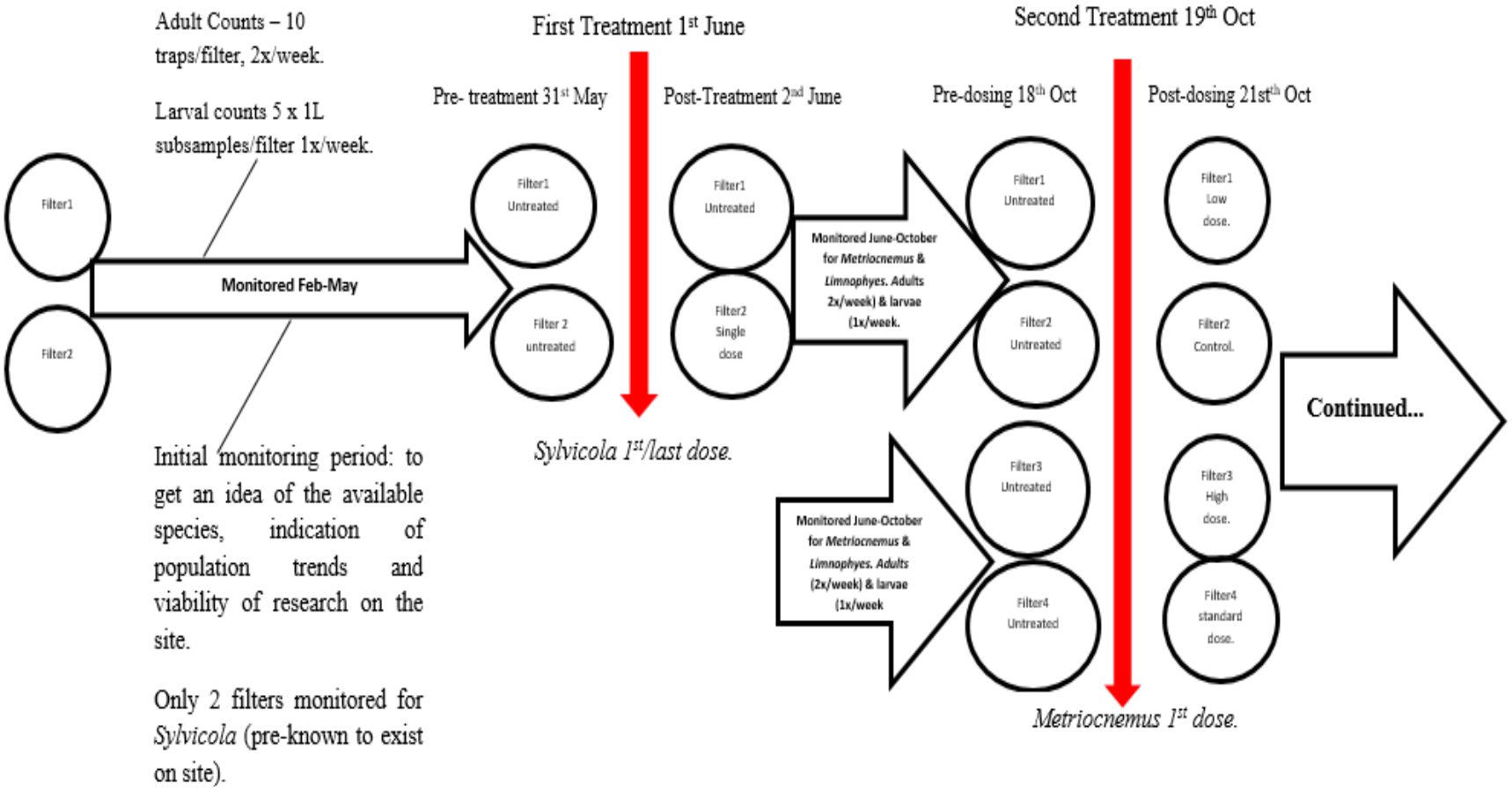


Fig. 8a: Summary of Helsby full experimental design. This ends on next page (Fig. 8b).

Helsby experimental deign continued…

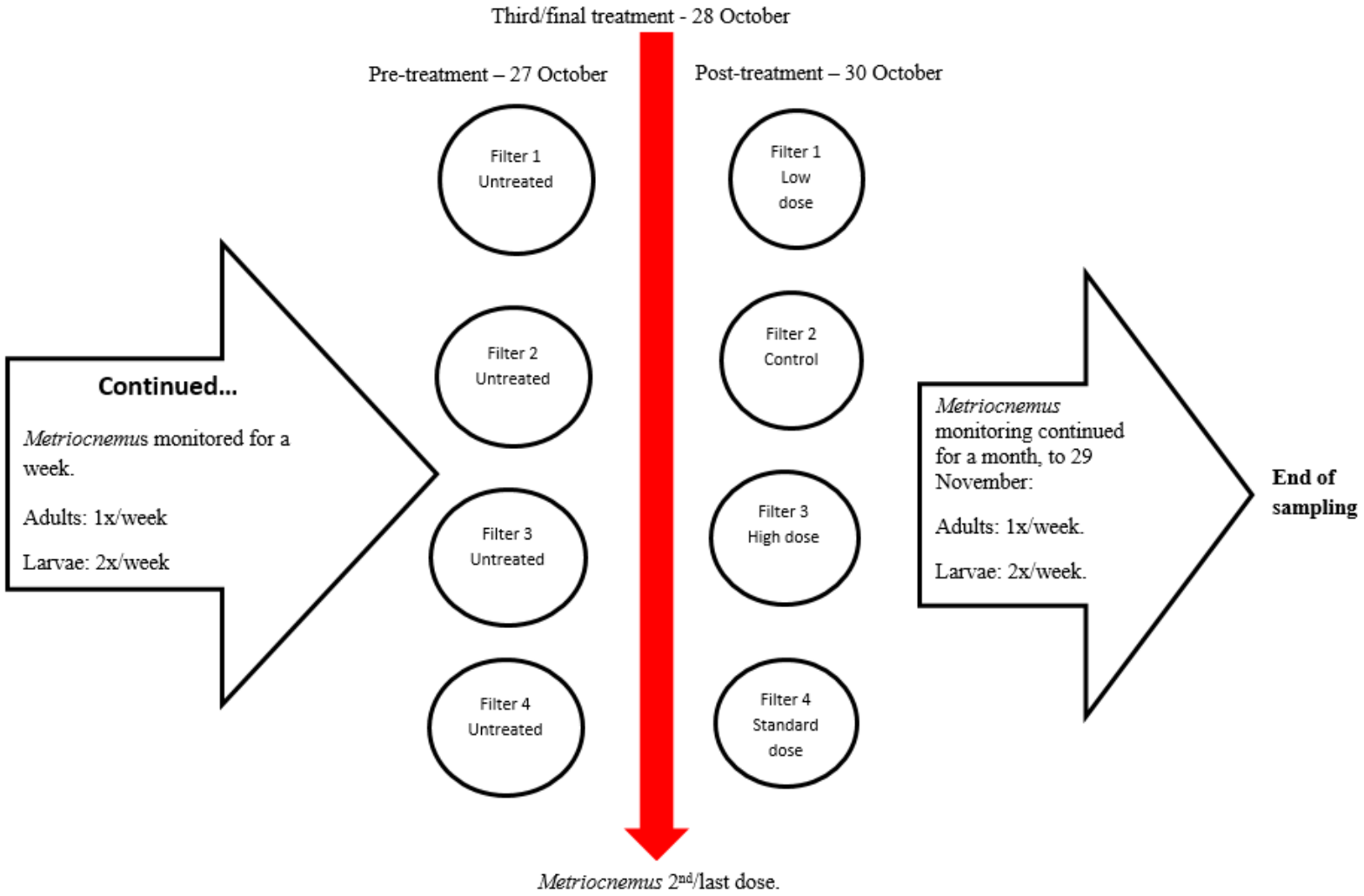


Fig. 8b: Continuation and last page of Helsby experimental design from Fig. 8a.

Macclesfield experimental design

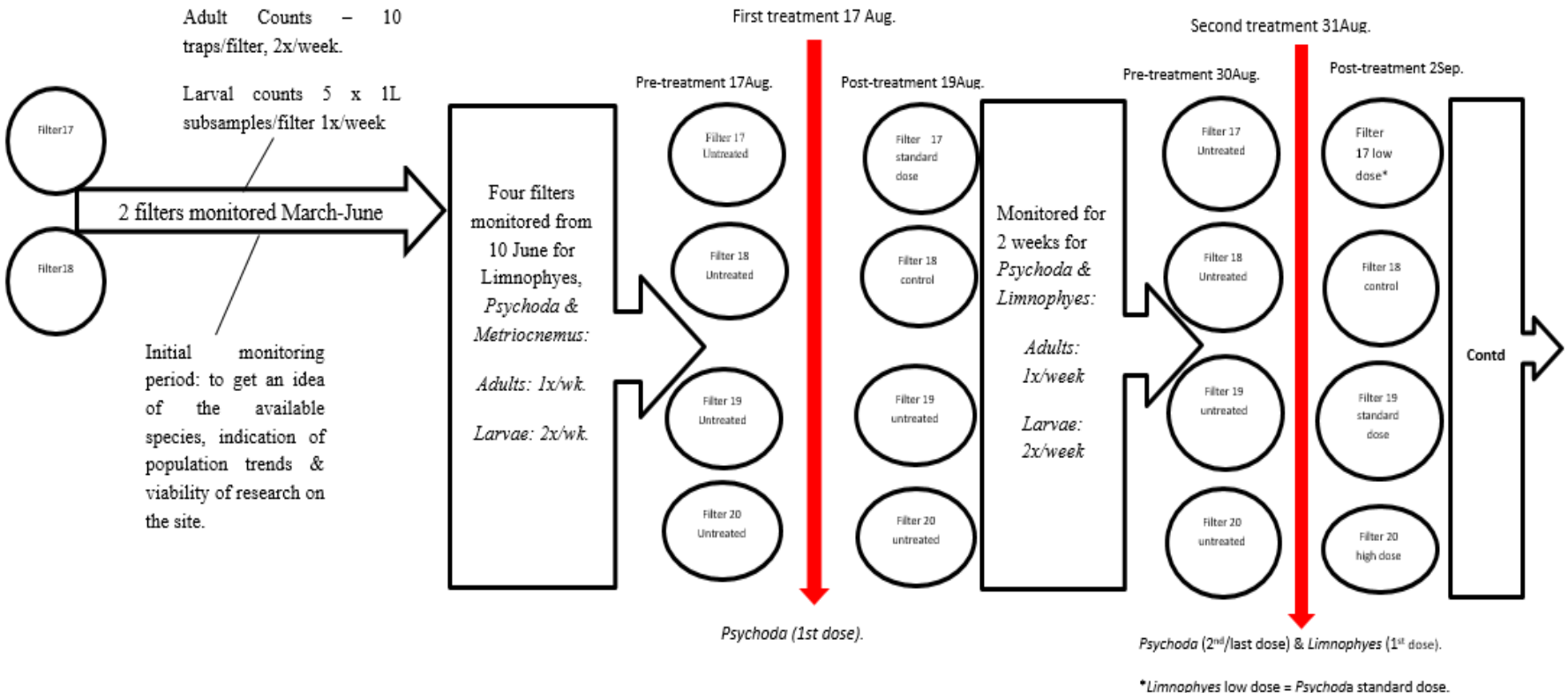


Fig. 9a: Summary of f Macclesfield full experimental design. This ends on next page as Fig. 9b.

Macclesfield experimental design continued….

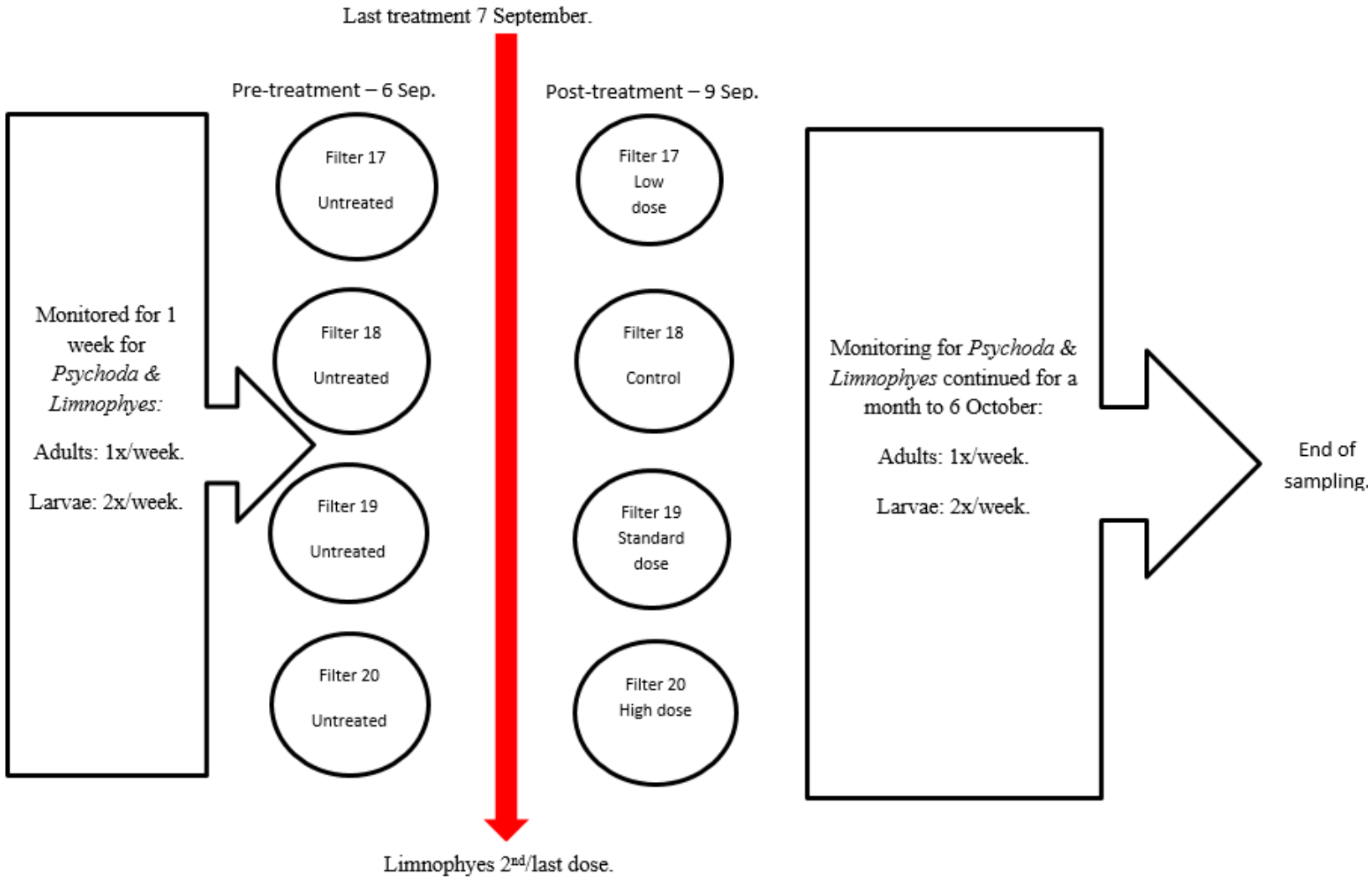


Fig. 9b: Continuation and last page of Macclesfield experimental design from Fig. 9a

2.3 Sampling and laboratory procedures

2.3.1 Sample collection: adult fly trapping

Adult flies were trapped using the same sampling techniques employed by Coombs (1997) and Cleworth, (2006). Rectangular adult emergence perspex boxes with an open bottom, a removable lid top and nylon-mesh ends and measuring 220mm x 120mm x 90mm each were used as fly traps. Solbe *et al*. (1967) are credited with the trap design which ensures conditions inside the trap remain constant with the rest of the filter bed surface.

At the very beginning of sampling, 10 emergence trap boxes were placed on each biological filter at least one meter from the edge and centre, and a few centimetres into the filter bed. In each case, enough clearance was left to avoid contact between flytrap and rotary effluent dispensers above. The trap boxes remained in place for the full duration of research. The removable components (flytrap lids) were marked with identification numbers linked to the biological filter it would be used on throughout. For example, 10 flytrap lids for Macclesfield filter 17 were marked 17_1 to 17_{10}.

Medium size (24.5 x 10cm) pesticide-free sticky traps were acquired from pest-control products suppliers, Agralan Limited, Swindon, UK. Each sticky trap was coated with 'dry' glue on both sides and protected with removable cover paper. One side of the cover paper had each corner cut off to affix the sticky trap to the bottom-facing side of each flytrap lid in advance of trapping. At the time of laying the traps and immediately before each lid was placed in position, the other cover paper was removed to fully expose the sticky under-surface.

Twenty four hours after each trapping session, the flytrap lids and their biological catch were

collected from each filter in medium-size buckets and transported to the laboratory where fly species were identified, counted and recorded. In the meantime, effluent reached the filter media around/below the trap unimpended (Solbe *et al.*, 1967) between the trapping periods.

2.3.2 Adult fly identification and counting

Each sticky flytrap was examined and fly species identified using literature-based morphological descriptions (subsections 1.6.1 – 1.6.4). Where possible, the flies were individually counted. With each sticky trap designed with five rows of 11 equal boxes embossed on its surface, when fly numbers were too high for individual counting, only a single box was counted and the subtotal multiplied by 55 to give an approximate total on the trap. This was repeated with each sticky flytrap with different species counts noted against the subsample numbers.

2.3.3 Disposal of adult flies

After identification and enumeration, the yellow sticky traps and their biological catch were binned. The flytrap lids were washed with tapwater and put aside in preparation for the next trapping cycle.

2.3.4 Sample collection: larvae and NTOs

For dipteran immatures and other filter macroinverebrates, subsampling techniques by Cleworth (2006) were employed. Five 1L plastic beakers were used to randomly collect filter media from each biological filter using a scooping trowel from at least 15-25cm below the surface and at least one metre away from the edge and centre of filterbed. Numbered and segregated according to

biological filter of origin, subsamples were put into 25-litre plastic buckets with lids and transportated to the laboratory.

2.3.5 Larval fly identification and counting

Target species were identified through literature-based morphological descriptions by Fair (1934), Lloyd (1937), Holtje (1943), Satchell (1947), Hawkes (1951), Tokunaga (1953), Oliver (1971), Delettre (1977), Redborg *et al.* (1983), Smith (1989), Coombs *et al.* (1997), Van Poppelen (1998), Robinson (2005) and El Bardicy *et al.* (2009),

The filter media (from each 1L subsample) was thoroughly washed with tap-water from a full 2-litre plastic beaker onto a lipped white plastic tray. Where necessary, a toothbrush was used to clean filter stones and dislodge all macrofauna from their crevices. To keep all biological contents visible on the tray, at least a quarter of the 2-litre tapewater was used each time.

The contents were poured into and mixed with the remaining water in the 2-litre plastic beaker and magnetically stirred to homogenise the distribution of biological media. When a vortex appeared and the circulation looked steady, the contents were pipetted in 25-mililitre (mL) quantities into five petri dishes (=125mL). Contents of each petri dish were examined under a Wild Heerbrugg M3 dissecting microscope (x40) with illumination. In such cases, the number for each species in all five petri dishes were added together before the sum was multiplied by 16 to give the total count in the 2-litre sub-sample. This was repeated with all subsamples for each filter.

2.3.6 Disposal of biological media

The biological media was discarded into the sink. The mechanical media was put aside in 25L

container and returned to original site at the end of each trapping and counting cycle.

2.4 Microscopy and photography

As explained in subsection 2.3.5, larval identification and counting involved use of the specified dissecting microscope whenever these could not be achieved through the naked eye. Developmental stages of target species were also photographed (Chapter 3, table 5) from single cavity well slides (in 90mm petri dishes) on graph paper background using a camera-mounted Leica MZ6 dissecting microscope.

2.5 Product application and treatment schedule

Before product application, rags and other wastewater-blocking material were removed from the outlet orifices of influent rotary distributor arms. From the treatment works' main inlet flow recorders, the tonnage of wastewater arriving at the site and distributed to each filter per second was noted. For each treatment strength (or for each test filter), a 110v Watson and Marlow peristaltic pump was calibrated to deliver the product within 30 minutes at the wastewater inflow. The total amount of product applied in each case was dependent on the desired treatment strength and amount of effluent being dosed onto the filter beds every second at time of treatment.

For example, in accordance with Valent (2016) recommended dosing protocol, the total amount of product required and used in one of the treatments against *Psychoda* at Macclesfield was calculated after monitoring the works inlet flow for 3mins. Main inlet feeding the works split into two lanes in such a way that 60% of the influent fed 20 filters in the section of the WWTW where the study was carried out and the other 40% fed the other 10 filters. Although the works' designed maximum

flow was 710L/second and the Dry Weather Flow (DWF) was 230L/second, main inlet flow recorders showed an average flow of 365L/second at the time of the trials. Therefore, flow to the works over 30 minutes: 365 (litres) x 1,800 (seconds) = 657,000 litres. With 60% of this going to the section of works concerned with this study, flow to each filter: 394,000 (L) / 20 (filters) = 19,710L. Therefore, at *Psychoda*'s recommended dose rate of 0.781mL per litre of influent, a single filter required: 19,710 x 0.781= 15.39L of the larvicide to be delivered over 30 minutes. A round figure of 15.5L was used to achieve a final product concentration of 80mg/L. Therefore, a different average flow rate would have required a different quantity of the product to achieve the same desired final concentration.

A total of four target fly species were monitored on both research sites in this study with two species targeted for larviciding per site. Three treatment strengths were used against Chironomid species, *L. minimus* and *M. eurynotus* on three different filters with the fourth untreated filter used as control. For the other species, *S. fenestralis* and *P. alternata*, one standard treatment strength was used on one filter with one untreated filter acting as a control for each. Treatment against *S. fenestralis* was done only once with each of the other three species targeted twice within one to two weeks. The treatment schedule (including biological filters and treatment strengths used) is shown below (table 3).

Table 3: Schedule of treatment against target species (in chronological order) also showing designated control and test-beds including treatment strengths used on them against each species.

Treatment dates (2016)	WWTW	Target species	Test filter no. (*)	Test filter no. (**)	Test filter no. (***)	Control filter no.
31-May	Helsby	*S. fenestralis*	-	2	-	1
17-Aug	Macclesfield	*P. alternata*	17	-	-	18
31-Aug	Macclesfield	*P. alternata*	17	-	-	18
31-Aug	Macclesfield	*L. minimus*	17	19	20	18
07-Sep	Macclesfield	*L. minimus*	17	19	20	18
19-Oct	Helsby	*M. eurynotus*	1	4	3	2
28-Oct	Helsby	*M. eurynotus*	1	4	3	2

Notes: *: Low dose, BTI concentration of 80mg/L

**: Medium dose, BTI concentration of 160mg/L (also only dose against *S. fenestralis*)

***: High dose, BTI concentration of 230mg/L

2.6 Recording data

Adult target species were counted and recorded per trap (10 per filter) (A1) before being averaged to give mean number for flies per trap (A2). Larval target species and NTOs were counted and recorded per litre of filter media, (5 x 1L-subsamples per filter) (A3) before also being averaged out to give a mean number per litre (A4). These were all rationalised for subsequent statistical analysis (A5-A10).

Only a few excel worksheet extracts from the huge body of data are attached at the end of this report to show how the population counts and the data generated was stored and how final statistics were handled.

2.7 Statistical analysis

Minitab 16 was the main statistical software package used with Excel also used for tests that could not be computed by the former. Fly counts before and after larviciding were tested for normal distributions and variances to help in choosing the appropriate statistical tests. Pre- and post-treatment counts of each test-bed and post-treatment counts of different filters were then statistically tested to gauge the impact of treatment and different treatment concentrations.

Paired observations from the same biological filter were compared using paired T-test with the Wilcoxon Signed rank test used as the non-parametric alternative. Two-Sample T-test was also used to compare the difference between means of samples from two different filters. To test the equality of means of more than two filter-populations, One-Way Anova was used. The non-parametric alternative, Kruskal-Wallis test was used to compare more than two filter population medians (not means). Where Kruskal-Wallis test yielded a significant result, it does not show where the difference(s) lie making it necessary for further tests. This was achieved through pairwise comparisons of filter population means using Mann-Whitney test, the non-parametric alternative to Two-sample t-test.

The choices between paired T-, two sample T-tests and their non-parametric alternatives were made based on the results of Kolmogorov-Smirnov normality test of each filter-population distribution.

For each test of normality, the output was in the form of a probability plot accompanied by descriptive statistics. Whenever data points fell close to the reference line, it meant the data was normally distributed and appropriate for parametric test if the other data set also followed a similar distribution. In such cases, the statistical outputs always comprised of a non-significant ($P > 0.05$) result~~s~~. The opposite is/was correct for alternative tests. On the other hand, the choice between One-Way Anova and the non-parametric alternative, Kruskal-Wallis test was decided by the output of Bartlett's and Levenes' (B and L) tests of equal variances of filter-population counts compared. Non-significant ($P > 0.05$) results in both B and L tests called for parametric test, One-Way Anova.

Differences between/among test-beds and control beds were evaluated before treatment was started (Table 4). All the biological filters were found to have no significant differences ($P > 0.05$) in larval and adult populations except in the case of the Chironomid species, *L. minimus and M. eurynotus* which showed significantly lower adult counts in the control filter prior to treatment ($P < 0.05$). This was not considered a major problem since the immediate target of the biocide under investigation is/was the larval stages.

Table 4: Adult and larval counts and statistical test results before first treatment against each target species. Filter mean counts are classified according to treatment strengths (low to high) planned for use on them.

Target species		Filter mean counts				Test used	T-value	DF	P-value	Meaning
		Control	Low (80mg/L)	Medium (160mg/L)	High (230mg/L)					
S. fenestralis	Adult	4.6	-	7.2	-	2-sample T-	2.01	18	0.06	NS
	Larvae	37.8	-	40.8	-	2-sample T-	0.16	8	0.879	NS
P. alternata	Adult	303.4	468.3	-	-	2-sample T-	1.98	13	0.069	NS
	Larvae	124.8	227.2	-	-	2-sample T-	0.93	7	0.385	NS
							H			
L. minimus	Adult	146.6	96.9	198.9	279	KWT	13.04	3	0.005	S
	Larvae	12.8	35.2	9.6	6.4	KWT	6.56	3	0.087	NS
M. eurynotus	Adult	46.0	65.6	101.5	87.9	KWT	12.67	3	0.005	S
	Larvae	115.2	208	198.4	211.2	KWT	4.54	3	0.209	NS

Notes: S: significant; NS: not significant; KWT: Kruskal-Wallis test.

Chapter 3. Results

3.1 All target species

At Helsby, a total of 184,851 adult flies made up of 129,586 *M. eurynotus;* 48,627 *L. minimus;* 3,718 and *P. alternata* 2,920 *S. fenestralis;* were recorded over the full research period. These are summarised (Fig. 10) as percentages of total count below.

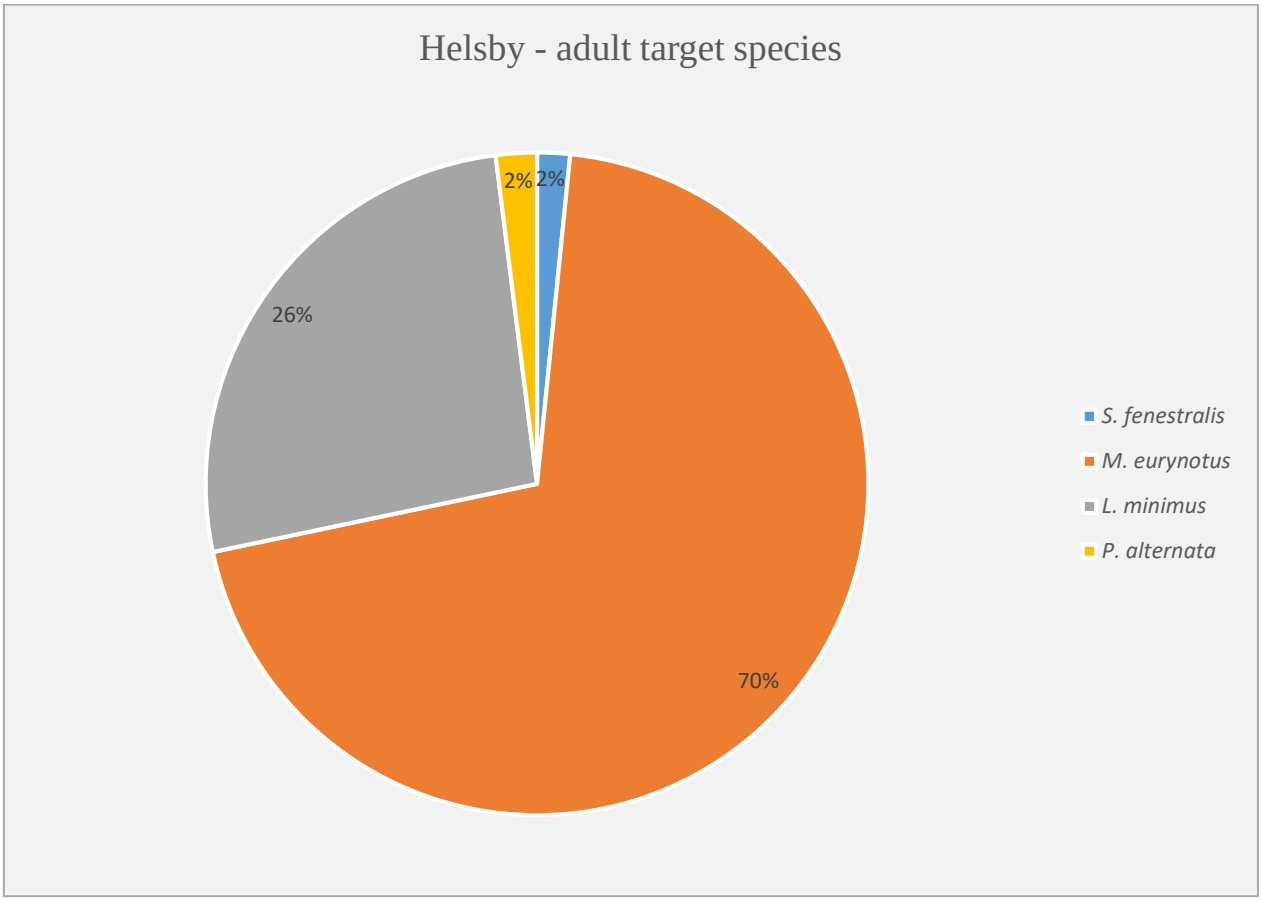


Fig. 10: Adult target flies recorded at Helsby between February and November 2016.

At the same site and over the same period, a total of 99,468 larval species composed of 10,257 *S. fenestralis*, 71,645 *M. eurynotus*, 16,843 *L. minimus* and 723 *P. alternata* larvae were recorded (Fig. 11).

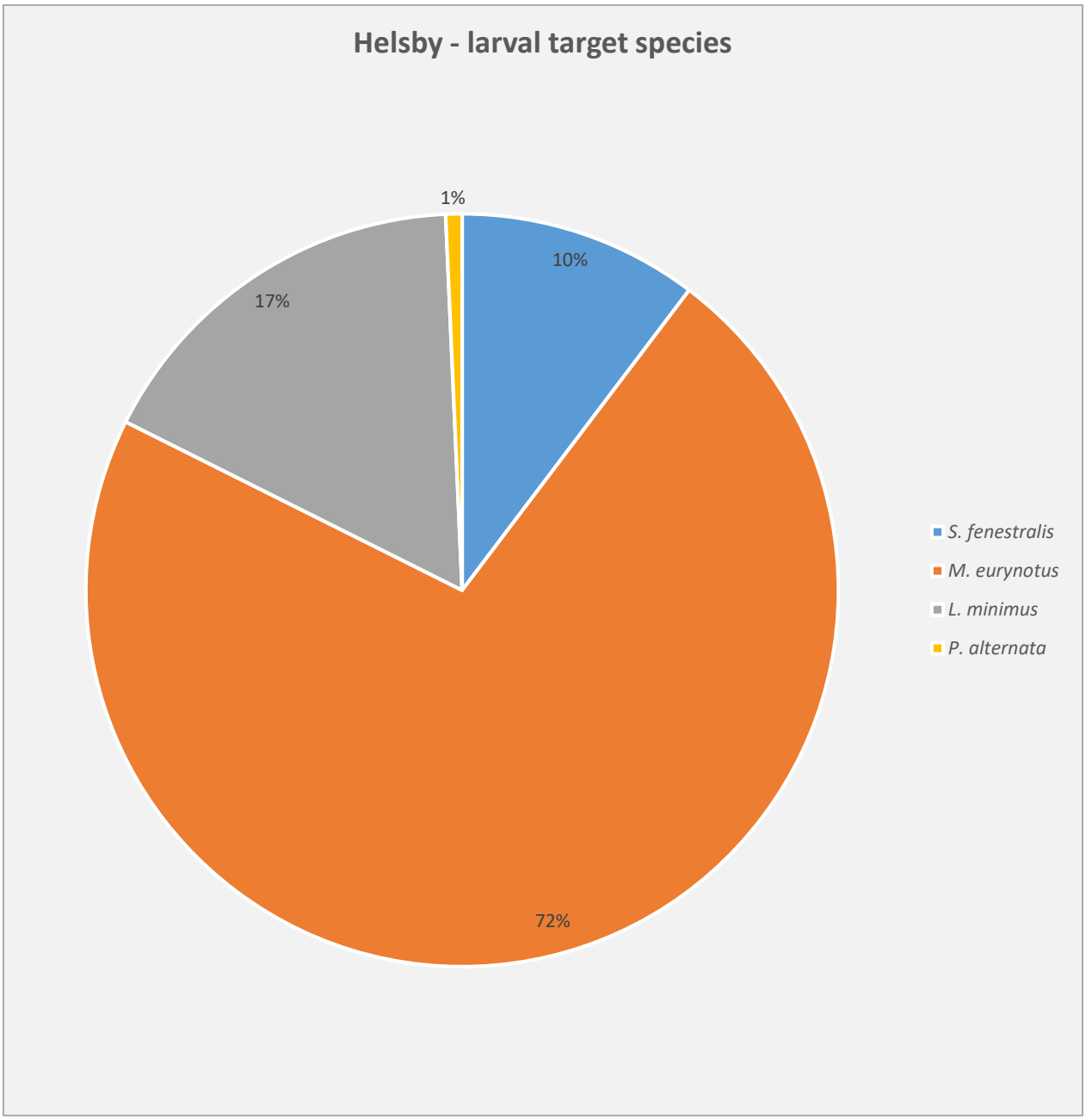


Fig 11: Target larval flies recorded at Helsby between March and November 2016.

At Macclesfield, a total of 447,254 adult flies were recorded over the full research period. This was made up of 1,831 were *S. fenestralis*, 83,484 *M. eurynotus*, 110,684 *L. minimus* and 251,255 *P. alternata*. These are also summarised (Fig. 12) as percentages of total count

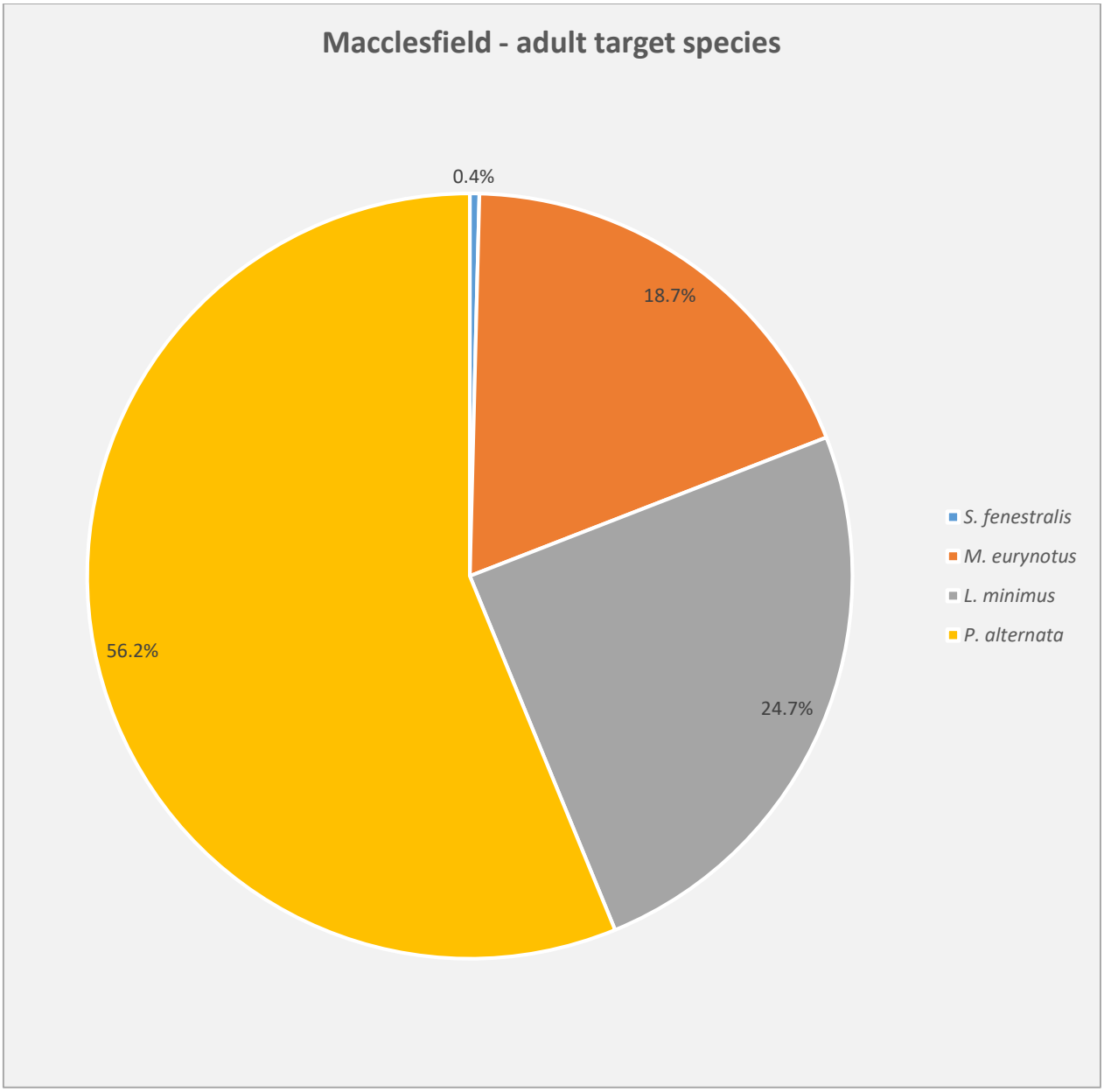


Fig 12: Target adult flies recorded at Macclesfield between March and November 2016.

At the same site and over the same period, a total of 170,162 larvae were recorded made up of 10,257 *S. fenestralis*, 71,645 *M. eurynotus*, 16,843 *L. minimus* and 723 *P. alternata*. These are summarised (Fig. 13) and expressed as percentages of total count.

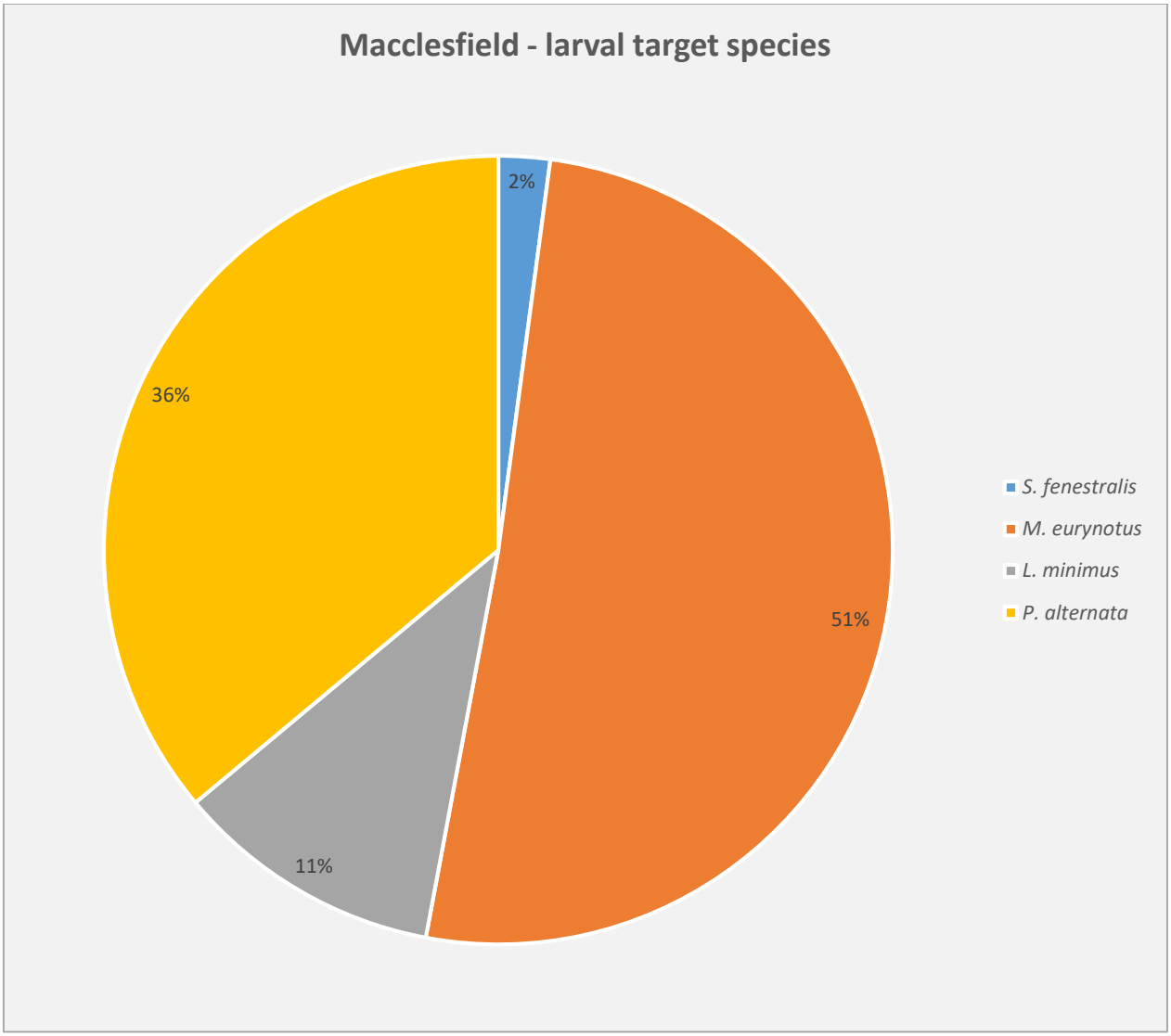


Fig 13: Larval species recorded at Macclesfield over full research period - March to November 2016.

The above total adult and larval populations for both sites were plotted together (Figs 14 and 15) below to show which site had more or less of each species.

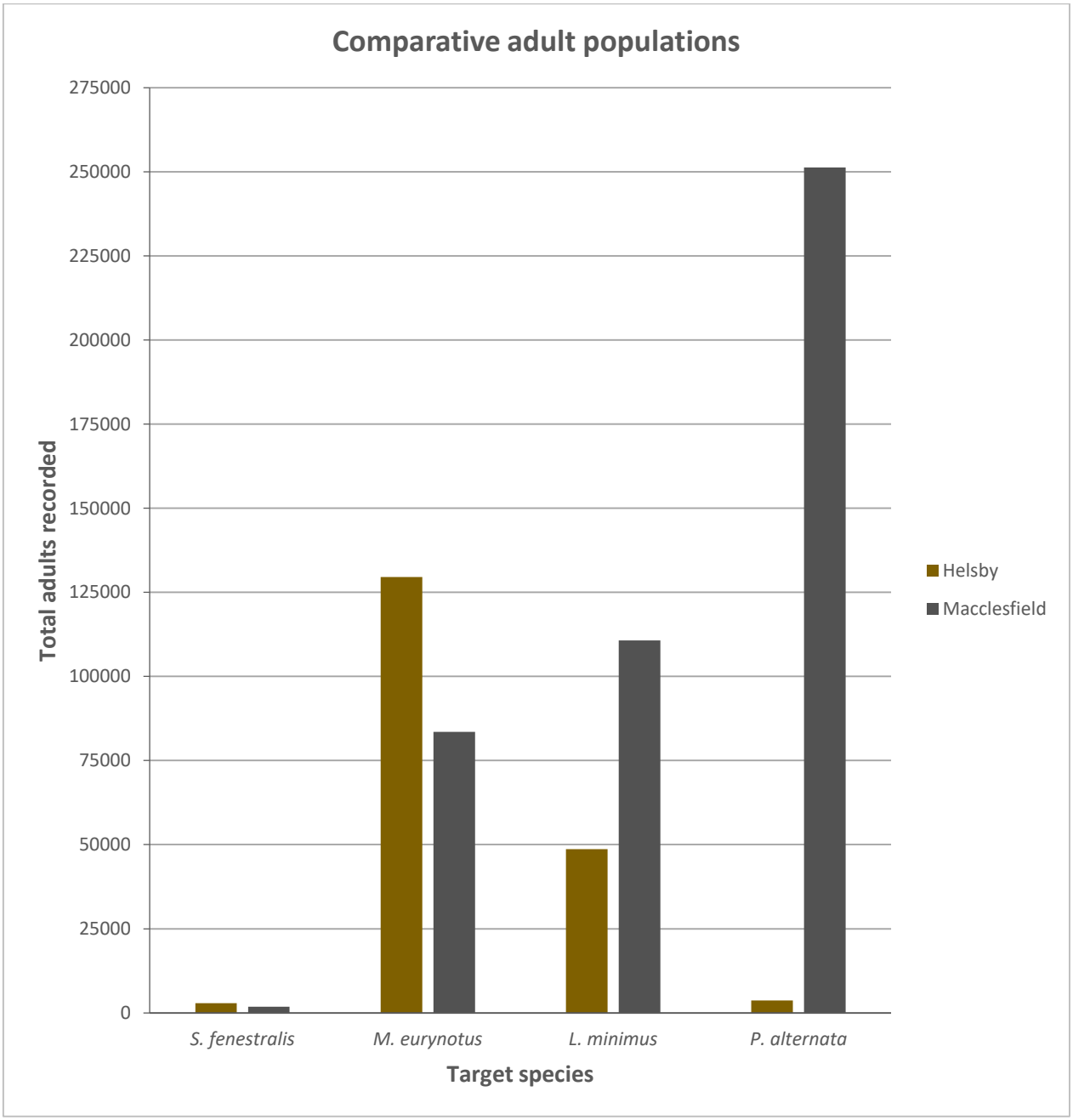


Fig. 14: Adult target species recorded at both sites over the full research period.

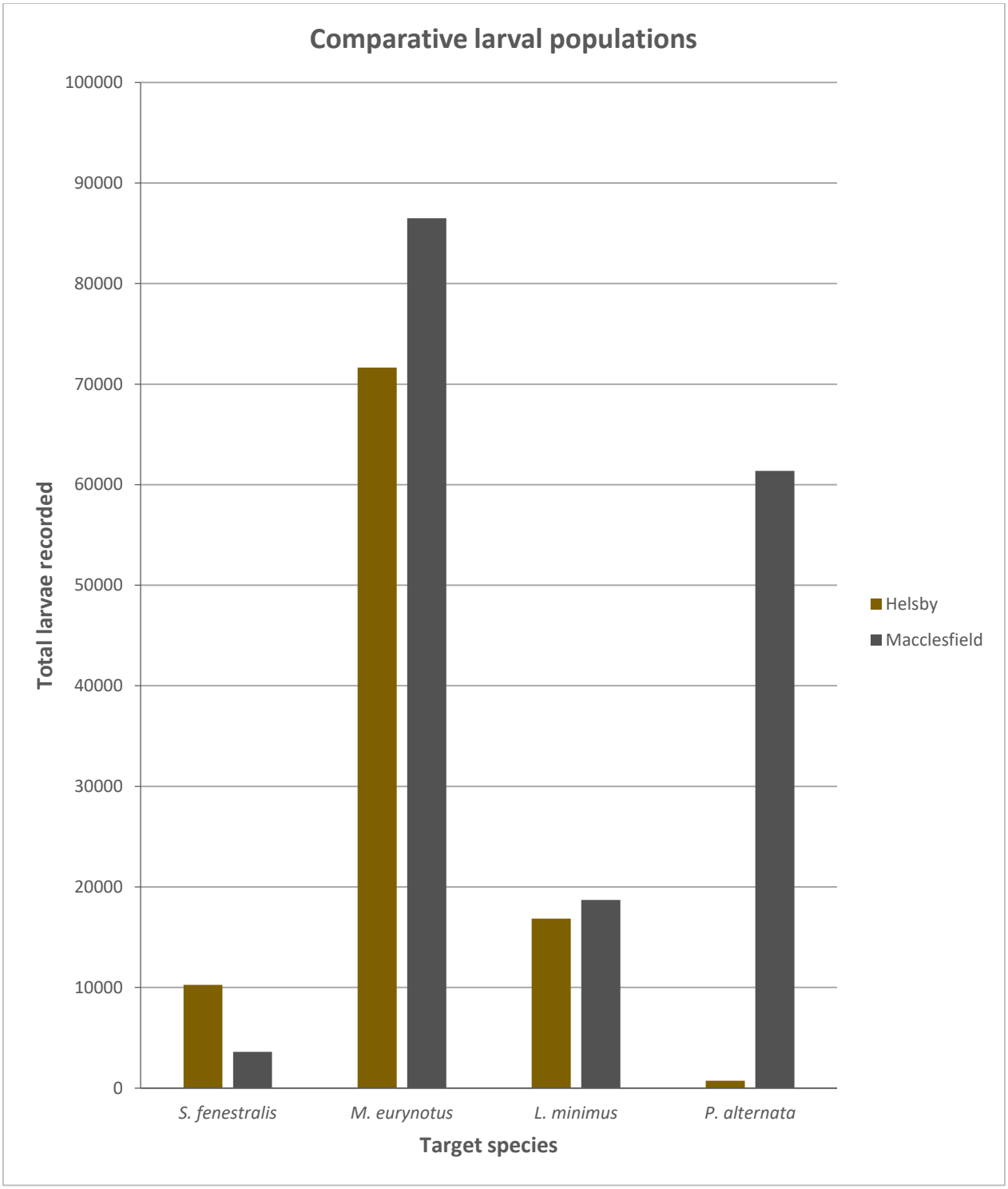
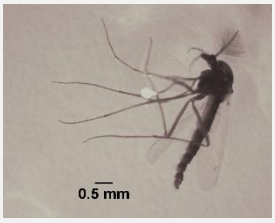
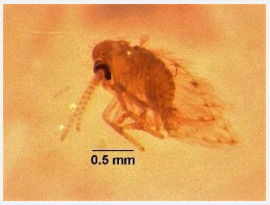
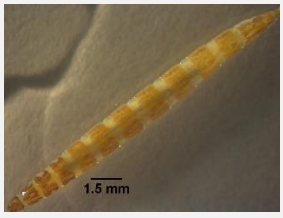
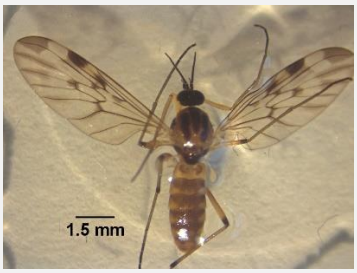


Fig 15: Larval target species recorded at both sites over the full research period.

Adult and larval stages were of particular interest to this study. The former are the ones that cause a public nuisance with the latter being the stage susceptible to the biolarvicide under investigation. The target species found and reported in this text had their developmental stages captured (Table 5).

Table 5: Examples of developmental stages of each target fly species captured in this study using a camera-mounted Leica MZ6 dissecting microscope (without distinguishing larval-instars).

Target species	Larvae	Pupae	Adult
L. minimus			No clear image
M. eurynotus			
P. alternata			
S. fenestralis			

3.1.1 *Sylvicola fenestralis* (window gnat) – Helsby WWTW

Population patterns of adult *S. fenestralis* (Fig 16) show that numbers dropped to nil 17 days after treatment and remained supressed for approximately three and half months. Adult population counts for the untreated filter were lower than the treated filter to start with but showed a similar pattern of decline, taking longer to reach low levels (21 days) indicating that there was a natural decline in fly numbers around the time of treatment.

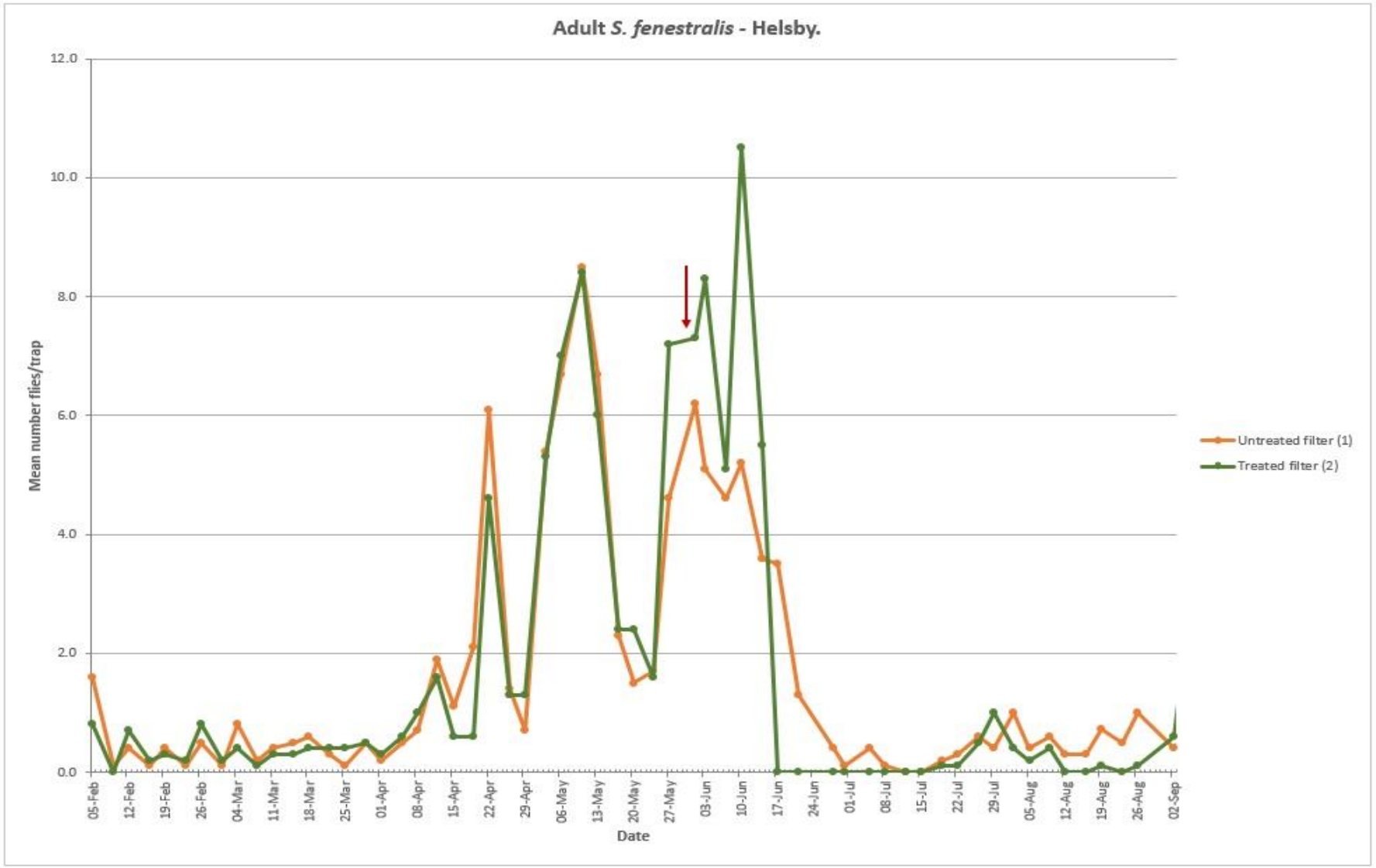


Fig. 16: Adult *S. fenestralis* population trends before and after treatment at Helsby. The red arrow indicates the date of single treatment with the recommended dose concentration of 160mg/L.

A summary of larval *S. fenestralis* population fluctuations (Fig 17) showed that larval numbers in the treated filter dropped quickly to zero within 48 hours of treatment and remained low for a further three and half months. In the untreated filter, larval numbers had a more gradual decline taking 27 days to reach zero.

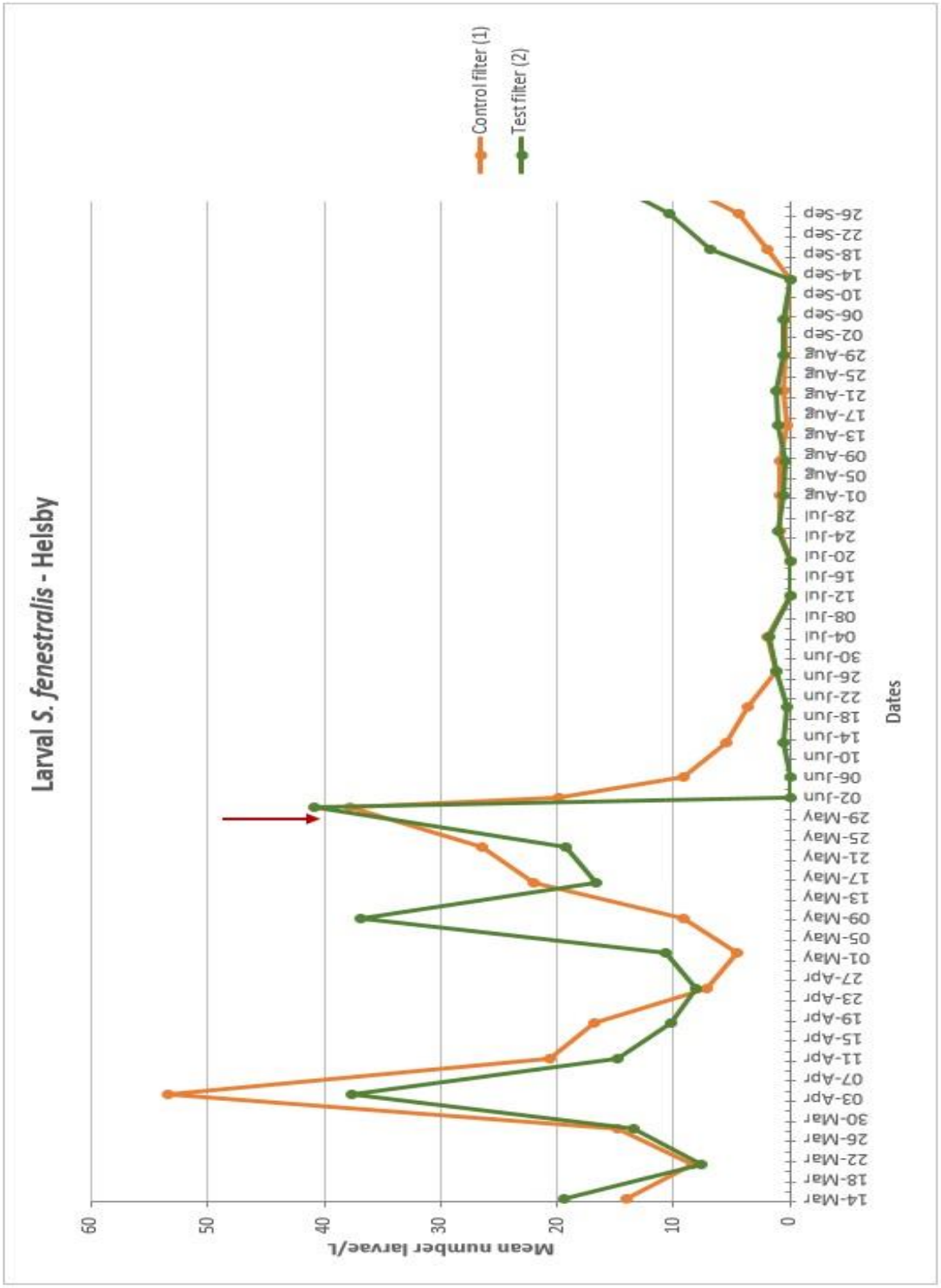


Fig. 17: Larval *S. fenestralis* population trends before and after treatment at Helsby. The red arrow indicates the first and last date of treatment with the recommended dose concentration of 160mg/L.

Twenty-three days after treatment against *S. fenestralis* at Helsby, the untreated filter (1) adult counts had dropped from a mean of 4.6 ± 0.86 to 3.5 ± 1.54 flies per trap which was not significant (Wilcoxon; P = 0.415). On the other hand, adult counts of the treated filter (2) dropped from 7.2 ±0.97 to zero flies per trap over the same period which was a significant drop (paired T-test; T = 7.47; P < 0.001). The adult counts of untreated and treated filters showed significant difference (two-sample T-test; T (18) = 2.28; P = 0.035) 23 days after treatment.

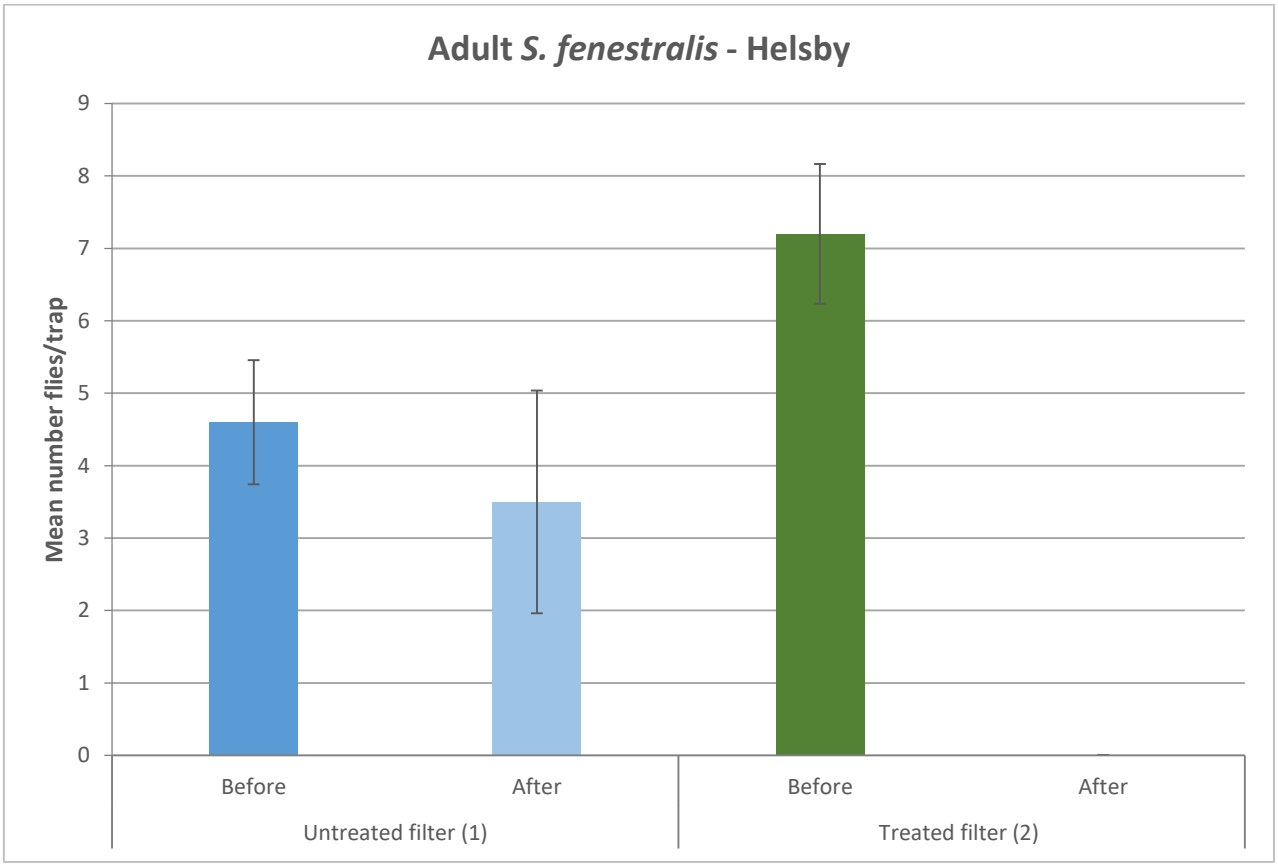


Fig. 18: Comparative pre- and post-treatment counts of treated and untreated filter beds for adult *S. fenestralis* at Helsby. This is based on mean number (±SE) of flies recorded on each adult emergence trap four days before treatment and 23 days after.

Forty-eight hours after treatment against *S. fenestralis* at Helsby (Fig. 19), untreated filter (1) larval counts had dropped from an average of 37.8 ±10.03 to 19.8 ±6.98 larvae per litre which was not a significant reduction (paired T-test; T = 2.68; P = 0.55). Over the same period, larval counts of treated filter (2) dropped from an average of 40.8 ±16.31 to 0 flies per litre which was not a significant drop (paired T-test; T=2.50, P=0.067). Although there was no significant difference (table 4) between the mean larval counts of the control and test filter-beds before product application, the two filters showed a significant difference (Mann-Whitney, P = 0.0075) was noted 48-hours post-treatment.

Fig. 19: Comparative pre- and post-treatment counts of treated and untreated filter beds for larval *S. fenestralis* at Helsby. This is based on mean number (±SE) of larvae found in each litre of filter media the day before product application and 48 hours after.

3.1.2 *Psychoda alternata* - Macclesfield WWTW

Adult *P. alternata* population fluctuations at Macclesfield (Fig. 20) indicate that the mean number of flies per trap in the control/untreated filter initially rose to around 400 immediately after the first treatment and dropped to around 300 after a week and rose again after the second treatment. They remained high (above 500 flies per trap) before dropping to below 300 after two weeks for approximately one month. In the treated filter however, the mean number of adult flies dropped from around 500 to 68 in 14 days after the first treatment. This was immediately followed by second treatment and mean number of flies remained at low levels (below 100 flies per trap) for about one month.

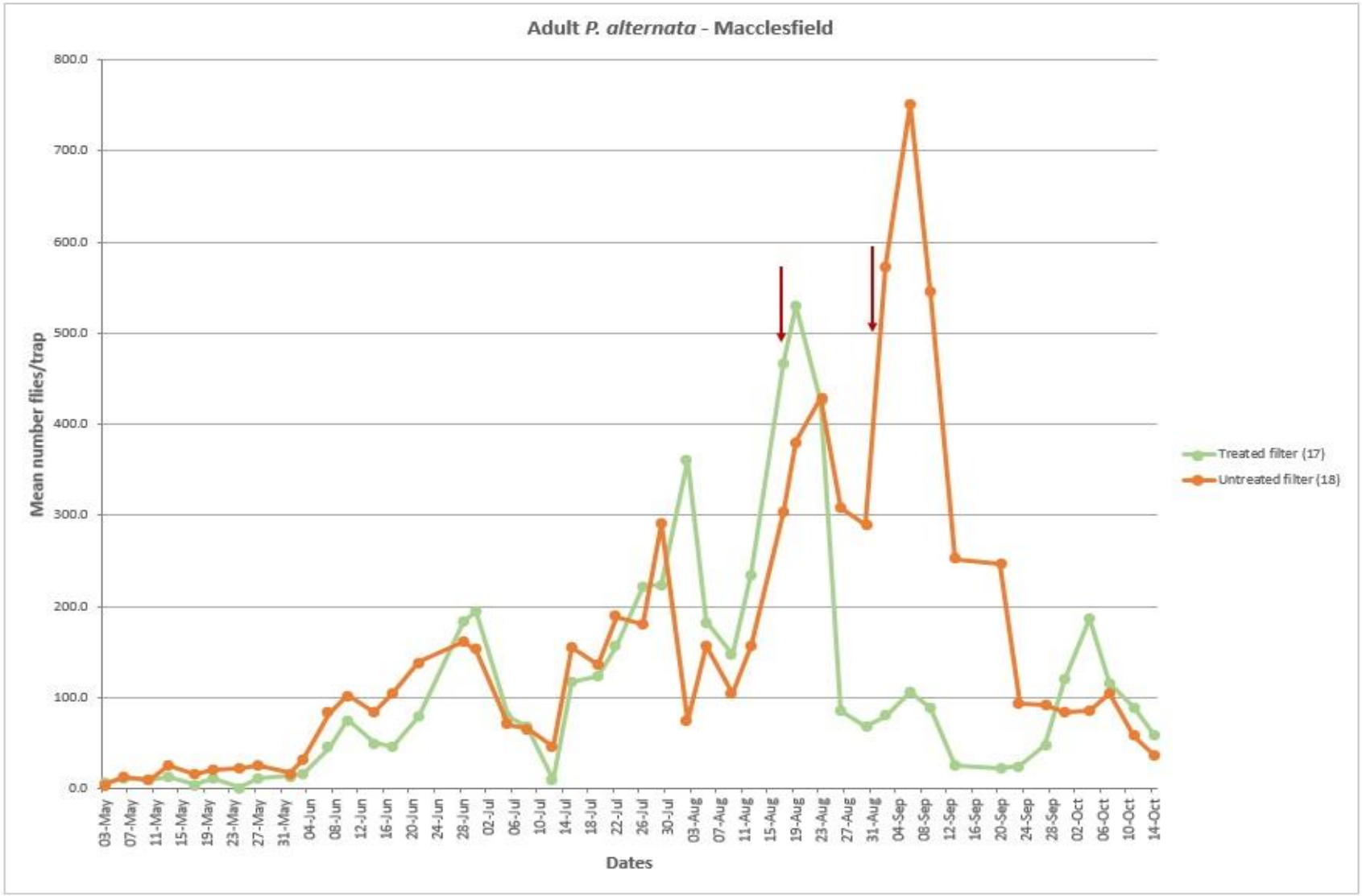


Fig. 20: Adult *P. alternata* population trends. Red arrows indicate points of first and second treatment with the recommended dose concentration of 80mg/L.

Larval numbers at Macclesfield (Fig. 21) showed an immediate drop within 48 hours after the first treatment and remained low for 12 days. After the second treatment, larval numbers remained low (below 50) for a further two weeks. In the untreated filter larval numbers fluctuated between 105 and 388 only dropping to below 100 almost one month after first treatment and/or about two weeks after the second treatment.

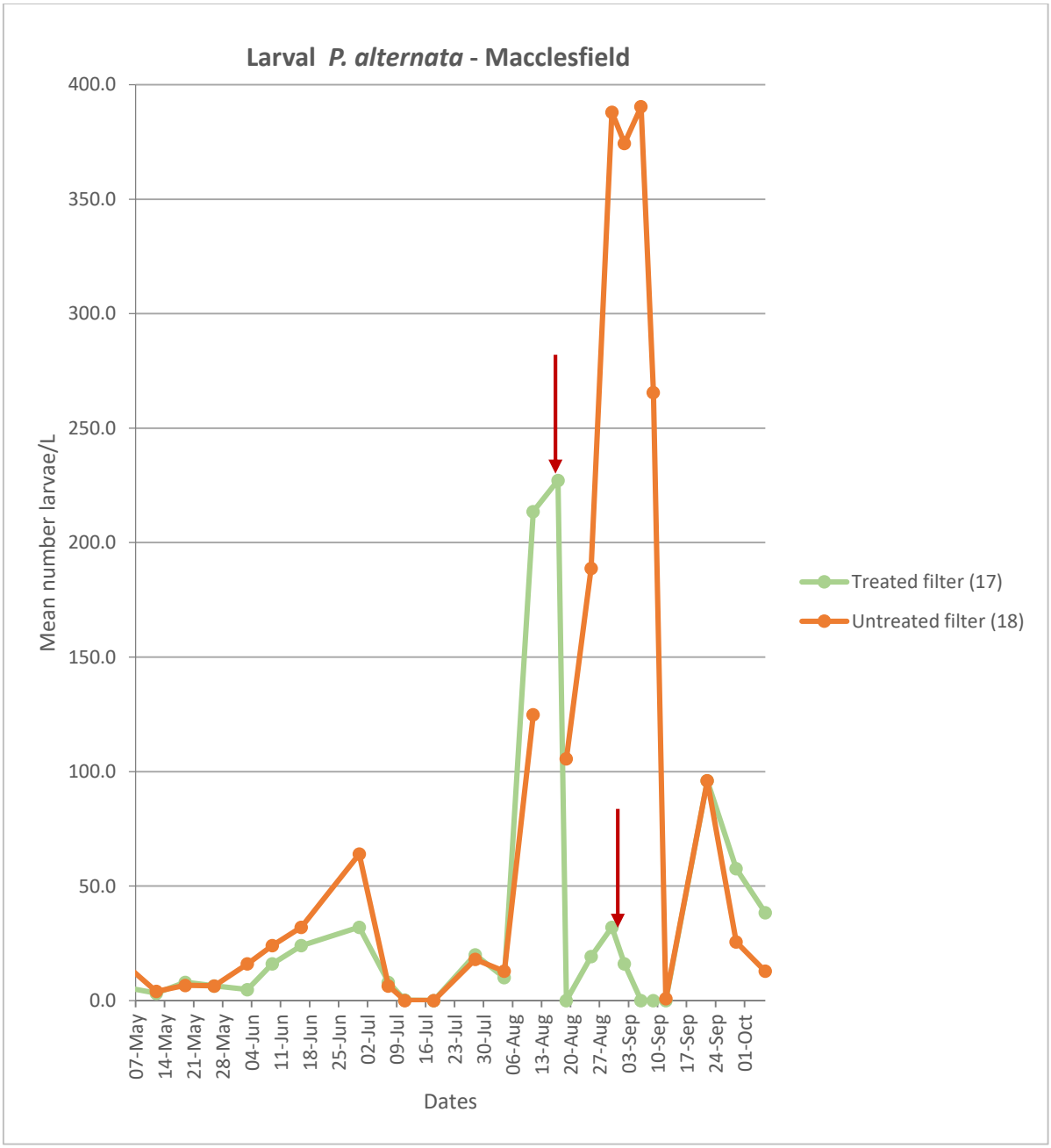


Fig. 21: Larval *P. alternata* population trends. Red arrows indicate points of first and second treatment with the recommended dose concentration of 80mg/L

Twenty-three days after first treatment against *P. alternata* at Macclesfield, the adult counts of the untreated filter (18) increased from an average of 303.4 ±36.39 to 545.7 ±62.98 flies per trap, representing a significant increase (paired T-test, T = 3.58, P = 0.0006) in fly numbers. However, a highly significant adult fly decrease (paired T-test, T = 5.40; P < 0.001) was noted in the treated filter over the same period after counts dropped from an average of 468.3 ±74.92 to 88.5 ±14.55 flies per trap. When the two filters adult counts from 23 days after the first treatment were compared against each other, the treated filter (17) showed an extremely significant reduction (two-sample T-test, T (9) = 7.07; P<0.001) in emerging flies compared to the untreated/control filter (18) (Fig. 22).

Twenty-three days after the second treatment, the control filter (18)) adult fly numbers had significantly dropped from an average of 290 ±36.77 to 93.1 ±9.23 flies per trap (paired T-test, T = 4.93; P = 001) indicating a natural population decline at this time. A high adult fly reduction (paired T-test, T = 5.82; P < 0.001) was also found in the treated filter, with counts dropping from an average of 67.7 ±9.68 to 24.8 ±4.59 per trap. This reduction in the mean number of emerging flies, was significantly greater (two-sample T-test, T (13) = 6.63; P < 0.001) than that observed in the control filter (Fig. 22).

When *P. alternata* emergence from the treated filter (17) was looked at across the full two-part treatment cycle, an extremely significant (paired T-test, T=6.01; P<0.001) fly reduction was observed after adult counts dropped from an average of 468.3, before the first treatment, to 24.8 per trap 23 days after the second/last treatment against the species (Fig. 22).

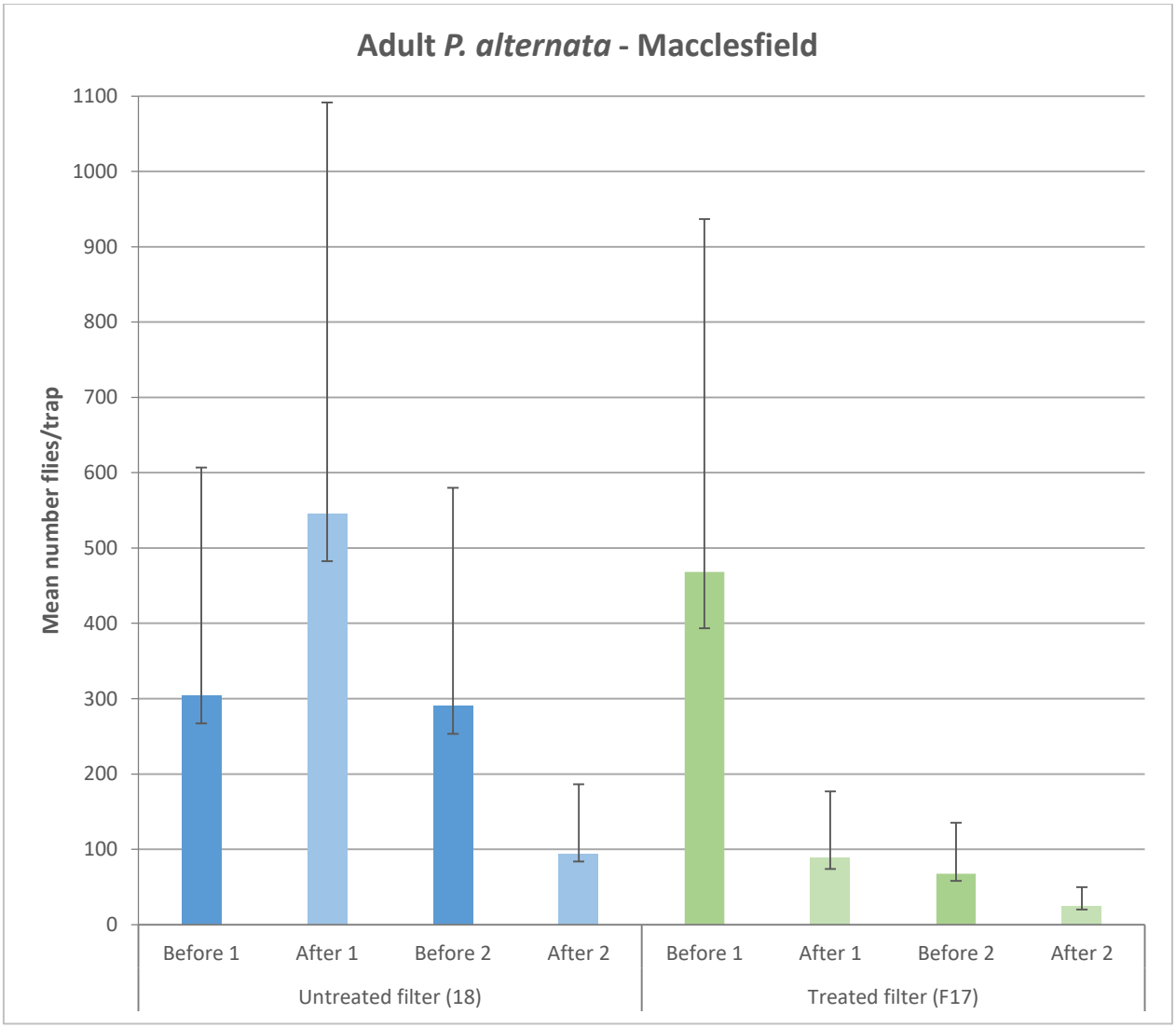


Fig. 22: Adult *P. alternata* pre- and post-treatment population counts at Macclesfield based on the mean number (±SE) of flies recorded from each adult emergence trap on day of (immediately before) first treatment and 24 hours prior to second treatment and 23 days after each treatment.

In relation to *P. alternata* larval counts at Macclesfield, (Fig. 23), the untreated filter showed little population changes (paired T-test, T = 0.231; P = 0.831) only dropping from a mean of 124.8 ±62.67 to 105.6 ±44.86 larvae per litre 48 hours after the first treatment. Over the same period, mean larval counts in the treated filter showed no significant statistical reduction (paired T-test, T = 2.49; P = 0.067) despite dropping from 227.2 ±91.13 to 0 larvae per litre. When the control and

treated filters were compared 48-hours after the first treatment, the treated filter (17), with zero larvae, had a significantly lower number than the control (18) (two-sample T-test, T (8) = 2.354; P = 0.046).

Prior to the second treatment, the untreated filter (18) larval counts had increased to a mean of 387.2 ±89.57 larvae / litre and showed no significant reduction (paired T-test, T = 0.10; P = 0.926) after recording a mean of 374.4 ±96.74 larvae/litre, forty-eight hours after the second treatment. At the same sampling points, the treated filter (17) showed a reduction in larval numbers from 32 ±20.86 to 16 ±7.16 but this was not significantly different (paired T-test, T = 0.61; P = 0.576). In comparing larval numbers over the two treatment cycle there was a highly significant difference between the control (374.4 ±96.74 larvae / litre) and the treated filter (16 ±7.16 larvae/ litre) at the end of the sampling period (two-sample T-test, T (4) = 3.69; P = 0.021) and in comparing the test filter, before (227) and after two treatments (16) (paired T-test, T = 2.31; P = 0.02).

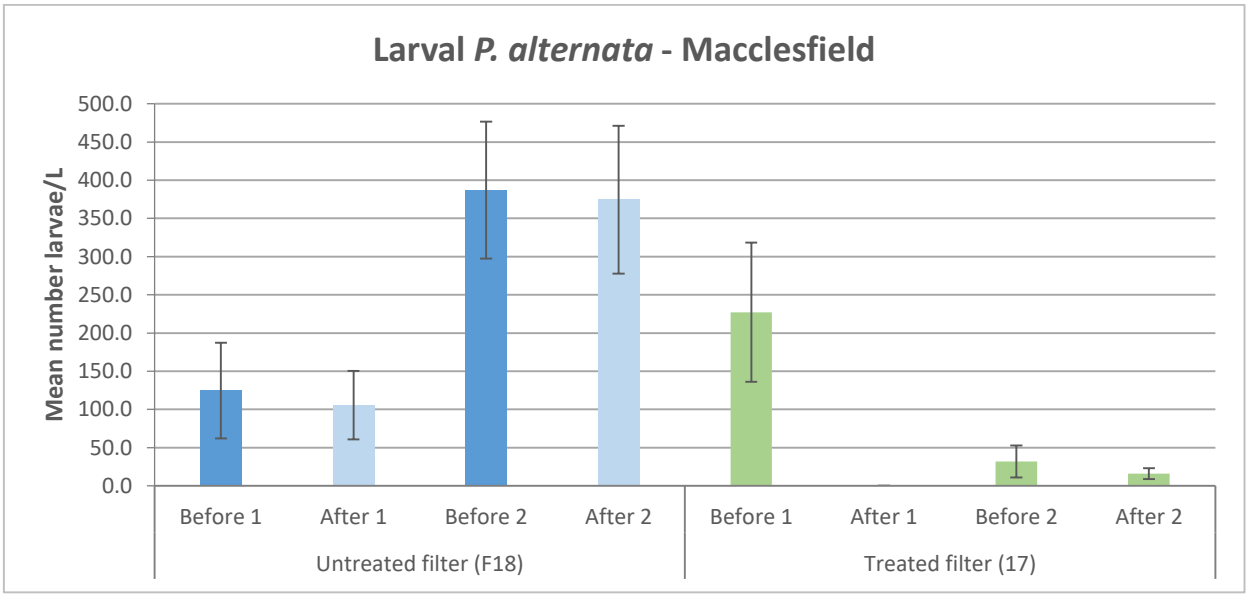


Fig 23: Larval *P. alternata* pre- and post-treatment mean larval counts (±SE) at Macclesfield. Data categories refer to counts done before or after first or second treatment. These were taken on the day of (and before) first treatment, 24 hours before the second treatment and 48 hours after each treatment.

3.1.3 *Limnophyes minimus* - Macclesfield WWTW

Adult *L. minimus* population patterns at Macclesfield (Fig. 24) showed that adult counts in all filters including the control dropped within a week of the first treatment and were all lower than pre-treatment counts 28 days after treatment. However, within that time period, counts in the control filter rose to over 80 flies per trap at a time when all treated filters had below 30.

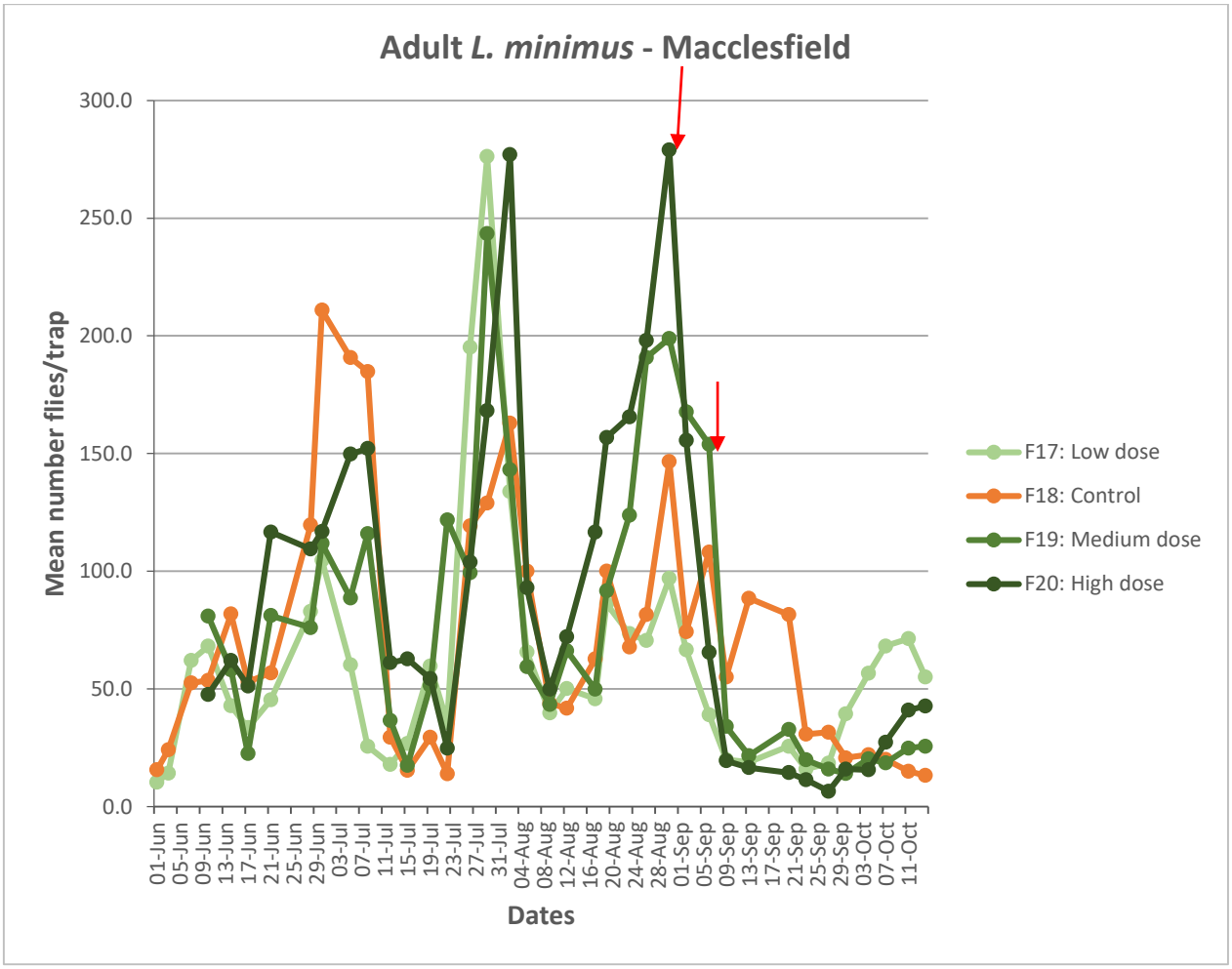


Fig. 24: Adult *L. minimus* population trends. Red arrows show the first and second treatment points with three different product concentrations on each of the three test filter beds - low dose (80mg/L), medium and recommended dose (160mg/L) and high dose (230mg/L).

Larval population patterns for *L. minimus* at Macclesfield (Fig. 25) indicated that mean counts were mostly below 50 larvae per litre prior to any treatment. However, after the first treatment, all filters except the control and medium-dose filter dropped to zero levels. The medium (recommended) dose filter counts only dropped to zero after the second treatment. Larval populations in all filters but one began to return to pre-treatment levels 22 days and 15 days after the first and second treatments. The high-dose filter was the exception taking four to five weeks after the two treatments to recover. Interestingly, the control populations dropped off 12 days after the first treatment but showed an increase after ten days.

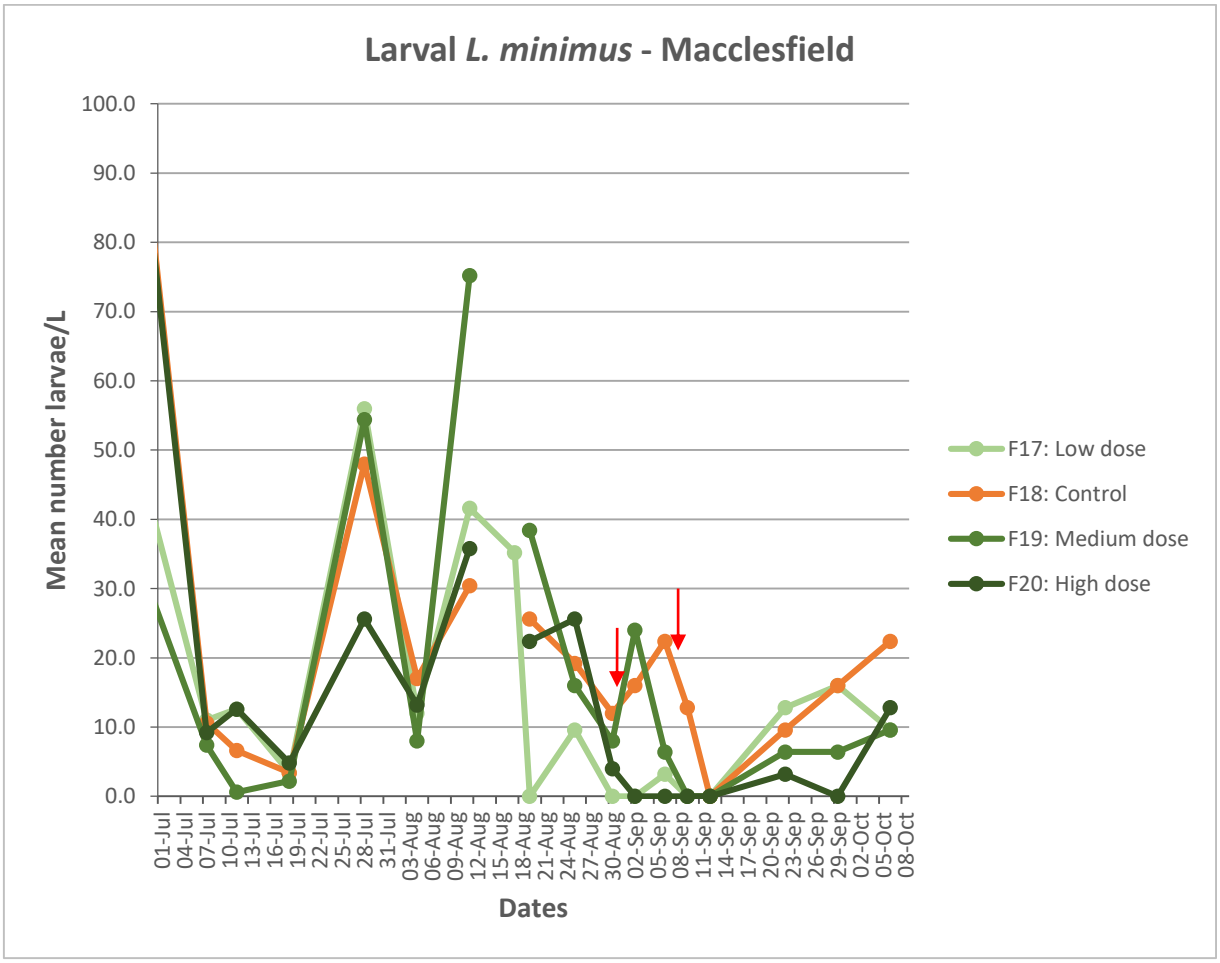


Fig. 25: Larval *L. minimus* population trends in the period close to treatment. Red arrows show the first and second treatment points with three different product concentrations on each of the three test filter beds - low dose (80mg/L), medium and recommended dose (160mg/L) and high dose (230mg/L).

Mean adult fly counts (±SE) (Fig. 26) before and after the first and second treatments against *L. minimus* at Macclesfield showed significant drops one month after each treatment except in the low dose-treated filter (17) which showed a one month after each treatment (tables 6-7).

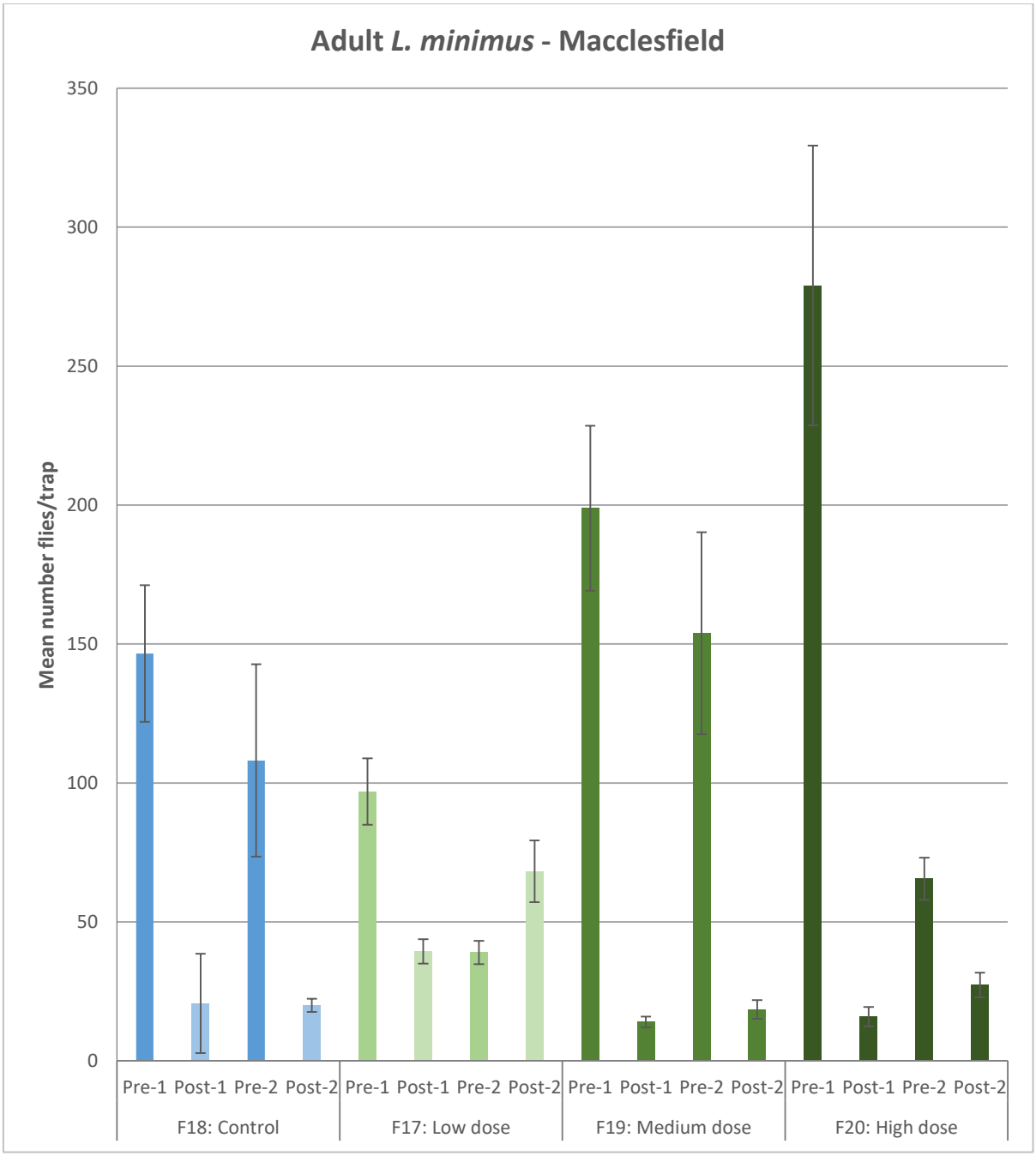


Fig. 26: Adult *L. minimus* pre- and post-treatment mean counts (±SE) at Macclesfield based on the mean number of flies recorded 24 hours before and one month after each treatment.

To compare outcomes of all treatment strengths against *L. minimus*, before and after the 1st treatment adult counts for all test filters were compared against each other. An extremely significant difference (one-way Anova, $F_{3, 36} = 13.56$; $P < 0.001$) in fly emergence was found among the filters. According to Turkey's Post hoc test, the low-dose-treated filter (17), differed most with the other three filters.

The achievement of individual treatment strengths against *L. minimus* at Macclesfield a month after the first application was also assessed. The control filter (18) and treated filters 17; 19 and 20 all showed significant *L. minimus* fly reductions ($P < 0.05$) (table 6). These trends are scrutinised in chapter 4.

Table 6: Statistical results of adult *L. minimus* reduction at Macclesfield - 24 hours before and one month after the first treatment.

	Pre-treatment-1	Post-treatment-1	Test used	Results
F18: Control	146.6 ±24.59	20.7 ±17.88	Paired T	T = 5.38; P < 0.001
F17: Low dose	96.9 ±11.91	39.4 ±4.37	Paired T	T = 5.14; P = 0.001
F19: Medium dose	198.9 ±29.67	14.0 ±1.91	Paired T	T = 6.26; P < 0.001
F20: High dose	279 ±50.37	15.9 ±3.51	Wilcoxon	W = 55.0; P = 0.006

A near-similar trend was seen between pretreatment-2 and posttreatment-2 counts. One-month after the second treatment against *L. minimus*, the control (18), medium (recommended) (19) and high

(20) dose treated filters showed significant adult fly reductions but the low dose-treated filter (17) showed a significant rise (table 7).

Table 7: Paired t- and Wilcoxon Rank test results of adult *L. minimus* population changes - 24 hours before and one month after second treatment at Macclesfield. The pre- and post-treatment counts are mean values of flies per trap.

	Pre-treatment-2	Post-treatment-2	Test used	Results
F18: Control	108.1 ±34.62	20 ±2.4	Wilcoxon	W = 55.0; P = 0.006
F17: Low dose	39 ±4.18	68.2 ±11.12	Paired t	T (9) = 2.71; P = 0.024
F19: Medium dose	153.9 ±36.32	18.5 ±3.33	Wilcoxon	W = 55.0; P = 0.006
F20: High dose	65.5 ±7.59	27.3 ±4.49	Paired t	T (9) = 5.50; P < 0.001

Comparisons between filters (or treatment strengths) at the end of all treatment against adult *L. minimus* (Table 8) indicated that fly reductions by both medium and high treatment strengths were significantly higher than by low concentration. On the other hand, the medium and high treatment strengths did not achieve significantly different outcomes. However, it is noteworthy that the level of reduction in all cases were no greater than the natural reduction in the control filters.

Table 8: Results of pairwise Mann-Whitney tests for adult *L. minimus* following significant result in Kruskal-Wallis test. The post-treatment counts are mean values of flies per trap.

	Post-treatment-2	Test significant at (P-value)	Result meaning
F18: Control vs F17: Low Dose	20 68.2	0.0008	Extremely significant difference with less flies in untreated filter.
F18: Control vs F19: Medium dose	20 18.5	0.3431	No significant difference in fly emergence.
F18: Control vs F20: High dose	20 27.3	0.3827	No significant difference in fly emergence.
F17: Low Dose vs F19: Medium dose	68.2 18.5	0.0008	Extremely significant difference with less flies in medium-dose treated filter.
F17: Low Dose vs F20: High dose	68.2 27.3	0.0058	Very significant fly reduction by high dose when compared to low dose.
F19: Medium dose vs F20: High dose	18.5 27.3	0.1394	No significant difference in treatment effect between medium and high doses.

In relation to evaluating the impact of the combined, two-treatment cycle on adult numbers, results showed that in both the medium and high concentrations, there were significant reduction in fly

numbers but this was not evident in the low concentration (table 9). However, notably, the untreated filter recorded an extremely high natural fly reduction over the same period.

Table 9: Results of Paired T-tests for adult *L. minimus* - 24 hours before the first treatment and one month after the second. The pre- and post-treatment counts are mean values of flies per trap.

	Pre-treatment-1	Post-treatment-2	Test results	Meaning in short
Filter 18: Control	146.6	20	T = 5.36; P < 0.001	Extremely significant reduction
Filter 17: Low	96.9	68.2	T = 1.61; P = 0.141	Non-significant fly reduction.
Filter 19: Medium	198.9	18.5	T = 6.34; P < 0.001	Extremely high fly reduction.
Filter 20: High	279.0	27.3	T = 5.01; P = 0.001	Extremely high fly reduction.

For statistical analyses of Macclesfield larval *L. minimus* counts, two points (Fig. 27) were chosen either side of each treatment date, 24 hours before and 48 hours after each treatment.

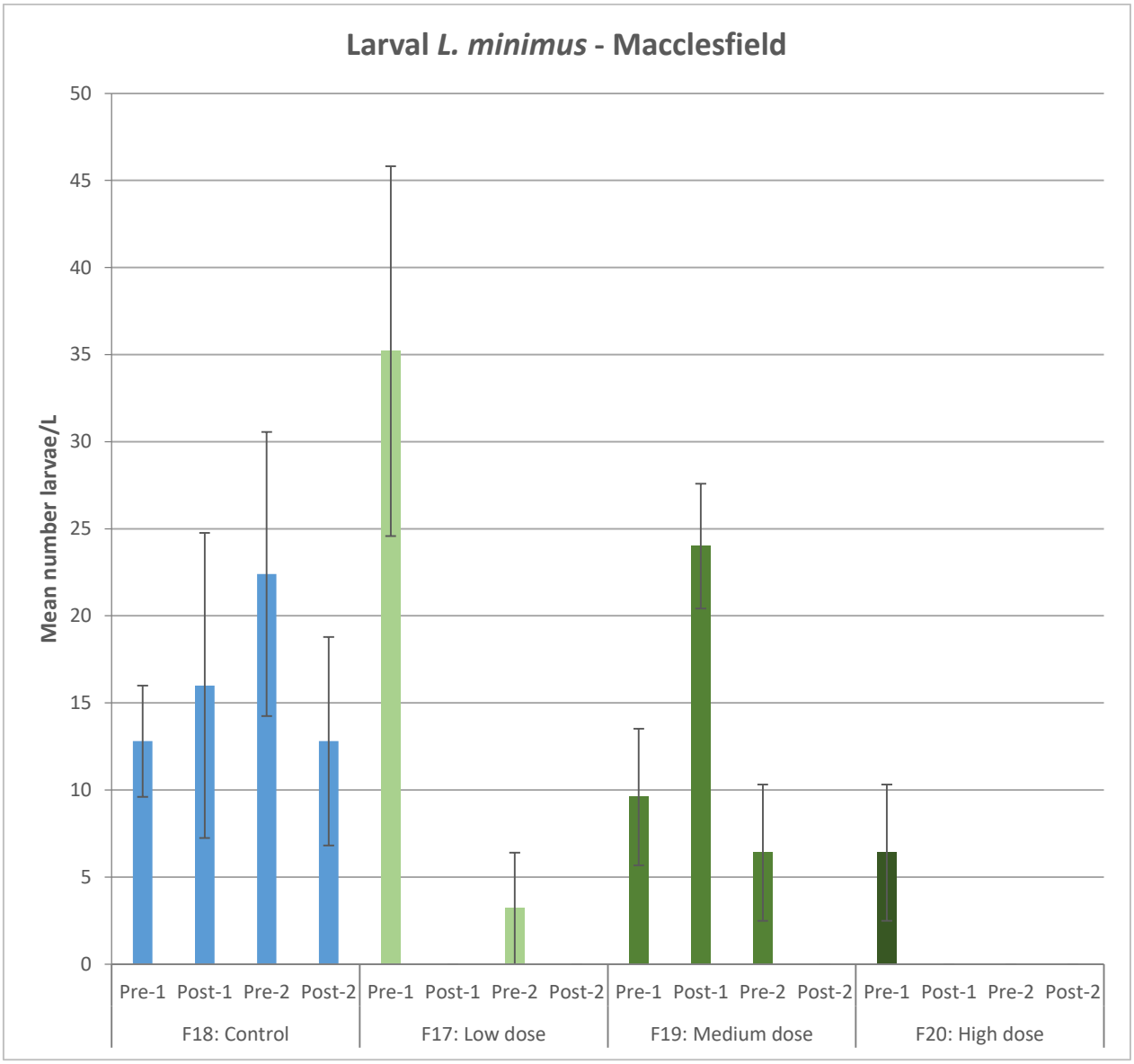


Fig. 27: Larval *L. minimus* pre- and post-treatment population counts at Macclesfield based on the mean number (±SE) of larvae found in each litre of filter media 24 hours before and 48 hours after each treatment. The data categories refer to counts before (pre) and after (post) treatment with numbers representing either first or second treatment.

Forty-eight hours after the first treatment against *L. minimus* at Macclesfield, the control and treated filters showed different changes in larval counts which were found to be non-significant reductions ($P > 0.05$) or increases even though in the low and high concentrations there were 0 larvae found in the traps (table 10).

Table 10: Statistical (Wilcoxon) results of larval *L. minimus* reduction at Macclesfield –24 hours before and 48 hours after the first treatment.

	Pre-treatment-1	Post-treatment-1	Results	Meaning
F18: Control	12.8	16	W = 3; P = 1.00	NS
F17: Low dose	35.2	0	W = 10; P = 0.100	NS
F19: Medium dose	9.6	24	W = 0; P = 0.181	NS
F20: High dose	6.4	0	W = 3; P = 0.371	NS

Notes: NS = No significant change

Assessment of the larval reduction by different BTI concentrations showed little evidence of any dose-dependent effects (table 11).

Table 11: Results of pairwise Mann-Whitney tests for larval *L. minimus* following significant result in Kruskal-Wallis test. The post-treatment counts are mean values of flies per trap.

	Post-treatment-1	Test significant at (P-value)	Result meaning
F18: Control vs F17: Low Dose	16 0	0.2619	Low dose reduced larvae but change was not significant compared to control which recorded an increase.
F18: Control vs F19: Medium dose	16 24	0.2781	Both filters showed larval increases which were not significantly different.
F18: Control vs F20: High dose	16 0	0.2619	High dose completely reduced larvae but change was not significant compared to control.
F17: Low Dose vs F19: Medium dose	0 24	0.0107	The total reduction of larvae by low dose was very significant compared to medium-dose.
F17: Low Dose vs F20: High dose	0 0	1.000	Treatment strengths achieved equal results 48-hours after first treatment.
F19: Medium dose vs F20: High dose	24 0	0.107	High dose achieved complete larval reduction nonetheless insignificant compared to medium dose.

Forty-eight hours after the second treatment against *L. minimus*, control filter recorded an average of 12.8 larvae per litre with no larvae recorded from any of the treated filters 17; 19 and 20. This translated to no significant difference (Kruskal-Wallis test, H = 4.40; DF = 3; P = 0.222) in post-treatment larval distribution or treatment outcomes when all (test and control) filters (treatment strengths) were compared against each other. Although no larvae were found in the three test filters after the 2-part treatment cycle, the statistical significance of this depletion was not identical across the filters (table 12).

Table 12: Results of paired T-tests for larval *L. minimus* - 24 hours before the first treatment and 48 hours after the second. The pre- and post-treatment counts are mean values of larvae per litre.

	Pre-treatment-1	Post-treatment-2	Test results	Meaning in short
Filter 18: Control	12.8	12.8	T = 0.00; P = 1.00	No change
Filter 17: Low Dose	35.2	0.0	T = 3.32; P =0.029	S
Filter 19: Medium dose	9.6	0.0	T = 2.45; P =0.070	NS
Filter 20: High dose	6.4	0.0	T = 1.63; P = 0.178	NS

Notes: S = Significant larval reduction; NS = No significant larval reduction

3.1.4 *Metriocnemus eurynotus* – Helsby WWTW

Adult *M. eurynotus* population patterns at Helsby (Fig. 28) showed that the mean counts across all treated filters dropped to zero or near-zero levels about 2 weeks after the first treatment with these low levels being maintained for more than one month after the second treatment. In the control filter adult fly numbers initially dropped but subsequently rose twice to levels higher than the pre-treatment counts at a time when all treated filters had very low counts.

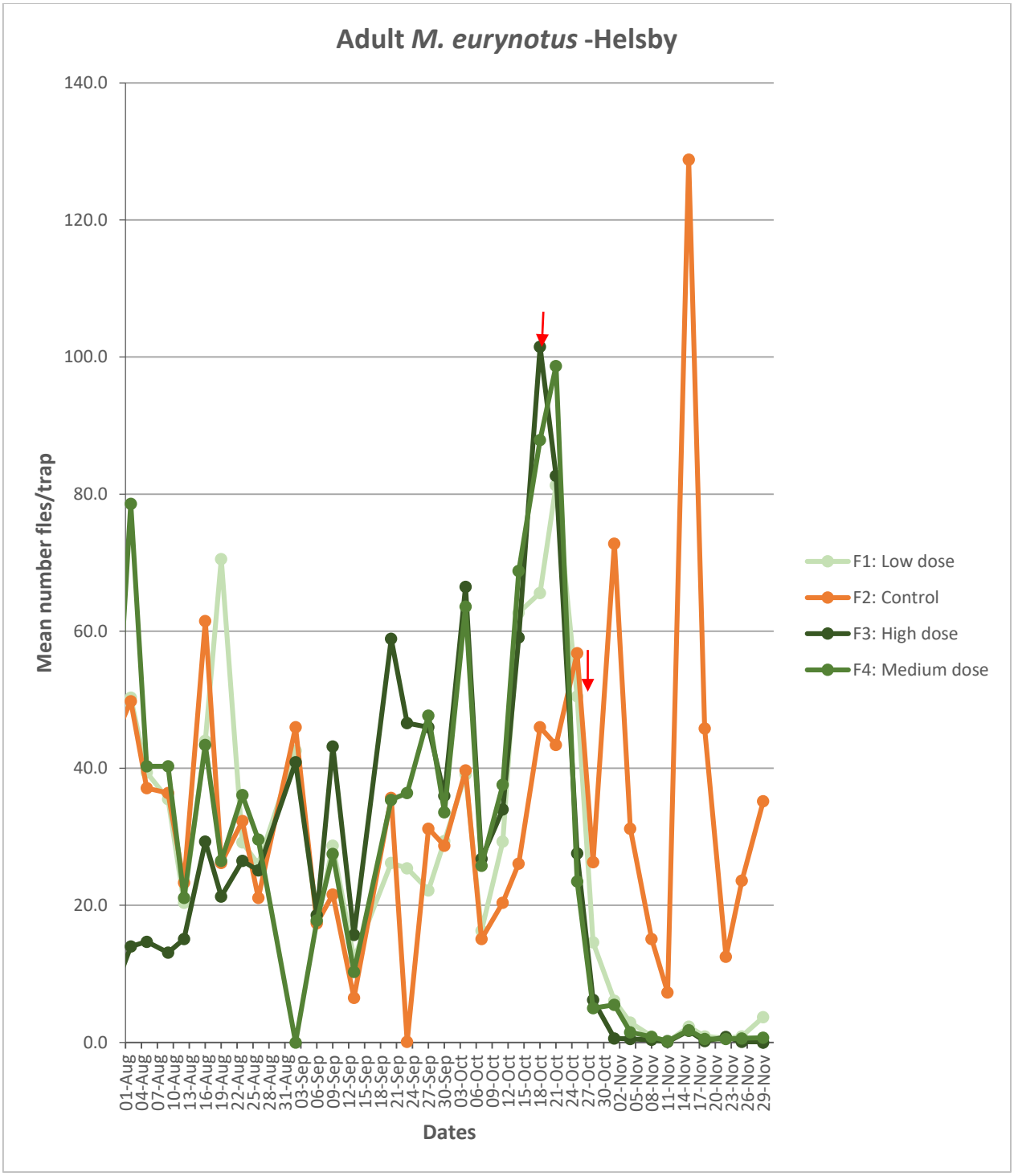


Fig. 28: Adult *M. eurynotus* population trends. Red arrows show the first and second treatment points with three different product concentrations on each of the three test filter beds - low dose (80mg/L), medium and recommended dose (160mg/L) and high dose (230mg/L).

Larval population patterns for *M. eurynotus* (Fig. 29) after the first treatment all treated filter populations dropped from nearly 200 to around 20 larvae per litre within 24 hours before further

reducing to zero within four days. Counts remained very low after the second treatment for about another three weeks. During this time control filters had in excess of 80 flies per trap for much of the time. Fly population recovery was evident in the control and low concentration filters 24 days after the second treatment but not in the medium and high concentration filters.

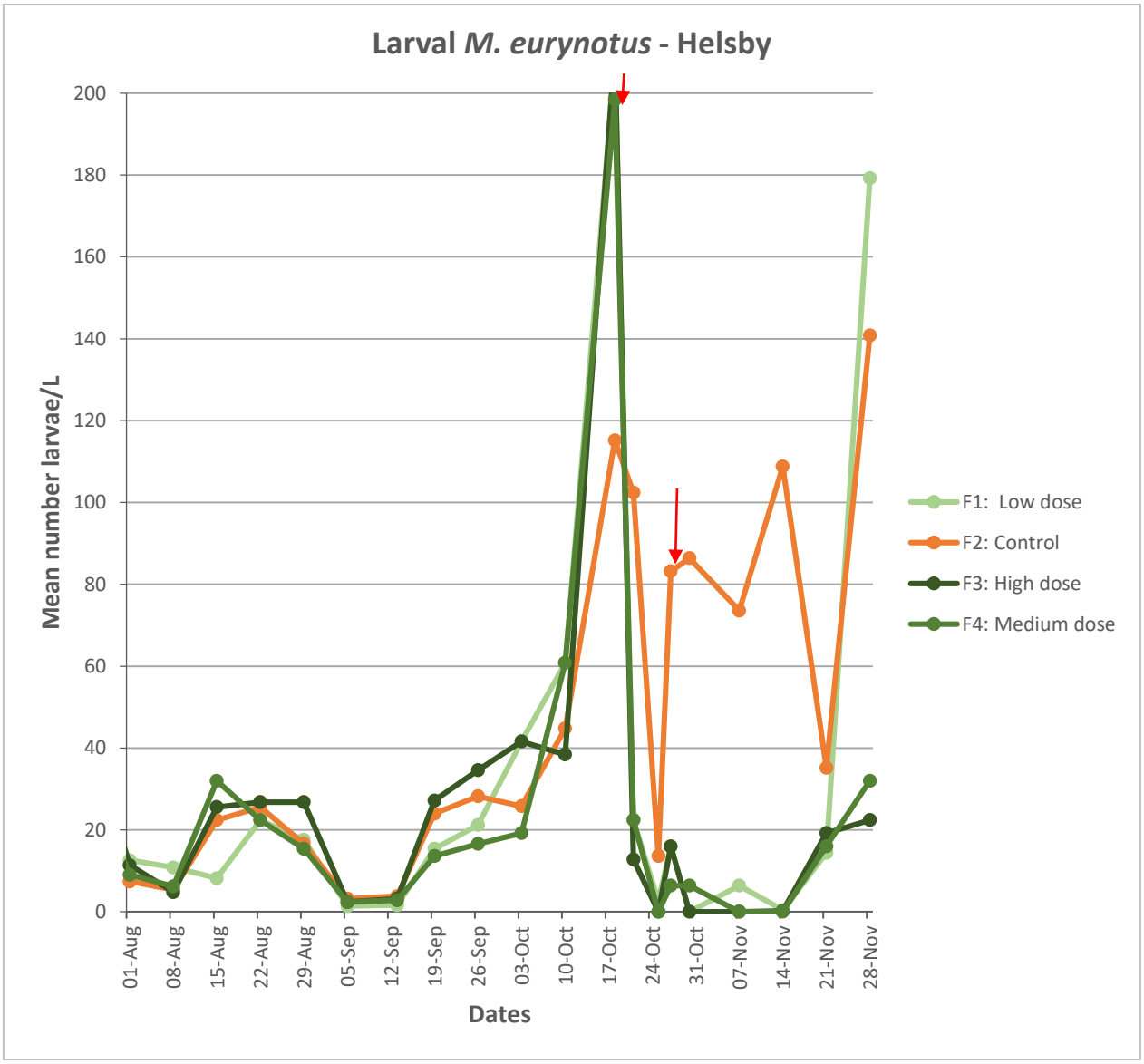


Fig. 29: Larval *M. eurynotus* population trends. Red arrows show the first and second treatment points with three different product concentrations on each of the three test filter beds - low dose (80mg/L), medium and recommended dose (160mg/L) and high dose (230mg/L).

For statistical analyses, *M. eurynotus* data from two adult and larval counts (Figs 30 and 31 respectively) from 24 hours before and one month after each treatment for adults and 24 hours before and 48 hours after each treatment for larvae were used.

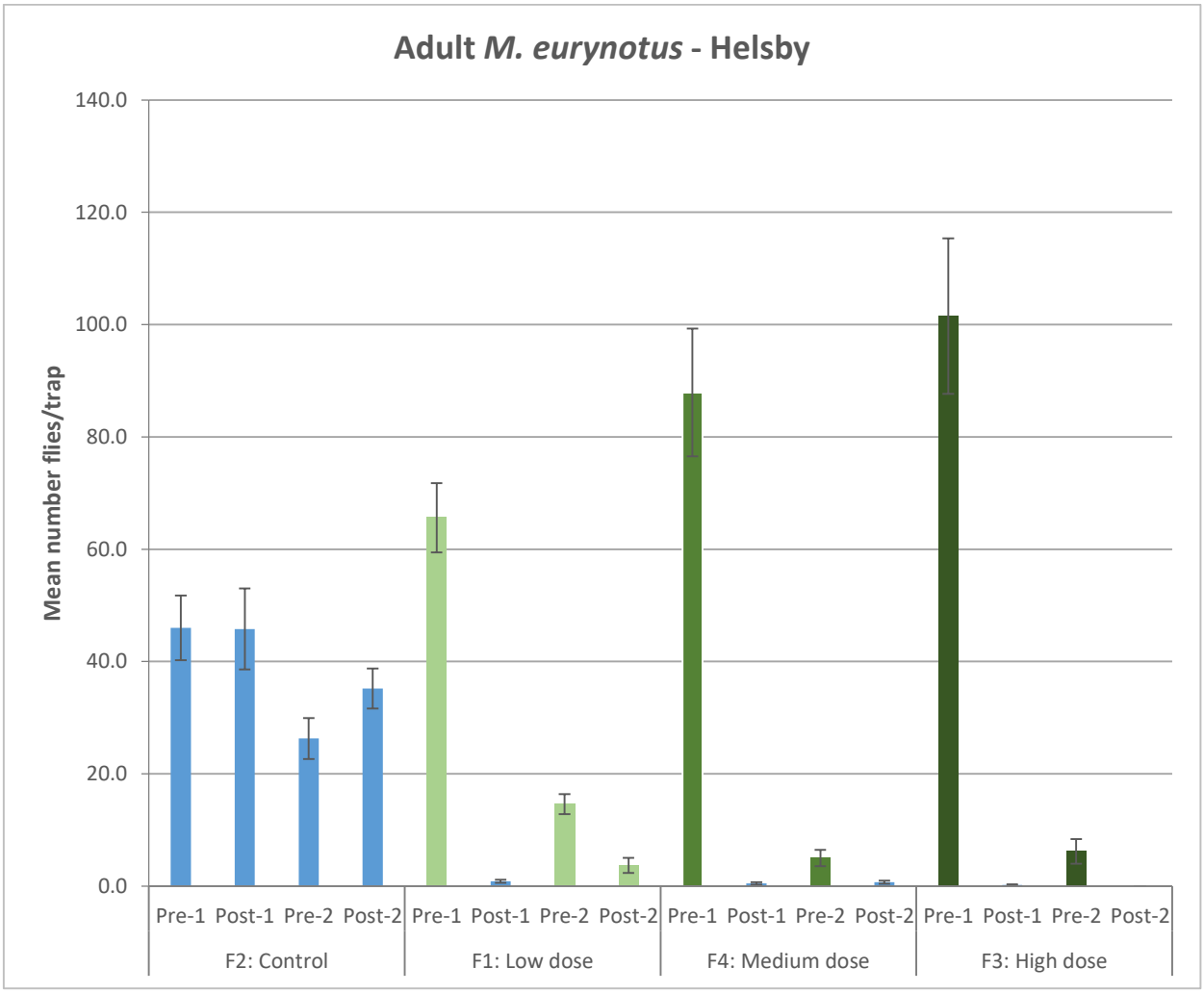


Fig. 30: Adult *M. eurynotus* pre- and post-treatment mean counts (± SE) at Helsby based on the mean number of flies recorded on each trap 24 hours before and 23 days after each treatment.

One month after the first treatment against *M. eurynotus*, the untreated control filter (2) showed no significant variation in adult counts (Paired t, T = 0.02; P = 0.981) dropping to a mean of 45.8 ±

7.22 from 46 ± 5.75 flies per trap. On the other hand, all treated filters (1; 3 & 4) recorded highly significant fly reductions (P > 0.05) with mean fly numbers below one in all cases (table 13).

Table 13: Statistical results of adult *M. eurynotus* at Helsby – based on mean counts (± SE) per trap, 24 hours before and one month after first treatment

	Pre-treatment-1	Post-treatment-1	Test used	Test results	Meaning in brief
F2: Control	46 ±5.75	45.8 ±7.22	Paired T	T = 0.024; P = 0.981	NS
F1: Low dose	65.6 ±6.15	0.9 ±0.28	Wilcoxon	W = 55; P = 0.006	S
F4: Medium dose	87.9 ±11.4	0.5 ±0.22	Wilcoxon	W = 55; P = 0.006	S
F3: High dose	101.5 ±13.8	0.2 ±0.13	Wilcoxon	W = 55; P = 0.006	S

Notes: S = Significant fly reduction; NS = No significant change.

To assess the effect of different dose strengths of larvicide on adult fly populations after the first treatment pair-wise comparisons were made (table 14).

Table 14: Results of pairwise Mann-Whitney tests for adult *M. eurynotus* following significant result in Kruskal-Wallis test. The post-treatment (1) counts are mean values of flies per trap.

	Post-treatment-1	Test significant at (P-value)	Result brief meaning
Filter 2: Control vs Filter 1: Low	45.8 0.9	0.0002	Extremely high fly reduction by low BTI dose compared to untreated filter.
Filter 2: Control vs Filter 4: Medium	45.8 0.5	0.0001	Extremely high fly reduction by medium BTI dose compared to untreated filter.
Filter 2: Control vs Filter 3: High	45.8 0.2	0.0001	Extremely high fly reduction by high BTI dose compared to untreated filter.
Filter 1: Low vs Filter 4: Medium	0.9 0.5	0.2596	No significant difference in treatments
Filter 1: Low vs Filter 3: High	0.9 0.2	0.0282	Significant fly reduction by high dose when compared to low dose.
Filter 4: Medium vs Filter 3: High	0.5 0.2	0.3222	No significant difference in treatments.

Comparative analyses indicated that there was little difference in fly reductions with the different concentrations of BTI used although there is a small indication that the larval killing was more effective in the high concentration compared to the low. One month after the second treatment (table 15), comparison of treatments indicated the medium and high concentrations were more

effective than the low concentration but that there was no difference between the high and the medium.

Table 15: Results of pairwise Mann-Whitney tests for adult *M. eurynotus* following significant result in Kruskal-Wallis test. The post-treatment (2) counts are mean values (±SE) of flies per trap recorded 1 month after second treatment.

	Post-treatment-2	Test significant at (P-value)	Result brief meaning
Filter 2: Control vs Filter 1: Low	35.2 ± 3.55 3.7 ± 1.32	0.0003	Extremely high fly reduction by low BTI dose compared to untreated filter.
Filter 2: Control vs Filter 4: Medium	35.2 ± 3.55 0.7 ± 0.26	0.0002	Extremely high fly reduction by medium BTI dose compared to untreated filter.
Filter 2: Control vs Filter 3: High	35.2 ± 3.55 0	0.0001	Extremely high fly reduction by high BTI dose compared to untreated filter.
Filter 1: Low vs Filter 4: Medium	3.7 ± 1.32 0.7 ± 0.26	0.0342	Significantly lower counts in the medium compared to low concentration.
Filter 1: Low vs Filter 3: High	3.7 ± 1.32 0	0.0010	Significantly lower counts in the high concentration compared to the low.
Filter 4: Medium vs Filter 3: High	0.7 ± 0.26 0	0.2292	No significant difference in treatments.

The overall effect of the two-dosing cycle on adult numbers (table 16) indicated highly significant reductions of flies to zero and near-zero in all treatment strengths.

Table 16: Statistical results of adult *M. eurynotus* at Helsby – based on mean counts per trap over the two-part treatment dosing cycle.

	Pre-treatment-1	Post-treatment-2	Test used	Test results	Meaning
F2: Control	46	35.2	Paired t	T = 2.17; P = 0.058	NS
F1: Low dose	65.6	3.7	Paired t	T =10.24; P<0.001	S
F4: Medium dose	87.9	0.7	Wilcoxon	W = 55; P = 0.006	S.
F3: High dose	101.5	0	Paired t	T =7.33; P<0.001	S

Notes: S = Significant fly reduction; NS = Non-significant reduction

In relation to larval *M. eurynotus* at Helsby all treated filters had significantly less larvae than the control (Fig. 31) after the first treatment with the second treatment reducing larval numbers further.

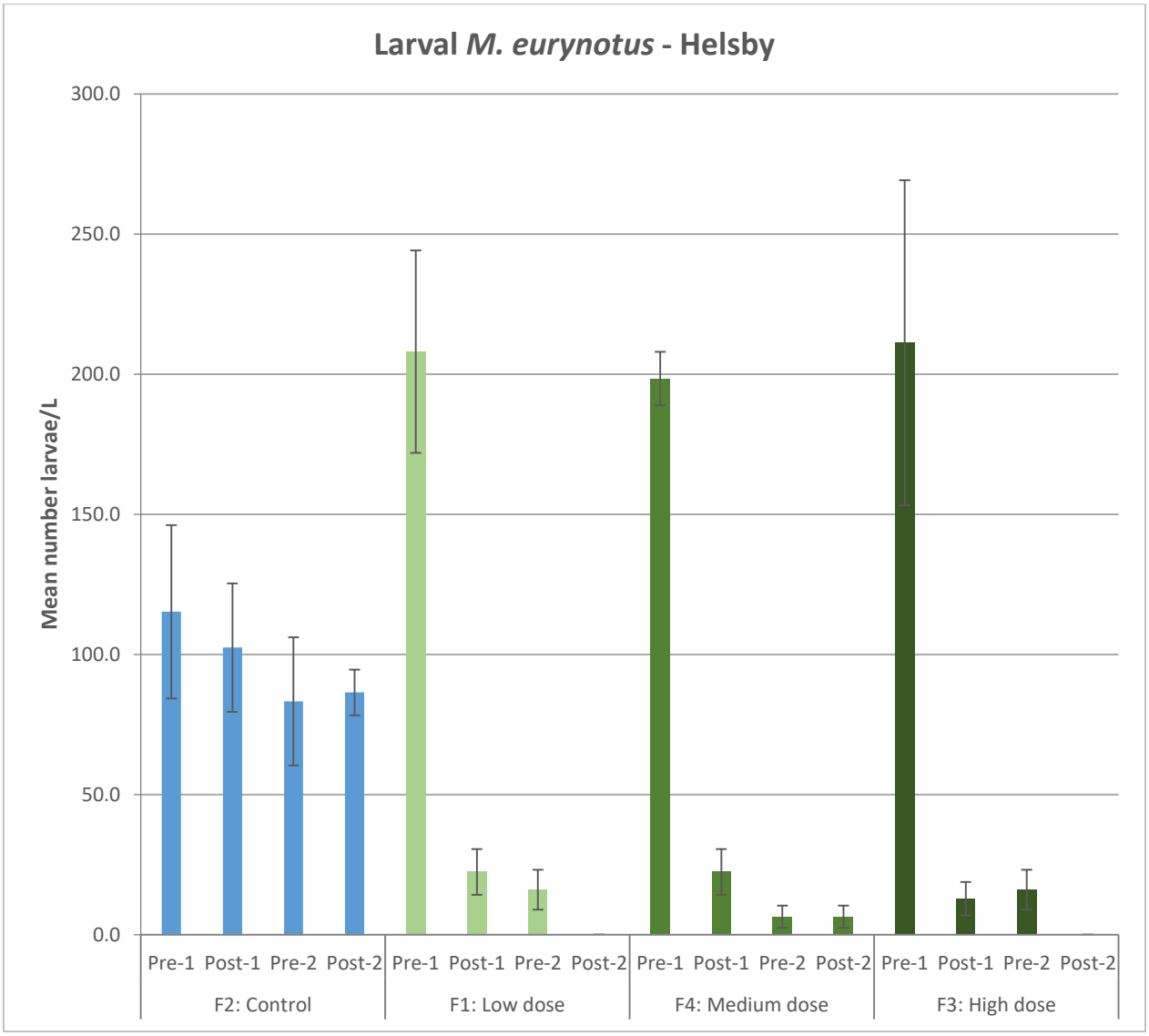


Fig. 31: Larval *M. eurynotus* pre- and post-treatment mean counts (±SE) at Helsby based on the mean number of larvae found in each litre of filter media 24 hours before and 48 hours after each treatment.

When pre- and post-treatment counts for first treatment against *M. eurynotus* were compared for each filter (or each treatment strength), highly significant larval reductions were noted across all treated filters except the control which showed no significant change (table 17).

Table 17: Statistical results of larval *M. eurynotus* at Helsby – based on mean counts (± SE) per litre, 24 hours before and 48 hours after first treatment

	Pre-treatment-1	Post-treatment-1	Test used	Test results	Meaning in brief
F2: Control	115.2 ± 30.94	102.4 ± 22.96	Wilcoxon	W = 4; P = 0.789	No significant change
F1: Low dose	208 ± 36.13	22.4 ± 8.16	Wilcoxon	W = 15; P = 0.059	Non-significant but notable reduction
F4: Medium dose	198.4 ± 9.6	22.4 ± 8.16	Paired t	T = 15.55; P < 0.001	High fly reduction
F3: High dose	211.2 ± 58	12.8 ± 5.99	Paired t	T = 3.12; P = 0.035	High fly reduction

At the same time, when all filters' larval counts 48-hours after the first product application were statistically tested against each other (table 18), teach treated filter showed an extremely high larval drop compared to the untreated filter. However, when larval counts of treated filters were compared against each other, there was no significant difference found. In other words, all three treatment strengths achieved near-similar treatment outcomes against *M. eurynotus* larvae.

Table 18: Results of pairwise Mann-Whitney tests for larval *M. eurynotus* following significant result in Kruskal-Wallis test. The post-treatment (1) counts are mean larvae per litre.

	Post-treatment-1	Test significant at (P-value)	Result meaning in brief
Filter 2: Control vs Filter 1: Low	102.4 22.4	0.0117	Extremely high larval reduction by low BTI dose compared to untreated filter.
Filter 2: Control vs Filter 4: Medium	102.4 22.4	0.0122	Extremely high larval reduction by medium BTI dose compared to untreated filter.
Filter 2: Control vs Filter 3: High	102.4 12.8	0.0114	Extremely high larval reduction by high BTI dose compared to untreated filter.
Filter 1: Low vs Filter 4: Medium	22.4 22.4	1.00	No significant difference in treatments
Filter 1: Low vs Filter 3: High	22.4 12.8	0.4432	No significant difference in treatments.
Filter 4: Medium vs Filter 3: High	22.4 12.8	0.4432	No significant difference in treatments.

Forty-eight (48) hours after the second treatment application, 86.4 $\pm$8.16; 0; 0 and 6.4 $\pm$3.92 larvae per litre were recorded from untreated filter 2, treated filters 1; 3 and 4 respectively. One-Way Anova confirmed a significant difference ($F_{3,16}$=87.12, P<0.001) in the filters' mean larval

counts at this sampling point. Turkey's Post hoc test (within Anova) revealed that only the larval mean counts of the untreated filter significantly differed from those of treated filters 1; 3 and 4.

To assess the overall impact of each treatment concentration on larval *M. eurynotus*, the first pre-treatment counts were statistically tested against those from 48-hours after second/last treatment (table 19). All treatment strengths achieved extremely high fly reductions at the end of treatments against *M. eurynotus* with a notable but nonetheless, non-significant population rise in untreated filter over the full treatment dosing cycle.

Table 19: Results for paired T-tests for larval *M. eurynotus* at Macclesfield – based on mean counts over the two-part treatment dosing cycle.

	Pre-treatment-1	Post-treatment-2	Test results	Meaning
F2: Control	115.2	86.4	T = 0.78; P = 0.48	Non-significant reduction.
F1: Low dose	208	0	T = 5.76; P = 0.005	High reduction.
F4: Medium dose	198.4	6.4	T = 16.97; P < 0.001	High reduction.
F3: High dose	211.2	0	T = 3.64; P < 0.02	High reduction.

3.2 Overall fly reductions

In this context, fly reduction refers to the actual drop in larvae and egressing adults of each target species after full treatment. In other words, in cases where treatments were applied twice, overall fly reduction was the population drop from the initial or first pre-treatment count to the post-treatment counts following the second/last product application. With the overall larval and adult pre- and post-treatment count drops already statistically tested and reported under the results of each species' above (subsections 3.1.1-3.1.4), this section only summarises fly reductions in percentage terms.

3.2.1 *Sylvicola fenestralis*

In the case of *S. fenestralis,* the single treatment registered 100% adult and larval population reductions.

3.2.2 *Psychoda alternata*

For *P. alternata* the first treatment reduced adult counts by 81% and larvae by 100%. The second treatment showed a further reduction in adult flies by 63% and larvae by 50%. However, over both treatments, adults and larvae of this species were reduced by 95% and 93% respectively.

3.2.3 *Limnophyes minimus*

The first low-dose treatment against *L. minimus* reduced adult populations by 59% with second application showing no further reduction. Overall, the low dose reduced adult egression by 30%

after the two product applications. At the same time, the first medium-dose treatment reduced adult populations by 88% with second treatment reducing the remaining flies by 81% to give an overall 91% over the full treatment period. However, the untreated filter showed adult population declines over the same periods. This is scrutinised further in chapter 4.

The first high-dose treatment against the same species reduced adult populations by 94% with second of the same treatment further reducing the remaining flies by 58% to give a 90% reduction overall after two treatments. The medium (recommended) and high (trial) doses did not achieve any significantly different fly reductions. However, the first and second treatments all showed 100% reductions in all dose-strength categories except for the first medium-dose treatment which showed no reduction in larvae initially but declining to 100% after second treatment. However, it was notable that mean counts for this fly species showed significant drop in both treated and untreated filters over the posttreatment sampling period.

3.2.4 *Metriocnemus eurynotus*

The low-dose treatment against *M. eurynotus* reduced mean adult counts initially by 99% after the first treatment and with a further 75% after the second treatment to achieve an overall, 94% fly reduction. At the same time, the medium dose reduced mean adult counts by 99% a further 86% after the first and second doses respectively to give 99% fly reduction overall. Finally, the high-dose treatment against the same species reduced the adult flies by 99% after the first treatment and then to 100% overall. There was no significant difference between efficacies of different treatment concentrations.

With *M. eurynotus* larval populations, the low-dose treatment reduced the mean numbers by 89% and 100% after the first and second treatments respectively to achieve an overall 100% larval reduction. The medium-dose treatment against the same species achieved 89% and 0% and an overall larval reduction of 97%. At the same time, high-dose treatment reduced mean larval counts by 94% and 100% after the first and second treatments to achieve an overall larval reduction of 100%. Again the differences between different treatment concentrations were not significant.

For ease of reference, overall adult and larval reductions above are summarised below (table 20).

Table 20: Summary of performance of VectoBac 12AS® strain AM65-52 (BTI AM65-52) in reducing filter-fly species at two sites monitored in this study. Blank (-) spaces show a treatment strength not trialled for particular species.

		Overall target filter-fly reduction (%)		
		Low dose 80mg/L	Medium dose 160 mg/L	High dose 230mg/L
L. minimus, Macclesfield	Adults	30	91	90
	Larvae	100	100	100
M. eurynotus, Helsby	Adults	94	99	100
	Larvae	100	97	100
P. alternata, Macclesfield*	Adults	95	-	-
	Larvae	93	-	-
S. fenestralis, Helsby*	Adults	-	100	-
	Larvae	-	100	-

3.3 Non-target organisms

No obvious NTO-population drops attributable to larviciding were observed in all treated filters after the full trials (Figs 32 and 33). Population trends in the treated and untreated filter showed similar random fluctuations regardless of period although nematodes appeared to flourish a little more in the treated filters after the end of treatments at both sites.

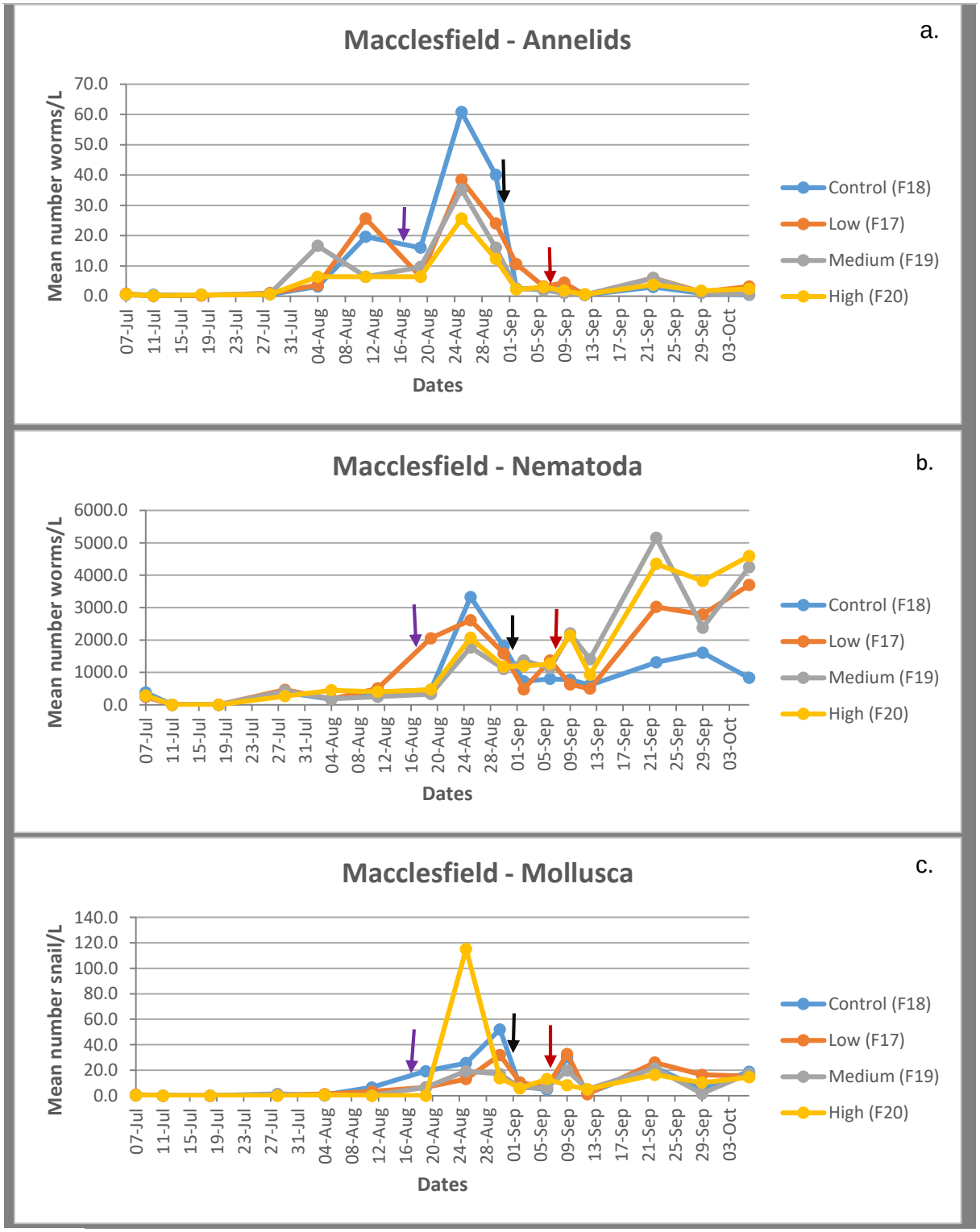


Fig. 32: (a.) Annelids, (b.) Nematodes and (c.) molluscs (snails and slugs) pre- and post-treatments population trends. Arrows show treatment dates – Purple (*P. alternata* 1st treatment), black (*P. alternata* 2nd treatment and *L. minimus* 1st treatment) and red (*L. minimus* 2nd treatment).

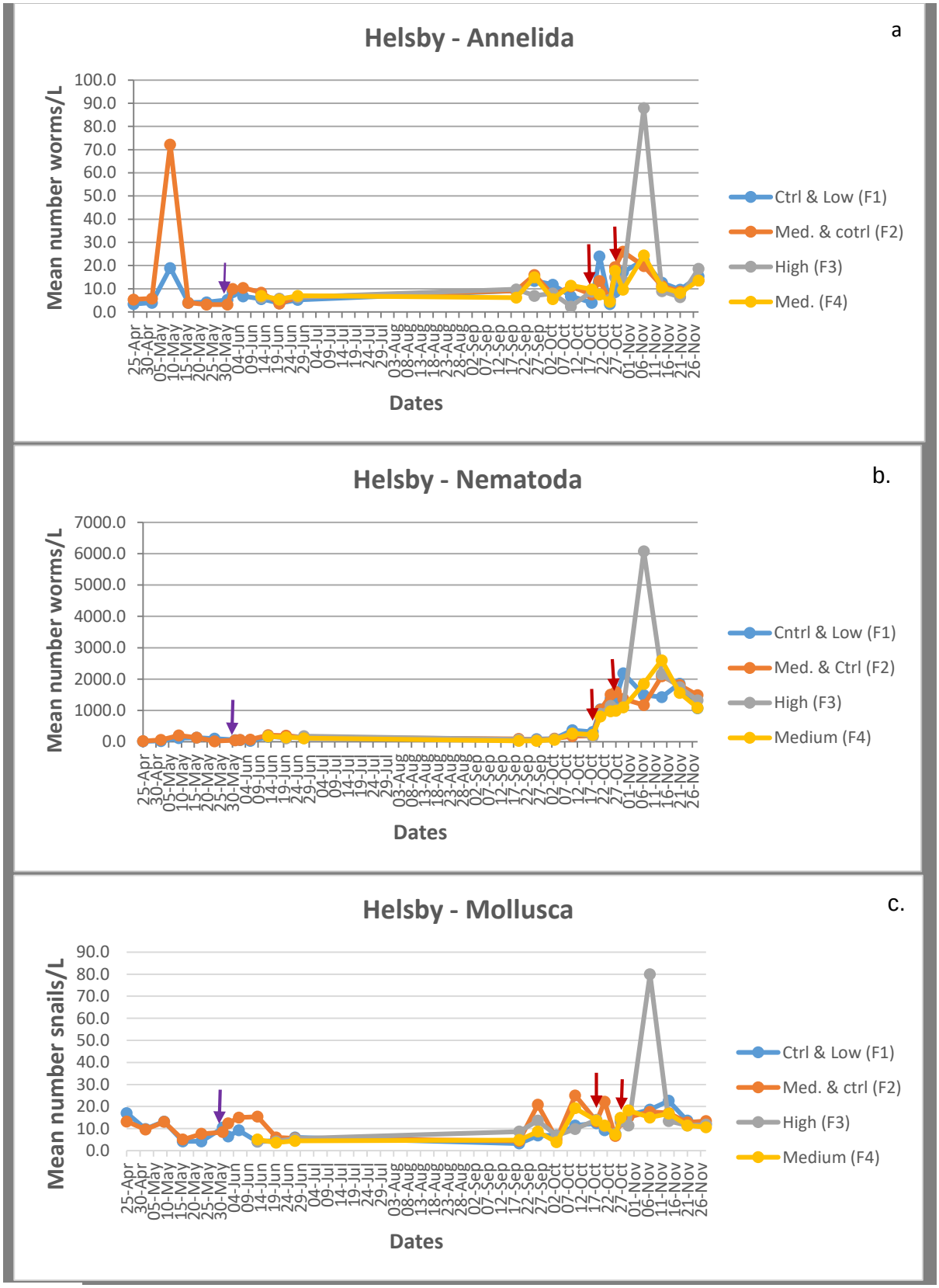


Fig. 33: (a.) Annelids, (b.) Nematodes and (c.) molluscs (snails and slug) pre- and post-treatments population trends for Helsby. Arrows show treatment dates – purple (*S. fenestralis*) and red (*M. eurynotus*).

Population counts taken over four weeks starting 48-hours after treatment against *S. fenestralis* at Helsby showed no significant population difference between aquatic annelids (two-sample t; T (6) = 1.18; P = 0.283), nematode (two-sample t; T (7) = 0.68; P = 0.520) and molluscan (two-sample t; T (5) = 2.09; P = 0.091).

Five months later, *M. eurynotus* was targeted using with two treatments at three different concentrations (or on 3 filters) within 9 days at the same site (Helsby). At Macclesfield, *P. alternata* and *L. minimus* were also targeted for treatment with three different product strengths (on 3 filters) with one week. Five and six weeks' post-treatment monitoring data for the respective sites' treated and untreated filters were tested using Kruskal-Wallis test and Anova which revealed that in all cases, NTOs suffered no significant population reductions or fluctuations (P > 0.05) as a result of the treatment (table 21).

Table 21: Statistical results of NTO population changes within 4-6 weeks of treatments against *M. eurynotus,*

	WWTW	Test used	H	DF	F-value	P-value	Meaning
Annelids	Helsby	KWT	1.00	3	-	0.802	NS
	Macclesfield	KWT	3.58	3	-	0.31	NS
Nematodes	Helsby	KWT	1.55	3	-	0.67	NS
	Macclesfield	KWT	7.63	3	-	0.054	NS
Molluscs	Helsby	KWT	1.82	3	-	0.61	NS
	Macclesfield	One-Way Anova		3, 24	0.44	0.728	NS

Notes: NS = no significant population change ; KWT: Kruskal-Wallis test

Chapter 4. Discussion

In considering the results from this study there are a number of important points to take into consideration. The larvicidal effect of VectoBac 12As® strain AM65-52 (BTI AM65-52) was evident within 48 hours but the subsequent effect on adult numbers is delayed for several weeks due to the developmental cycle of each species. The overall effect on adult numbers however may be confounded by immediate recolonization after the treatment cycle. The natural recolonisation and population declines occurring in the fly populations also make comparisons with control filters more problematic. Weather condition-induced pupal diapause could also mislead when drawing conclusions on adult egression. In several cases in this study, the timing of the first treatment has coincided with natural declines in the fly populations. In hindsight (allowing for trial protocol delays and operational difficulties), slightly earlier commencement of treatment may have made comparisons more relevant.

4.1 The differences in target species populations between/among control and test filters and between/among the test filters before and after treatment with BTI, VectoBac 12As® strain AM65-52 (BTI AM65-52)

4.1.1 *Sylvicola fenestralis*

It was shown that there was a 100% treatment success against *S. fenestralis* at Helsby after zero adults and larvae were recorded in the test-bed after treatment with reduced number recorded in the control filter. The significant drop in post-treatment numbers in the control filter suggested that treatment coincided with natural reduction of the species populations. There could also have been

some product feedback into the four—way effluent distribution chamber during treatment. However, this could be discounted if one looks at the period it took for the species population to recover after treatment. Notable *S. fenestralis* populations only started to return three and half (3.5) months post-treatment.

The sudden and prolonged population drop could be attributed to treatment being followed by natural reduction. In that case, there was no point of a second product application.

Although post treatment counts for treated and untreated filters showed immediate, prolonged and unequal drops, the effect of treatment was observable. The untreated filter's adult and larval reductions after treatment were there but non-significant whilst those of treated filter showed highly significant reduction. Interestingly, the 100% larval reduction was not found to be a significant result. This is one instance that brings to the fore the observation that the P-value is a guidance only. In fact, the P-value does not always "address the questions that scientific research requires" (Taroni *et al.,* 2016) through its limitations at providing "probabilities of competing hypotheses". The P-value is used as part of drawing conclusions from scientific observations in conjunction with background information (Dewey and Schlattmann, 2015). This position is supported by the observation that the two filters showed adult and larval population homogeneity before treatment and this changed after treatment (and higher population drops in treated filter) with significant difference noted between the two filters.

4.1.2 *Psychoda alternata*

As explained elsewhere in this report, this species was targeted for two bi-weekly treatments in Macclesfield's filter 17 with number 18 as control. With no significant difference between control and test-bed in both larval and adult first pre-treatment and post-treatment populations, it was interesting to find that the same filters showed different statistical results post-treatment. The two filters showed a very significant reduction in adult populations whilst the larval variables could not be successfully tested statistically due to the total absence of larvae in the test bed post-treatment one.

Because the filters started with no differences, the adult and larval results confirmed treatment effectiveness. Furthermore, even though there was no significant difference between adult and larval populations in control and filter beds before the first treatment, it was interesting to note that when the second pre-treatment counts for the same filters were compared, high significant differences were noted meaning results of the first treatment were clearly manifest. Furthermore, product effectiveness was confirmed by the very high significant differences between adult populations in both filters post-treatment two. Changes to test filter bed populations from the first pre-treatment counts to the last post-treatment counts looked obvious at face value. However, only adults showed significant differences between the first pre- and post-treatment counts, post-treatments one and two plus pre-treatments one and two. This confirmed progressive drops in fly egressions. However, the same cannot be said for similar comparisons for larval data. The fact that other three paired t-tests for larval changes before and after the first treatment, after treatments one and two plus those before the first and after the second treatments showed no significant difference was also surprising. These tests compared the mean larval counts: 227.2 against 0; 0 against 80 and

227.2 versus 16 larvae per litre. This could be down to the fact that two of the tests had one column each with zero larvae and impossible to test for normality in the first place. At the same time, the same could not be tested using non-parametric equivalent, Wilcoxon Signed rank test which require six pairs of data. However, the total depletion of larvae in two filters contributing to 93% larval reduction was an impressive treatment outcome.

The best result to be relied on could still be the adult egressions comparisons. When the two treatment cycles are treated as a single operation, we note that the very first adult pre-treatment count versus the last/second post-treatment count achieved an extremely significant reduction (of 95%) in mean number of *P. alternata* egressing from the filters.

4.1.3 *Limnophyes minimus*

Although there was significant difference between *L. minimus* adult populations in the control and three test-beds before the first treatment, this was diminished by the fact larvae, which are the stages targeted by treatment, showed no significant difference across all four filters. Therefore, treatments were applied on filters with no major larval differences.

As reported earlier, there were significant differences in both adult and larval population medians across filters after the first treatment. This is interesting considering there had not been significant difference in larval populations prior to treatment. Adult *L. minimus* pairwise differences revealed by Mann-Whitney tests confirmed that low-dose treatment (filter 17) was least effective as it differed most against the other two (standard- and high-dose) treated and even filters. It is also

clear that the standard and high doses achieved significantly equal impact as they showed no difference post-treatment one.

Adult and larval *L. minimus* did not show a clear response to treatment. The untreated filter adult counts dropped slightly more than those from one of the treated filters. On the other hand, larval counts in control rose together with those of one of the treated filters after the 1st treatment but showed its effect after 2nd product application compared to the untreated filter. These observations could be attributed to two things. Firstly, these could suggest that the timing of dosing coincided with a natural decline in the species population. At the same time, the rise in larval numbers in the medium (recommended) dose-treated filter could be inferred as evidence that chironomid species of Orthocladiinae subfamily are not highly susceptible to the larvicide (Stephens *et al*., 2004).

The extremely high significant differences in adult populations across all four filters just before the second treatment is a confirmation of a treatment-induced shift in the species populations as the significant difference was slightly higher before the first treatment. It is also a confirmation of treatment effectiveness that before the second treatment larval populations had shifted from being not significant to being significant.

Although significant difference was noted between the low-dose filter 17 and the other three filters, the control filter 18 had no significant difference with both the medium-dose filter 19 and high-dose filter 20 before the second treatment. This adult egression in treated filters underlines the importance of a second treatment against *L. minimus*. Pre-treatment two, the pairwise population differences between the four filters showed that control differed most with the high-dose filter with the low and medium-dose filters having the least difference. However, the three test beds had no

significant difference meaning all three treatment strengths were effective against *L. minimus* larvae.

The low dose gave a significant result between the low-dose test-filters and the other 2 test-beds meaning, treatment differed significantly with strength. However, the absence of a significant difference between the standard and high-dose filter adult populations is a confirmation that there was nothing to separate the effectiveness of those two treatment strengths. The 100% larval reduction after the second treatment across all test beds meant there was no difference (Kruskal-Wallis, H = 0.00; DF = 2; P=1) across all treated filters (or treatment strengths) at the end of all treatment against *L. minimus*.

4.1.4 *Metriocnemus eurynotus*

Although no significant difference in *M. eurynotus* adult populations across all four filter beds, at least the product target, larval species showed no significant difference prior to treatment. After the first treatment, it was notable that 11 of 12 pairwise tests showed that larval and adult fly counts only differed between the untreated filter and treated ones. This suggests treatment was effective to near-equal levels across all concentrations.

The significant difference in mean counts across all filters before the second treatment resulted in pairwise comparisons that revealed the highest significant differences in adult counts to between the untreated filter and all treated filters with the highest difference being shown to be against the medium-dose filter. It was interesting that the standard and high-dose filters did not have much difference in both adult and larval counts. Larval counts were more pronounced in showing

differences between untreated filter and all treated filters whilst the treated filters did no show much difference among themselves before second treatment. There was nothing to separate the low- and high-dose-treated filters before the second treatment. This suggests that there were some residual *M. eurynotus* larvae that had either survived the first treatment or had developed from early non-feeding stages. Therefore, no matter which treatment strength, this species needed more than one treatment. Most importantly, post-treatment two, the significant difference among all test-beds was subsequently shown to have arisen from the low-dose and untreated filters against the other filters. However, the total depletion of larvae from the high-dose filter at the end of treatment meant it was not feasible to look for statistical significance against the other 3 filters. The importance of this result is that despite the huge population drops, the species could not be totally wiped out by the low and medium doses even after second treatment.

4.2 Effectiveness of first and/or second treatment in filter-fly reduction and how the different treatment concentrations compared.

4.2.1 *Sylvicola fenestralis*

As shown in the results section, only one treatment targeting *S. fenestralis* was applied at Helsby. The treatment was effective with 100% fly reduction in both larval and adult flies.

4.2.2 *Psychoda alternata*

The first treatment was more effective than the second treatment at fly reduction. However, the quick re-colonisation of test-bed by the species could be a sign of the importance of second treatment to wipe them out.

4.2.3 *Limnophyes minimus*

The first low-dose treatment one was more effective than second in reducing *L. minimus* adult flies but this dose was still poor on the overall compared to the higher doses. The larval rise 48 hours' post-treatment-1 with the medium (recommended) dose could be attributed to two things. Firstly, that there could have been a batch of non-feeding stages (Dhadialla *et al.,* (1998, Coombs *et al.*, 1997) coming through after the extremely short window of product activity and a confirmation that larvicidal susceptibility is not uniform across larval instars (Coombs *et al.,* 1997. Last but not least this species belongs to the not-so-susceptible Chironomid subfamily Orthocladiinae (Stevens et al., 2004, 2013).

4.2.4 *Metriocnemus eurynotus*

For the other species from Chironomid subfamily Orthocladiinae, *M. eurynotus*, the second treatment appeared most effective at drastically reducing the fly nuisance. However, although the high dose exhibited a higher fly reduction immediately after each treatment than the medium dose, there was hardly anything to separate their overall impact of fly reduction. It is noteworthy that like in the case of the other species of the same subfamily immediately above, it only took a second treatment to achieve satisfactory fly reduction.

4.3 How long VectoBac® reduced fly numbers after treatment

S. fenestralis populations remained supressed to near-zero levels for about seven weeks. However, the recovery period itself is hard to solely attribute to one factor. The target species' life-cycles are

temperature-dependent (Peng *et al.,* 1992) with light also having a positive influence on adult *S. fenestralis* egression (Jenkins *et al.,* 1949). Atmospheric temperature aside, filter bed temperature is also affected by effluent dosing frequency (Coombs, 1997) of the biological filters and operational challenges like equipment breakdown affecting different filters. Pend *et al.* (1992) found out that "different families respond differently to … the conditions" prevailing at any given time.

Therefore, there is always more than one factor acting upon the filter bed biological systems at any given time. On the other hand, *P. alternata adult and larval* populations remained suppressed at bottom levels for just above 2 weeks before showing signs of really slow recovery. Both *L. minimus* adult flies and larvae appeared to remain suppressed for close to a month before starting to show signs of recovery. Like *L. minimus*, *M. eurynotus* populations appeared to be suppressed by treatment for around a month.

4.4 Treatment effect on NTOs

No evidence was found to suggest that at the operational and trial doses, the biolarvicide had any deleterious effect on filter invertebrate NTOs in this study. This was consistent with findings by various earlier workers.

Merritt *et al*. (1989) and Molloy (1990), cited in Jackson *et al.* (1994) concluded "that *B.t.i.* had little or no effect on most non-target macroinvertebrates." Lacey and Siegel (2000) also reported that delta-endotoxin-based insecticidal activity of the BTI larvicide had no "direct" effect on invertebrate NTOs. Gunasekaran *et al.* (2002) and Lagadic *et al.* (2016) reported similar findings

although the latter went a step further to warn against larviciding above the recommended treatment thresholds. Citing a myriad of peer reviewed papers, Gray and Fusco (2017) recently reported that the BTI formulation has "been repeatedly shown to be safe to non-target organisms."

In the current study, some slight but statistically insignificant rise of nematode worms were observed in treated filters at both sites. However, this could be attributed to reduced feeding competition following dipteran larval depletion.

Summary and conclusions

Treatment outcome

The current study provides strong evidence that VectoBac 12As® strain AM65-52 (BTI AM65-52) is an effective larvicide against some nuisance dipteran flies at waste water treatment sites. Although treatment was found to be effective and satisfactory on both adult and larval reduction at 91-100% across target species, its effect on *L. minimus* adult egression was not easy to determine as the untreated filter also showed adult population decline post-treatment. However, treatment could still be regarded as effective against this species based on larval reduction across the 2-treatment cycle. The biocidal immediacy of treatment against larvae eliminates the onset of any misleading influences of other biotic and abiotic factors on adult egression.

There was significant larval depletion across all four species within 48 hours at the recommended treatment strengths (160mg/L for *S. fenestralis, L. minimus*, and *M. eurynotus,* and *80*mg/L for *P.*

alternata). The lower treatment strength was also effective against *M. eurynotus* but not *L. minimus.* No additional benefit was seen in increasing the dose to 230mg/L.

It was also clear that a single treatment with a medium-dose was very effective against *S. fenestralis.* The single dose used against *P. alternata* was also very effective although a second treatment enabled any initially surviving non-feeding larvae to be eliminated. A combination of treatment-induced changes and apparent natural population declines made interpretation more difficult in the case of *L. minimus*. However, together with the other Chironomid species, *L. M. eurynotus,* the *L. minimus* proved difficult to eliminate with a single dose. These required second doses for more significant reduction in numbers because of the fairly low susceptibility (subsection 6.2.3).

When all the academic literature reviewed and laws looked at in this study were taken together, it was clear that the intention of manufacture and use of pesticides is to control and not to totally eliminate pestiferous, phytophagous and nuisance insects. Unsurprisingly, anecdote reports from previous workers suggested that fly reduction of +80% is considered satisfactory. With different recommended doses against different target species achieving fly reductions of 91-100% in this study, it was conclusive that the biolarvicide, VectoBac® was effective at controlling target fly species.

No evidence of collateral damage on other vital filter fauna like aquatic earthworms, nematode worms and, snails and slugs by the biocidal product was found.

Weakness of study

Larval subsampling using a scooping trawl does not guarantee working at identical depths throughout the filters and throughout the study. However, if this resulted in a few errors, these would have been constant and smoothed out by use of mean counts of collected samples.

The product efficacy is not only dependent on its own properties - there are other biotic and abiotic factors (Stevens et al., 2004, 2013) that can compromise treatment success. However, since filters on the same site would be subject to the same conditions, the study treatment outcomes can be relied on.

Many aspects of wastewater treatment are only covered in old academic literature.

Areas of future research

5.3.1 With "the effect of repeated applications on most ecosystems ... relatively unknown" (Lacey and Steigel, 2000) and at the same time, with product specificity in successive treatment being brought into question (Boisvert and Boisvert, 2000), it could be a good idea to study the biological and ecological phenomena under controlled conditions.

5.3.2 Could different trophic levels sustained by the rich biodiversity of WWTW pose unforeseen danger to human or animal health in future and could vertebrates that inhabit or frequent these facilities be part of a food web made up of common parasites, viruses and bacteria?

5.3.3 Could modern biological tools disprove findings made more than 100 years ago (Fair, 1934) that the sewage-associated but non-biting nuisance flies covered in this study are incapable of mechanical transmission of pathogens?

5.3.4 There is glaring gap on the biology of Chrinonomid species, *M. eurynotus* and *L. mninimus* despite their ecological importance on a local and global scale.

5.3.5 Since the ultimate aim of wastewater treatment is to discharge known quality final effluent into receiving waters, it could be prudent to develop automatic detection systems of abnormal levels of known or common pollutants in the final effluent before discharge into the inland watercourses.

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Appendices

List of appendices

A1: Daily adult bi-weekly count extract – Helsby

Helsby, Filter 1: July 26th, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae		
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other
1.1	2	28	4	9		0
1.2	0	32	5	3		3
1.3	1	61	0	1		0
1.4	0	13	1	0		1
1.5	0	17	13	3		0
1.6	1	16	25	2		3
1.7	0	22	13	6		0
1.8	1	38	4	8		0
1.9	0	21	19	5		0
1.10	1	61	7	6		1
Totals	**6**	**309**	**91**	**43**		**8**
Mean	**1**	**30.9**	**9.1**	**4.3**		**0.8**

Helsby, Filter 2: July 26th, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae		
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other
2.1	0	66	7	5		0
2.2	0	25	1	4		0
2.3	0	41	12	2		0
2.4	0	6	9	0		0
2.5	0	31	7	6		1
2.6	0	7	2	1		0
2.7	1	85	7	7		1
2.8	3	47	5	6		0
2.9	1	10	3	4		1
2.10	0	61	5	1		1
Totals	**5**	**379**	**58**	**36**		**4**
Mean	**0.5**	**37.9**	**5.8**	**3.6**		**0.4**

Helsby, Filter 3: July 26th, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae			18.0;18.5
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other	
3.1	0	7	14	6		4	
3.2	1	8	17	2		0	
3.3	0	11	29	4		1	
3.4	0	6	5	0		0	
3.5	0	5	2	4		2	
3.6	1	13	14	0		0	
3.7	0	17	3	3		0	
3.8	0	6	8	2		1	
3.9	0	22	11	1		0	
3.10	0	16	8	4		1	
Totals	**2**	**111**	**111**	**26**		**9**	
Mean	**0.2**	**11.1**	**11**	**2.6**		**0.9**	

Helsby, Filter 4: July 26th, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae		
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other
4.1	0	28	16	4		1
4.2	0	30	38	1		0
4.3	0	37	7	1		0
4.4	0	26	6	0		0
4.5	1	21	2	0		0
4.6	1	11	0	1		0
4.7	0	60	18	12		0
4.8	0	58	31	3		1
4.9	1	9	8	2		0
4.10	1	49	58	1		1
Totals	**4**	**329**	**184**	**25**		**3**
Mean	**0.4**	**33**	**18.4**	**2.5**		**0.3**

Helsby, Filter 1: July 29th, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae		
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other
1.1	0	44	7	39		0
1.2	0	46	6	25		1
1.3	2	41	6	17		0
1.4	0	26	9	3		0
1.5	0	14	15	8		0
1.6	0	32	15	4		1
1.7	2	15	9	9		0
1.8	0	87	3	27		1
1.9	0	50	32	10		0
1.10	0	81	17	14		2
Totals	**4**	**436**	**119**	**156**		**5**
Mean	**0.4**	**43.6**	**11.9**	**###**		**0.5**

Helsby, Filter 2: July 29th, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae		
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other
2.1	5	103	9	25		0
2.2	1	28	5	5		0
2.3	1	69	74	17		1
2.4	0	12	5	2		0
2.5	0	45	7	9		1
2.6	1	5	9	3		0
2.7	2	35	8	0		1
2.8	0	34	9	7		1
2.9	0	35	1	11		0
2.10	0	60	8	2		0
Totals	**10**	**426**	**135**	**81**		**4**
Mean	**1.0**	**42.6**	**13.5**	**8.1**		**0.4**

Helsby, Filter 3: July 29th, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae			18.1;18.2
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other	
3.1	0	7	9	17		3	
3.2	0	2	4	3		0	
3.3	0	11	12	6		0	
3.4	0	6	7	11		1	
3.5	1	6	6	2		0	
3.6	0	12	11	4		1	
3.7	0	6	7	3		0	
3.8	0	1	3	2		1	
3.9	1	4	7	2		0	
3.10	0	3	4	7		0	
Totals	**2**	**58**	**70**	**57**		**6**	
Mean	**0.2**	**5.8**	**7.0**	**5.7**		**0.6**	

Helsby, Filter 4: July 29th, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae		
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other
4.1	1	31	8	2		0
4.2	0	22	5	2		0
4.3	1	39	17	9		0
4.4	1	18	10	3		0
4.5	0	30	6	4		1
4.6	0	7	5	1		0
4.7	2	54	19	2		0
4.8	0	22	14	6		0
4.9	0	24	18	4		0
4.10	3	27	18	6		2
Totals	**8**	**274**	**120**	**39**		**3**
Mean	**0.8**	**27**	**12.0**	**3.9**		**0.3**

Helsby, Filter 1: August 2nd, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae		
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other
1.1	2	48	15	28	0	0
1.2	1	55	4	8	0	0
1.3	2	108	6	8	0	4
1.4	1	20	9	11	0	1
1.5	2	15	16	7	0	1
1.6	0	31	28	4	0	0
1.7	0	18	31	4	0	0
1.8	0	112	12	8	0	2
1.9	0	36	14	12	0	0
1.10	2	60	6	14	0	0
Totals	**10**	**503**	**141**	**104**	**0**	**8**
Mean	**1**	**50.3**	**14**	**10**	**0**	**0.8**

Helsby, Filter 2: August 2nd, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae			18.3; 19.1
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other	
2.1	2	128	8	11	0	0	
2.2	1	28	2	7	0	0	
2.3	1	61	5	5	0	1	
2.4	0	5	6	0	0	0	
2.5	0	70	4	7	0	0	
2.6	0	29	3	4	0	0	
2.7	0	81	5	7	0	0	
2.8	0	40	12	6	0	0	
2.9	0	22	2	8	0	0	
2.10	0	34	8	5	0	1	
Totals	**4**	**498**	**55**	**60**	**0**	**2**	
Mean	**0.4**	**49.8**	**5.5**	**6**	**0**	**0.2**	

Helsby, Filter 3: August 2nd, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae		
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other
3.1	0	9	8	8	0	0
3.2	0	10	4	3	0	1
3.3	0	8	6	8	0	0
3.4	0	28	2	5	0	2
3.5	0	3	2	6	0	2
3.6	0	24	3	5	0	0
3.7	1	16	4	4	0	1
3.8	0	8	16	11	0	1
3.9	2	16	7	4	0	0
3.10	0	18	5	6	0	0
Totals	**3**	**140**	**57**	**60**	**0**	**7**
Mean	**0.3**	**14**	**5.7**	**6**	**0**	**0.7**

Helsby, Filter 4: August 2nd, 2016.

Fly Trap No.	Anisopodidae	Chironomidae		Psycodidae		
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other
4.1	0	40	17	9	0	0
4.2	0	99	4	8	0	1
4.3	1	80	6	4	0	1
4.4	0	126	16	8	0	0
4.5	0	112	24	7	0	0
4.6	0	8	3	4	0	0
4.7	0	129	8	3	0	0
4.8	0	64	6	5	0	0
4.9	0	16	5	3	0	2
4.10	0	112	45	5	0	0
Totals	**1**	**786**	**134**	**56**	**0**	**4**
Mean	**0.1**	**79**	**13.4**	**5.6**	**0**	**0.4**

A2: All adult fly bi-weekly mean count extract – Helsby

	Filter 1 - all adult means						Filter 2 - all adult means						Filter 3 - all adult means						Filter 4 - all adult means					
	S. fenestralis	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other	*S. fenestralis*	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other	*S. fenestralis*	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other	*S. fenestralis*	*M. eurynotus*	*L. minimus*	*P. alternata*	*P. cinerea*	Other
26-Jul	0.6	30.9	9.1	4.3		0.8	0.5	37.9	5.8	3.6		0.4	0.2	11.1	11.1	2.6		0.9	0.4	32.9	18.4	2.5		0.3
29-Jul	0.4	43.6	11.9	15.6		0.5	1.0	42.6	13.5	8.1		0.4	0.2	5.8	7.0	5.7		0.6	0.8	27.4	12.0	3.9		0.3
02-Aug	1.0	50.3	14.1	10.4	0.0	0.8	0.4	49.8	5.5	6.0	0.0	0.2	0.3	14.0	5.7	6.0	0.0	0.7	0.3	78.6	13.4	5.6	0.0	0.4
05-Aug	0.4	39.4	4.4	5.5	0.0	0.6	0.2	37.1	4.6	3.9	0.0	0.3	0.6	14.7	10.1	7.3	0.0	1.1	0.6	40.3	9.6	5.0	0.0	0.9
09-Aug	0.6	35.5	11.3	6.5	0.0	0.4	0.4	36.4	4.3	4.7	0.0	0.2	0.4	13.1	10.1	7.3	0.0	1.1	0.4	40.3	9.6	5.0	0.0	0.9
12-Aug	0.3	20.4	15.5	7.0	0.2	1.0	0.0	23.3	7.6	10.0	0.0	0.6	0.4	15.1	20.8	6.1	0.0	0.9	0.7	21.1	17.9	4.4	0.0	0.6
16-Aug	0.3	44.0	28.1	4.6	0.0	0.0	0.0	61.5	10.8	4.6	0.0	0.4	0.9	29.3	35.3	2.5	0.0	0.9	0.3	43.4	22.2	1.3	0.0	1.0
19-Aug	0.7	70.5	24.0	6.0	0.0	1.8	0.1	26.2	6.8	2.4	0.0	0.3	0.9	21.3	17.6	3.1	0.0	0.5	0.5	26.5	14.6	1.1	0.0	0.5
23-Aug	0.5	29.2	7.5	2.2	0.0	1.1	0.0	32.3	9.4	1.2	0.0	0.8	0.5	26.5	18.1	2.2	0.0	0.7	0.7	36.1	21.4	1.1	0.0	1.0
26-Aug	1.0	26.0	9.4	1.9	0.0	0.1	0.1	21.1	9.5	2.6	0.0	0.4	0.3	25.1	13.6	2.4	0.0	1.4	0.3	29.6	14.7	1.2	0.0	0.4
02-Sep	0.4	42.6	15.3	1.9	0.0	0.3	0.6	46.0	14.6	1.7	0.0	0.1	0.0	40.9	51.7	2.3	0.0	2.4	0.3	0.0	0.0	0.6	0.0	0.4
06-Sep	0.8	17.6	28.1	1.5	0.0	0.8	3.2	17.4	14.3	1.4	0.0	0.2	0.4	18.6	64.6	2.2	0.0	0.4	0.6	17.8	76.1	0.5	0.0	0.7
09-Sep	0.5	28.7	38.8	2.1	0.0	0.0	0.6	21.6	13.9	2.6	0.0	0.1	0.4	43.2	70.6	1.2	0.0	0.4	0.4	27.5	58.1	0.8	0.0	0.4
13-Sep	0.9	12.2	12.6	1.3	0.0	0.4	0.7	6.5	8.6	0.7	0.0	0.1	0.3	15.7	19.0	0.1	0.0	0.3	0.0	10.3	24.4	0.1	0.0	0.3
20-Sep	1.0	26.2	30.7	2.2	0.0	0.0	0.3	35.7	20.1	2.0	0.0	0.0	1.3	58.9	106.9	1.0	0.0	0.2	1.0	35.4	85.5	0.5	0.0	0.0
23-Sep	0.5	25.4	0.2	23.9	1.2	0.0	0.0	0.1	35.6	24.7	1.9	0.0	1.6	46.6	125.5	0.7	0.0	0.2	1.5	36.4	62.3	0.1	0.0	0.3
27-Sep	0.6	22.2	19.0	1.0	0.0	0.3	0.0	31.2	33.0	1.8	0.0	0.0	0.3	46.0	86.0	0.8	0.2	0.2	0.6	47.7	93.2	0.8	0.0	0.9
30-Sep	0.3	29.4	37.7	0.9	0.0	0.1	0.2	28.7	38.5	0.0	0.7	0.0	0.7	36.0	76.0	7.3	0.1	0.0	1.0	33.6	79.1	0.1	0.0	0.0
04-Oct	0.4	39.1	48.2	1.3	0.0	0.6	0.0	39.7	50.5	2.8	0.0	0.0	0.4	66.5	115.7	1.2	0.0	1.0	0.3	63.6	110.5	0.1	0.0	0.5
07-Oct	0.1	16.3	34.9	2.6	0.0	0.0	0.0	15.1	34.3	1.0	0.0	0.0	0.2	26.8	62.9	0.8	0.0	0.1	0.1	25.8	59.2	0.1	0.0	0.1
11-Oct	0.5	29.3	46.8	1.8	0.0	0.4	0.6	20.4	28.0	0.1	0.0	0.0	0.6	34.0	86.7	0.8	0.0	0.0	0.1	37.6	64.2	0.1	0.1	0.0
14-Oct	0.8	62.7	66.6	2.7	0.0	0.0	1.4	26.1	27.7	1.4	0.0	0.0	1.1	59.1	101.4	0.6	0.0	0.2	0.5	68.8	86.3	0.4	0.0	0.0
18-Oct	0.9	65.6	60.8	2.3	0.0	0.0	2.6	46.0	35.2	0.9	0.0	0.0	1.2	101.5	93.3	0.8	0.0	0.4	1.0	87.9	95.8	0.2	0.0	0.3
21-Oct	2.0	81.3	80.4	1.3	0.0	0.0	3.8	43.4	33.2	0.5	0.0	0.1	1.1	82.7	93.3	0.6	0.0	0.0	1.0	98.7	143.5	0.0	0.0	0.0
25-Oct	0.8	50.5	50.7	0.2	0.0	0.0	1.2	56.8	19.8	0.8	0.0	0.0	0.5	27.6	28.1	0.5	0.0	0.0	0.5	23.5	19.5	0.2	0.0	0.0
28-Oct	1.0	14.6	90.2	5.3	0.0	0.0	2.3	26.3	36.1	1.4	0.0	0.0	1.9	6.2	43.7	5.5	0.0	2.2	0.9	5.0	48.3	0.0	0.0	0.1
01-Nov	1.1	6.1	39.4	0.5	0.0	0.7	1.3	72.8	51.3	1.7	0.0	0.2	1.8	0.6	14.1	0.2	0.0	0.6	1.5	5.5	23.3	0.1	0.0	0.1
04-Nov	0.7	2.9	25.4	0.1	0.0	0.0	0.7	31.2	18.5	1.6	0.0	0.1	0.8	0.5	16.6	0.0	0.0	0.3	0.2	1.5	0.0	25.0	0.0	0.0
08-Nov	0.7	0.9	12.1	0.1	0.0	0.0	0.4	15.1	17.0	0.3	0.0	0.0	0.7	0.4	3.9	0.0	0.0	0.3	0.5	0.8	14.0	0.0	0.0	0.0
11-Nov	0.1	0.1	2.2	0.0	0.0	0.0	0.1	7.3	5.8	0.5	0.0	0.0	0.2	0.1	0.6	0.0	0.0	0.2	0.1	0.2	1.4	0.0	0.0	0.0
15-Nov	0.1	2.3	20.4	0.3	0.0	0.3	0.3	128.8	59.9	1.6	0.0	0.7	0.1	1.7	7.7	0.0	0.0	1.9	0.2	1.8	23.0	0.4	0.0	1.7
18-Nov	0.0	0.9	10.1	0.0	0.0	0.0	0.0	45.8	28.4	0.7	0.0	0.0	0.0	0.2	3.2	0.0	0.0	0.1	0.0	0.5	6.4	0.0	0.0	0.0
22-Nov	0.2	0.6	4.1	0.0	0.0	0.0	0.1	12.5	10.2	0.6	0.0	0.0	0.0	0.8	7.3	0.0	0.0	0.0	0.0	0.5	2.7	0.0	0.0	0.0
25-Nov	0.0	0.9	2.6	0.0	0.1	0.0	0.3	23.6	12.0	0.2	0.0	0.1	0.0	0.1	0.7	0.0	0.0	0.1	0.0	0.6	1.8	0.0	0.0	0.0
29-Nov	0.0	3.7	0.9	0.0	0.0	0.0	0.0	35.2	14.9	1.1	0.0	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0	0.7	2.3	0.0	0.0	0.0

A3: Daily larval & NTO weekly counts extract data – Helsby

Filter 1 **Date: 01 August 2016**

	----------Target species: larval & pupal stages----------											-----Non-target macrofauna----		
Sub-samples	S-L1	S-L2	S-L3	S-L4	SP	EM	LM	MP	Lim	Psy.a1	Psy.a2	Annelida	Nematoda	Mollusca
1.1	0	0	0	1	2	5	6	3	0	0	0	8	144	21
1.2	0	0	1	1	1	13	19	10	2	0	0	18	640	14
1.3	0	0	1	0	1	12	12	13	3	0	0	5	144	5
1.4	0	0	0	0	0	15	21	1	2	0	0	0	400	0
1.5	0	0	2	2	4	18	17	9	1	0	0	15	16	5
Totals	**0**	**0**	**4**	**4**	**8**	**63**	**75**	**36**	**8**	**0**	**0**	**46**	**1344**	**45**
Mean	**0.0**	**0.0**	**0.8**	**0.8**	**1.6**	**13**	**15.0**	**7.2**	**1.6**	**0.0**	**0.0**	**9.2**	**268.8**	**9.0**

Filter 2 **Date: 01 August 2016**

	----------Target species: larval & pupal stages----------											-----Non-target macrofauna----		
Sub-samples	S-L1	S-L2	S-L3	S-L4	SP	EM	LM	MP	Lim	Psy.a1	Psy.a2	Annelida	Nematoda	Mollusca
2.1	0	0	1	1	0	5	5	3	1	0	0	11	1440	21
2.2	0	0	0	0	1	7	9	8	2	0	0	0	80	3
2.3	0	0	0	0	0	11	18	2	4	0	0	5	160	5
2.4	0	0	2	1	3	6	5	7	3	0	0	1	0	2
2.5	0	0	0	1	1	8	9	8	0	0	0	0	160	4
Totals	**0**	**0**	**3**	**3**	**5**	**37**	**46**	**28**	**10**	**0**	**0**	**17**	**1840**	**35**
Mean	**0.0**	**0.0**	**0.6**	**0.6**	**1.0**	**7.4**	**9.2**	**5.6**	**2.0**	**0.0**	**0.0**	**3.4**	**368.0**	**7.0**

Filter 3 **Date: 01 August 2016**

	----------Target species: larval & pupal stages----------											-----Non-target macrofauna----		
Sub-samples	S-L1	S-L2	S-L3	S-L4	SP	EM	LM	MP	Lim	Psy.a1	Psy.a2	Annelida	Nematoda	Mollusca
3.1	0	0	2	0	0	13	17	7	0	0	0	0	480	13
3.2	0	0	0	0	1	21	28	15	7	0	0	18	640	0
3.3	0	0	0	2	6	8	8	4	2	0	0	5	160	0
3.4	0	0	0	1	1	12	9	5	1	0	0	14	80	19
3.5	0	0	0	0	2	3	2	0	1	0	0	0	320	11
Totals	**0**	**0**	**2**	**3**	**10**	**57**	**64**	**31**	**11**	**0**	**0**	**37**	**1680**	**43**
Mean	**0.0**	**0.0**	**0.4**	**0.6**	**2.0**	**11**	**12.8**	**6.2**	**2.2**	**0.0**	**0.0**	**7.4**	**336.0**	**8.6**

Date: 01 August 2016

Filter 4	----------Target species: larval & pupal stages----------											-----Non-target macrofauna----		
Sub-samples	S-L1	S-L2	S-L3	S-L4	SP	EM	LM	MP	Lim	P.a1	P.a2	Annelida	Nematoda	Mollusca
4.1	0	0	0	0	0	11	7	2	2	0	0	13	640	18
4.2	0	0	0	0	0	9	13	3	2	0	0	8	96	9
4.3	0	0	1	0	2	18	23	11	3	0	0	9	400	1
4.4	0	0	0	1	3	5	8	3	1	0	0	11	320	3
4.5	0	0	1	1	1	2	6	4	6	0	0	1	480	1
Totals	**0**	**0**	**2**	**2**	**6**	**45**	**57**	**23**	**14**	**0**	**0**	**42**	**1936**	**32**
Mean	**0.0**	**0.0**	**0.4**	**0.4**	**1.2**	**9**	**11.4**	**4.6**	**2.8**	**0.0**	**0.0**	**8.4**	**387.2**	**6.4**

Key

S-L1 to S-L4: *Sylvicola* larval stages 1-4
SP: *Sylvicola* pupae
EM: Metronecmus - Early Larval stage
LM: Metrocnemus - Late Larval stage
MP: Metronecmus pupae
Lim: *Limnophyes* larvae
P.a1: Psychoda alternata larvae - early stage
P.a2: Psychoda alternata larvae - late stage

A4: Larval and NTO mean weekly counts extract Helsby

	Helsby, Filter 1:													
	----------Target species: larval & pupal stages----------											-----Non-target organisms-----		
Day	S-L1	S-L2	S-L3	S-L4	SP	EM	LM	MP	Lim	Psy.a1	Psy.a2	Annelida	Nematoda	Mollusca
14-Mar	6.8	7.8	6.2	6.6	1.6	59.6	58.0	1.6	0.0		0.0	3.8	0.4	6.6
21-Mar	7.0	3.4	5.0	6.2	1.8	54.6	53.0	2.2	0.0		0.0	2.8	0.2	9.6
28-Mar	7.4	8.0	6.8	10.6	1.6	50.8	41.6	3.0	0.0		0.0	5.8	0.4	10.4
04-Apr	20.4	13.4	40.0	64.4	11.4	52.2	52.2	10.2	47.8		0.0	4.2	7.6	16.2
11-Apr	6.4	6.4	14.2	19.2	4.8	15.6	15.6	23.0	4.8		0.0	4.4	5.6	4.4
18-Apr	3.2	3.2	13.6	8.8	3.6	36.2	36.2	29.6	12.6		1.8	8.6	1.2	6.2
25-Apr	1.6	1.8	5.4	11.6	5.6	20.8	20.8	8.4	19.8		0.4	3.4	18.4	17.0
02-May	1.2	1.4	3.2	4.2	2.6	51.2	53.2	22.4	51.2		0.0	4.0	28.8	9.8
09-May	3.2	2.6	6.6	7.2	6.4	313.0	317.6	188.8	166.4		0.0	19.0	118.4	13.2
16-May	6.6	7.0	15.0	24.6	12.6	244.8	251.2	99.2	134.4		0.0	4.0	134.4	4.2
23-May	8.4	12.0	14.4	21.4	18.8	283.0	293.0	204.8	117.2		0.0	4.2	105.6	4.2
31-May	14.4	17.2	20.6	26.6	12.0	21.0	23.2	30.4	15.6		0.0	5.2	60.8	10.4
02-Jun	6.4	7.2	12.6	11.4	12.6	35.8	39.4	52.8	23.2		0.0	8.6	56.0	6.4
06-Jun	3.2	3.6	5.6	7.0	10.6	70.8	73.2	22.4	9.6		0.0	6.8	32.0	9.2
13-Jun	1.6	2.0	3.4	4.2	9.2	135.6	139.6	34.0	52.6		0.0	5.6	163.2	4.0
20-Jun	1.6	1.8	1.8	1.6	3.8	81.6	84.8	40.0	19.2		0.0	3.8	188.8	4.6
27-Jun	1.0	0.6	0.6	0.8	1.8	48.4	52.4	12.2	8.6		0.0	5.2	118.4	6.0
04-Jul	0.6	1.4	0.6	0.8	2.0	16.8	20.4	6.6	6.2		0.0	2.6	118.4	3.6
12-Jul	0.0	0.0	0.0	3.0	0.4	3.2	3.8	2.4	4.2		0.0	2.6	1.4	1.0
19-Jul	0.0	0.0	0.0	1.2	0.6	3.2	2.6	3.2	3.6		0.0	1.6	0.0	0.8
25-Jul	0.0	0.2	0.6	1.6	1.8	18.2	22.4	10.4	13.8		3.2	9.4	124.0	9.2
01-Aug	0.0	0.0	0.8	0.8	1.6	12.6	15.0	7.2	1.6	0.0	0.0	9.2	268.8	9.0
08-Aug	0.0	0.4	0.4	0.2	3.4	10.8	14.2	4.8	5.2	0.0	0.4	5.2	656.0	15.8
15-Aug	0.0	0.0	0.2	1.0	1.6	8.2	9.2	4.0	1.0	0.0	0.0	2.6	467.8	7.2
22-Aug	0.0	0.2	0.4	1.0	1.4	22.4	32.0	19.2	0.8	0.0	0.0	4.4	584.0	6.6
29-Aug	0.0	0.2	0.2	0.6	0.8	17.6	21.2	8.2	0.4	0.0	0.0	7.0	320.0	6.8
05-Sep	0.0	0.2	0.2	0.8	0.4	1.4	2.6	0.8	0.6	0.2	0.0	8.4	86.4	3.2
13-Sep	0.0	0.0	0.0	1.2	0.2	1.6	3.0	0.4	0.6	0.0	0.0	6.6	83.2	2.6
19-Sep	0.0	0.2	1.8	17.4	5.6	15.4	43.4	12.8	16.0	0.0	0.0	9.2	73.6	3.2
26-Sep	0.0	0.2	4.2	35.2	8.4	21.2	76.0	16.8	28.4	0.0	0.2	13.4	89.6	6.8
03-Oct	0.0	4.0	6.4	35.2	6.4	41.6	80.0	25.6	32.0	6.4	3.2	11.8	99.2	7.0
10-Oct	0.0	3.2	6.4	19.2	16.0	60.8	64.0	35.2	48.0	0.0	0.0	6.8	374.4	11.4
18-Oct	3.2	12.8	9.6	16.0	22.4	208.0	182.4	67.2	102.4	12.8	9.6	4.0	320.0	12.4
21-Oct	0.0	0.0	0.0	9.6	25.6	22.4	41.6	150.4	9.6	0.0	0.0	24.0	1043.2	9.2
25-Oct	0.0	0.0	0.0	0.0	0.0	2.6	10.4	4.6	0.0	0.0	0.0	3.4	1379.2	7.6
27-Oct	3.2	6.4	0.0	32.0	9.6	16.0	6.4	6.4	0.0	0.0	0.0	8.6	1356.8	14.6
30-Oct	0.0	0.0	0.0	0.0	30.2	0.0	0.0	19.2	0.0	0.0	0.0	17.2	2185.6	16.0
07-Nov	0.0	0.0	0.0	0.6	3.4	6.4	0.0	0.0	0.0	3.2	0.0	22.2	1494.4	18.6
14-Nov	0.0	0.0	0.2	0.4	1.4	0.4	0.0	0.4	12.8	0.0	0.0	12.6	1427.2	22.6
21-Nov	6.4	6.4	0.0	0.0	1.2	14.4	31.2	0.4	20.4	3.2	0.0	9.6	1859.2	13.6
28-Nov	0.0	6.4	0.0	0.0	3.0	179.2	144.0	3.2	89.6	0.0	0.0	15.4	1068.8	12.0
Key														
S-L1 - S-L4	Sylvicola larval stages 1-4						MP	Metriocnemus pupae						
SP	Sylvicola pupae						Lim	Limnophyes larvae						
EM	Metriocnemus early larval stage						Psy	Psychoda larvae						
LM	Metriocnemus late larval stage													

A5: *S. fenestralis* rationalised pre- and post-treatment data – Helsby

	Control filter (1)				Test filter (2)			
	Adult counts		Larval counts		Adult counts		Larval counts	
	Before	After	Before	After	Before	After	Before	After
	2	3	15	8	8	0	21	0
	4	3	14	13	8	0	10	0
	10	0	44	5	9	0	17	0
	2	0	52	34	4	0	96	0
	2	1	64	39	3	0	60	0
	4	1			7	0		
	8	1			14	0		
	5	13			5	0		
	6	1			7	0		
	3	12			7	0		
Totals	**46**	**35**	**189**	**99**	**72**	**0**	**204**	**0**
Mean	**4.6**	**3.5**	**37.8**	**19.8**	**7.2**	**0**	**40.8**	**0**
SE	**0.8589**	**1.5366**	**10.0319**	**6.9814**	**0.9638**	**0**	**16.3138**	**0**

A6: *P. alternata* rationalised pre- and post-treatment data – Macclesfield

Pre- and post-treatment-1.

	Control Filter 18: 1st dose.				Test Filter 17: 1st dose.			
	Adult counts		Larval counts		Adult counts		Larval counts	
	Before	After	Before	After	Before	After	Before	After
	550	476	112	256	275	41	96	0
	275	770	368	48	225	52	480	0
	363	880	80	16	880	100	416	0
	220	660	32	160	330	72	48	0
	280	511	32	48	495	160	96	0
	198	201			660	65		
	143	423			440	165		
	395	491			605	117		
	330	385			113	38		
	280	660			660	75		
Totals	**3034**	**5457**	**624**	**528**	**4683**	**885**	**1136**	**0**
Mean	**303.4**	**545.7**	**124.8**	**105.6**	**468.3**	**88.5**	**227.2**	**0**
SE	**36.3863**	**62.9769**	**62.6661**	**44.8571**	**74.9163**	**14.5505**	**91.1298**	**0**

Pre- and post-treatment-2.

	Control Filter 18: 2nd dose.				Test Filter 17: 2nd dose			
	Adult counts		Larval counts		Adult counts		Larval counts	
	Before	After	Before	After	Before	After	Before	After
	239	73	176	176	51	16	16	0
	413	127	688	448	36	16	0	32
	230	117	448	192	40	23	112	0
	161	57	240	704	65	20	0	32
	385	85	384	352	51	42	32	16
	495	57			60	18		
	249	106			128	51		
	330	63			116	41		
	279	121			60	8		
	119	125			70	13		
Totals	**2900**	**931**	**1936**	**1872**	**677**	**248**	**160**	**80**
Mean	**290**	**93.1**	**387.2**	**374.4**	**67.7**	**24.8**	**32**	**16**
SE	**36.7671**	**9.2285**	**89.571**	**96.744**	**9.6782**	**4.5869**	**20.861**	**7.1554**

A7: *L. minimus* rationalised pre- and post-treatment data – Macclesfield

Pre- and post-treatment-1 data

	Control F18_1st				Test F17: low dose_1st				Test F19: standard dose_1st				Test F20: high dose_1st			
	Adult counts		Larval counts		Adult counts		Larval counts		Adult counts		Larval counts		Adult counts		Larval counts	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
	65	15	16	0	178	54	48	0	99	5	0	32	63	12	16	0
	167	13	16	0	85	31	0	0	299	11	16	32	134	19	0	0
	70	15	16	16	92	56	32	0	141	19	16	16	660	6	0	0
	207	24	0	48	120	21	64	0	352	17	0	24	330	24	0	0
	250	32	16	16	80	28	32	0	176	20	16	16	275	9	16	0
	90	23			120	44			310	7			225	6		
	230	18			80	45			212	22			198	14		
	160	31			96	46			90	7			291	43		
	199	22			70	18			112	16			336	10		
	28	14			48	51			198	16			278	16		
Totals	**1466**	**207**	**64**	**80**	**969**	**394**	**176**	**0**	**1989**	**140**	**48**	**120**	**2790**	**159**	**32**	**0**
Mean	**146.6**	**20.7**	**12.8**	**16.0**	**96.9**	**39.4**	**35.2**	**0.0**	**198.9**	**14.0**	**9.6**	**24.0**	**279.0**	**15.9**	**6.4**	**0.0**
SE	**24.5928**	**17.8831**	**3.2000**	**8.7636**	**11.9137**	**4.3670**	**10.6132**	**0**	**29.6728**	**1.9149**	**3.9192**	**3.5777**	**50.3686**	**3.5070**	**3.9192**	**0**

Pre- and post-treatment-2 data – Macclesfield.

	Control F18_2nd				Test F17: low dose_2nd				Test F19: standard dose_2nd				Test F20: high dose_2nd			
	Adult counts		Larval counts		Adult counts		Larval counts		Adult counts		Larval counts		Adult counts		Larval counts	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
	55	12	16	16	30	25	0	0	404	7	16	0	61	22	0	0
	61	20	16	0	25	71	0	0	259	30	0	0	35	42	0	0
	75	25	32	0	44	90	0	0	85	17	16	0	90	29	0	0
	112	26	48	32	62	35	16	0	60	18	0	0	90	34	0	0
	408	27	0	16	43	63	0	0	112	23	0	0	51	23	0	0
	60	24			45	95			55	18			41	9		
	70	31			55	135			261	11			34	12		
	61	12			29	90			91	11			95	47		
	140	11			36	27			102	9			80	11		
	39	12			21	51			110	41			78	44		
Totals	**1081**	**200**	**112**	**64**	**390**	**682**	**16**	**0**	**1539**	**185**	**32**	**0**	**655**	**273**	**0**	**0**
Mean	**108.1**	**20**	**22.4**	**12.8**	**39.0**	**68.2**	**3.2**	**0.0**	**153.9**	**18.5**	**6.4**	**0.0**	**65.5**	**27.3**	**0.0**	**0.0**
SE	**34.6193**	**2.4037**	**8.1584**	**5.9867**	**4.1793**	**11.1194**	**3.2**	**0**	**36.3234**	**3.3275**	**3.9192**	**0**	**7.5942**	**4.4871**	**0**	**0**

A8: *M. eurynotus* rationalised pre- and post-treatment data – Helsby.

Pre- and post-treatment-2 data

	Control filter 2_1st				Test filter 1: low dose_1st				Test Filter 3: high dose_1st				Test Filter 4: standard dose_1st			
	Adult counts		Larval counts		Adult counts		Larval counts		Adult counts		Larval counts		Adult counts		Larval counts	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
	25	49	176	80	55	1	256	16	151	0	64	32	79	0	176	0
	80	36	32	80	47	1	272	48	60	1	288	0	45	0	208	48
	59	92	192	192	56	0	80	32	43	0	384	0	104	0	176	32
	31	6	64	64	47	0	256	0	116	0	112	16	85	0	224	16
	49	39	112	96	84	1	176	16	118	1	208	16	73	1	208	16
	48	37			81	3			50	0			50	1		
	51	50			36	1			143	0			129	1		
	22	34			80	1			88	0			85	0		
	61	45			78	0			80	0			66	2		
	34	70			92	1			166	0			163	0		
Totals	**460**	**458**	**576**	**512**	**656**	**9**	**1040**	**112**	**1015**	**2**	**1056**	**64**	**879**	**5**	**992**	**112**
Mean	**46.0**	**45.8**	**115.2**	**102.4**	**65.6**	**0.9**	**208.0**	**22.4**	**101.5**	**0.2**	**211.2**	**12.8**	**87.9**	**0.5**	**198.4**	**22.4**
SE	**5.7484**	**7.2200**	**30.9425**	**22.9643**	**6.1521**	**0.2769**	**36.1331**	**8.1584**	**13.8470**	**0.1333**	**57.9986**	**5.9867**	**11.3837**	**0.2236**	**9.6**	**8.1584**

Pre- and post-treatment-2 data.

	Control filter 2_2nd				Test filter 1: low dose_2nd				Test Filter 3: high dose_1st				Test Filter 4: standard dose_1st			
	Adult counts		Larval counts		Adult counts		Larval counts		Adult counts		Larval counts		Adult counts		Larval counts	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
	27	31	112	80	9	8	32	0	5	0	32	0	1	0	16	16
	41	45	144	112	15	4	32	0	0	0	0	0	4	0	0	16
	39	47	16	80	9	1	0	0	1	0	0	0	9	1	0	0
	12	8	48	96	8	2	0	0	17	0	16	0	6	0	0	0
	40	32	96	64	21	13	16	0	0	0	32	0	4	1	16	0
	31	37			18	6			2	0			5	0		
	22	31			24	1			7	0			3	2		
	13	41			19	1			5	0			1	1		
	26	44			12	0			5	0			16	0		
	12	36			11	1			20	0			1	2		
Totals	**263**	**352**	**416**	**432**	**146**	**37**	**80**	**0**	**62**	**0**	**80**	**0**	**50**	**7**	**32**	**32**
Mean	**26.3**	**35.2**	**83.2**	**86.4**	**14.6**	**3.7**	**16.0**	**0.0**	**6.2**	**0.0**	**16.0**	**0.0**	**5.0**	**0.7**	**6.4**	**6.4**
SE	**3.6394**	**3.5522**	**22.8526**	**8.1584**	**1.7839**	**1.3170**	**7.1554**	**0**	**2.1949**	**0.0**	**7.1554**	**0**	**1.4606**	**0.2603**	**3.9192**	**3.9192**

A9: NTOs complete mean counts –Helsby

	ANNELIDA: HELSBY				NEMATODA:- HESLBY				MOLLUSCA: HELSBY			
	Ctrl/ Low	Med./cotr	High (F3)	Med. (F4)	Ctrl/ Low	Med./cotr	High (F3)	Med. (F4)	Ctrl/ Low	Med./cotr	High (F3)	Med. (F4)
14-Mar	3.8	4.8			0.4	0.6			6.6	9.8		
21-Mar	2.8	2.2			0.2	0.0			9.6	8.8		
28-Mar	5.8	2.8			0.4	0.4			10.4	6.8		
04-Apr	4.2	5.0			7.6	48.0			16.2	7.4		
11-Apr	4.4	5.0			5.6	5.8			4.4	21.2		
18-Apr	8.6	7.8			1.2	21.8			6.2	7.0		
25-Apr	3.4	5.4			18.4	24.0			17.0	13.2		
02-May	4.0	5.8			28.8	64.0			9.8	9.6		
09-May	19.0	72.2			118.4	208.0			13.2	13.0		
16-May	4.0	4.0			134.4	137.6			4.2	5.0		
23-May	4.2	3.2			105.6	19.2			4.2	7.6		
31-May	5.2	3.2			60.8	54.4			10.4	8.4		
02-Jun	8.6	10.0			56.0	70.4			6.4	12.4		
06-Jun	6.8	10.4			32.0	75.2			9.2	15.0		
13-Jun	5.6	8.4	6.2	7.0	163.2	214.4	182.4	176.0	4.0	15.4	4.2	5.0
20-Jun	3.8	3.6	5.8	5.4	188.8	198.4	115.2	147.2	4.6	6.0	4.2	3.6
27-Jun	5.2	6.4	6.4	7.0	118.4	144.0	182.4	115.2	6.0	5.6	5.4	4.4
19-Sep	9.2	9.2	9.8	6.2	73.6	96.0	76.8	33.6	3.2	4.4	8.6	4.8
26-Sep	13.4	16.0	7.0	15.0	89.6	57.6	56.8	33.6	6.8	20.8	13.6	8.6
03-Oct	11.8	7.2	8.0	5.6	99.2	105.6	92.8	67.2	7.0	5.2	6.8	3.8
10-Oct	6.8	11	2.4	11.4	374.4	182.4	275.2	265.6	11.4	25	9.8	19.2
18-Oct	4.0	7.6	8.8	9.8	320.0	185.6	166.4	227.2	12.4	13.2	14.0	13.6
21-Oct	24.0	13.4	7.6	7.6	1043.2	1023.2	899.2	809.6	9.2	22.2	11.0	11.4
25-Oct	3.4	5	5.4	4.4	1379.2	1507.2	1152.0	976.0	7.6	6.6	7.6	7.2
27-Oct	8.6	19.4	15.0	17.8	1356.8	1592.8	1043.2	995.2	14.6	13	13.4	14.8
30-Oct	17.2	26	13.4	9.6	2185.6	1369.6	1108.8	1104.0	16.0	15.2	11.4	18.2
07-Nov	22.2	19.8	88.0	24.4	1494.4	1174.4	6080.0	1849.6	18.6	17.6	80.0	15.0
14-Nov	12.6	11.2	9.0	10.6	1427.2	2092.8	2156.8	2604.8	22.6	17	13.4	16.8
21-Nov	9.6	8.8	6.4	8.2	1859.2	1814.4	1742.4	1561.6	13.6	12.8	11.4	11.4
28-Nov	15.4	13.6	18.6	13.6	1068.8	1491.2	1328.0	1091.2	12.0	13.4	11.8	10.6

A10: NTOs complete mean counts – Macclesfield

	ANNELIDA: MACCLESFILED				NEMATODA MACCLESFIELD				MOLLUSCA: MACCLESFIELD			
	Ctrl (F18)	Low (F17)	Med. (F19	High (F20)	Ctrl (F18)	Low (F17)	Med. (F19	High (F20)	Ctrl (F18)	Low (F17)	Med. (F19	High (F20)
17-Mar	4.4	2.0			9.4	9			0.0	0.0		
22-Mar	0.0	4.2			0.0	125			0.0	0.0		
31-Mar	1.8	2.2			76.8	138			0.0	0.0		
07-Apr	1.2	0.4			155.2	58			0.6	0.6		
14-Apr	2.0	1.6			200.0	580			0.0	0.0		
21-Apr	0.8	1.6			635.2	269			1.2	0.4		
28-Apr	0.8	0.8			6.4	19			0.2	0.0		
05-May	0.2	0.4			102.4	355			0.2	0.8		
12-May	1.8	0.6			144.0	26			0.2	0.0		
19-May	0.8	0.6			210.0	214			0.8	0.8		
26-May	1.4	1.2			241.6	202			0.6	0.6		
03-Jun	2.4	0.6			310.4	136			0.2	0.6		
09-Jun	0.4	0.8			72.0	51			0.4	0.2		
16-Jun	0.5	0.6	1.4	1.2	140.0	93	108.8	64.0	0.4	1.2	0.8	0.4
30-Jun	1.0	0.8	1.2	1.2	276.0	69	192.0	137.6	1.2	0.8	0.6	1.0
07-Jul	0.6	0.8	0.4	0.6	377.6	242	272.0	288.0	0.4	0.8	0.2	0.4
11-Jul	0.4	0.0	0.2	0.2	0.0	0	0.0	0.0	0.0	0.0	0.0	0.0
18-Jul	0.4	0.2	0.4	0.4	0.0	0	0.0	0.0	0.0	0.0	0.0	0.0
28-Jul	0.6	1.0	0.8	0.6	383.4	461	425.6	265.6	0.2	0.6	1.4	0.0
04-Aug	3.2	3.6	16.6	6.4	173.0	175	184.8	451.2	0.6	1.2	0.2	0.6
11-Aug	19.6	25.6	6.4	6.4	390.4	506	246.4	400.0	6.4	3.2	0.2	0.0
17-Aug		16.0				483				3.2		
19-Aug	16.0	6.4	9.6	6.4	454.4	2054	329.6	464.0	19.2	6.4	6.4	0.0
25-Aug	60.8	38.4	35.2	25.6	3328.0	2611	1766.4	2064.0	25.6	13.0	19.2	115.2
30-Aug	40.0	24.0	16.0	12.3	1804.0	1584	1108.0	1180.0	52.0	32.0	17.0	13.8
02-Sep	2.4	10.6	2.6	2.2	723.2	477	1369.6	1200.0	7.0	10.4	6.2	5.8
06-Sep	2.6	3.2	2.0	3.0	800.0	1370	1136.0	1260.8	4.8	6.2	6.0	13.0
09-Sep	4.2	4.4	1.0	1.8	771.2	627	2201.6	2137.6	30.2	32.8	19.8	8.2
12-Sep	0.4	0.4	0.4	0.6	608.0	496	1408.0	928.0	1.6	1.2	5.0	5.0
22-Sep	3.0	6.0	6.0	3.8	1308.8	3021	5155.2	4348.8	22.0	26.0	21.4	16.4
29-Sep	1.0	1.4	1.0	1.8	1606.4	2787	2374.4	3824.0	5.2	16.4	1.6	10.2
06-Oct	0.4	3.2	0.6	2.4	832	3696	4246.4	4588.8	18.8	15.2	17.4	14.4