At any given stage of the life cycle, some of these snails would die. Thus 1 cubic metre of plastic medium could contain approximately 0.25 kg of decomposing mollusc. The Stage 4 towers had much heavier snail infestation than the Stage 2 towers. In the former, biofilm was almost non-existent, whereas in the latter, there was a healthy 2-3mm thick biofilm.

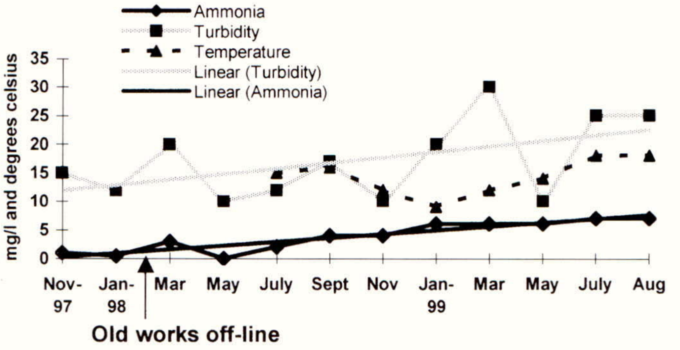

Figure 2. Changes in ammonia and turbidity results in the period after refurbishment of the filters.

The concrete surfaces of the Stage 3 clarifiers had a much thinner biofilm than the Stage 5 clarifiers, suggesting that the snails could seriously be affecting the partially cleaned effluent coming out of the Stage 3 clarifiers. Gut content analysis suggested that snails were grazing on *Zoogloea* spp. biofilm. Using hydrochemical data from Dussart (1979) and from past measurements at Berkhamsted, a forward stepwise multiple regression suggested the following factors were significant to the distribution of *L.peregra* (P=0.00378):-

Lymnaea peregra abundance = 946 - 53.9K + 15.55Cl^- - 0.44Ca^{2+} - 0.99HCO_3^-

Biotic factors were not included. Non-significant factors included pH, total hardness, Mg^{2+}, Na^+, NO_3^-, NH_3^+, NO_4^-, PO_4^{3-}, SO_4^{2-}, and O_2, though any of these could, of course be limiting at extreme concentrations. Table 2 compares the composition of two natural sites with the artificial circumstances afforded by Berkhamsted. The latter represent a large upward projection of the AGE data.

Table 2 Comparison of water chemistry data for Berkhamsted with data from natural sites in North West England (Dussart,1976).

mg/L	Site with least snails ULLSWATER	Site with the most abundant snails AGECROFT	Berkhamsted second tower 4b(22/10/99)
Ca^{2+}	9.5	48.8	122
HCO_3^-	20.5	92	272
Mg^{2+}	4.4	14.76	2.8
NO_3^-	3.3	0.49	52.7
PO_4^{3-}	0.04	0.15	4.6
Na^+	5.3	14.2	61.4
K^+	0.92	6.7	9.8
Cl^-	13.3	32	71
pH	6.93	7.6	7.5

After treatment with copper sulphate, the snail populations in the Stage 4 towers were completely eliminated. The foaming stopped and the works returned to full efficiency within three months from the re-seeding of the filters with effluent from a nearby works. Figure 2 suggests that it took 9 months for the situation to reach a situation where snail densities were causing failure to reach the consent standards. Starting from a near-zero snail population, and with information based on the life cycle of *L. peregra* (Dussart, 1979), it could therefore take a minimum of 6-8 months for the snail problem to develop again. Figure 3 shows that six months later in July 2000, ammonia concentrations had indeed risen to consent levels. However, when the Stage 4 filters were examined in March 2002, no snails were found on the top of the filter.

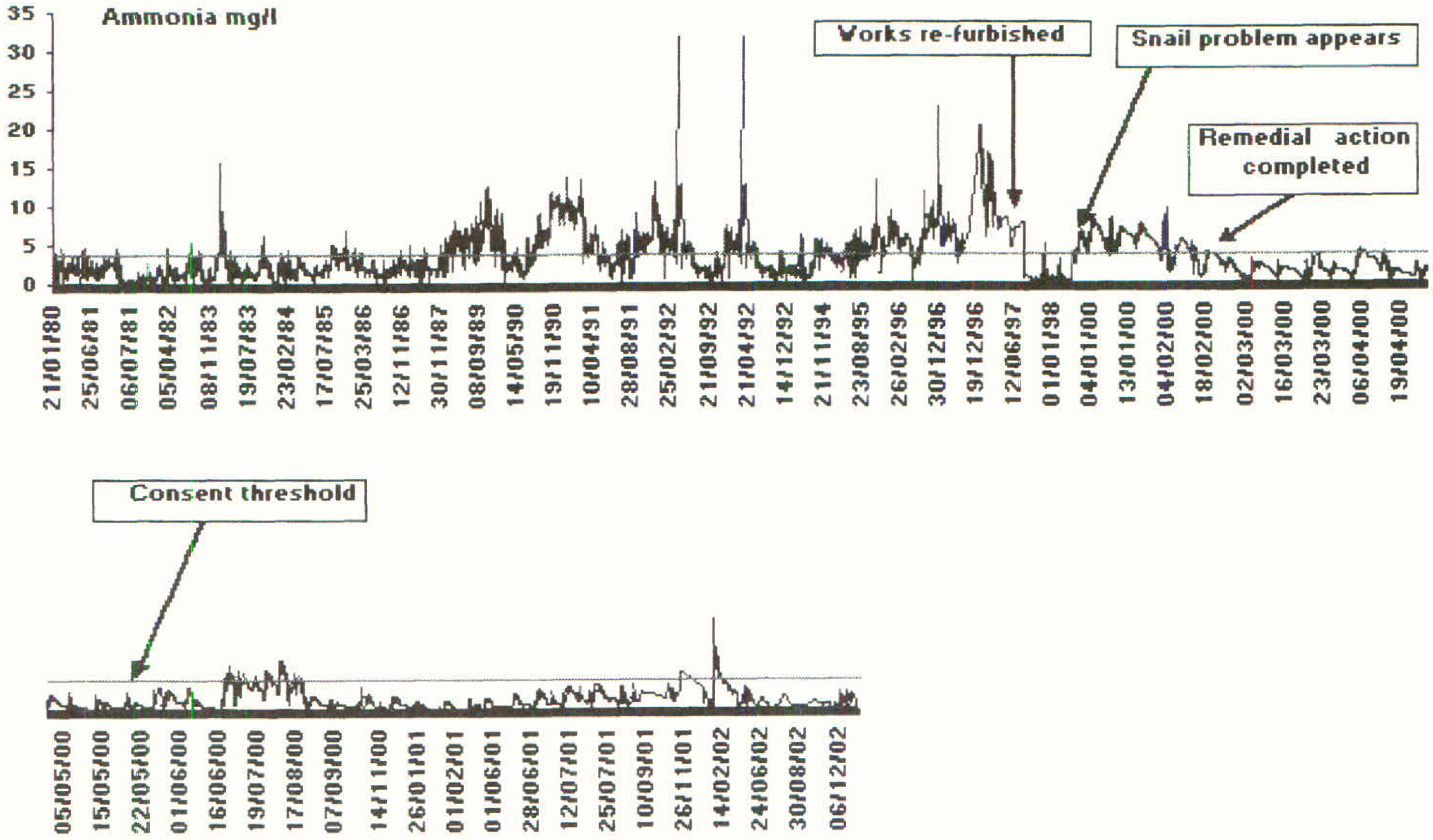

Figure 3 Long term performance of the Berkhamsted effluent treatment works

DISCUSSION

It is important to be certain about the taxonomy of the culprit. If the offender had been an alien snail such as *Physa acuta, a* one-off action could cure the problem. Conversely, with an indigenous snail such as *L. peregra,* a one-off action might only be successful in the short term, since immediate re-invasion of the works would be expected. Although the offending snail was *L. peregra* under current classification criteria, the real identity may be uncertain. *L. peregra* is one of the most ubiquitous pulmonate snails in temperate Eurasian freshwaters and has a wide range of morphology. The currently accepted nomenclatural revision was by Hubendick in 1951. However, on the continent, two major types are recognised - *Radix ovata* and *L. peregra* (e.g. Gloer, *et al*., 1990). The snails found in the works would be called *L. peregra* by most British malacologists, but *Radix ovata* by most continental workers. Indeed, there is a recent suggestion that the British *L. peregra* should be called *Radix balthica* (Linnaeus 1758) and the continental *R.ovata* should be called *Radix labiata* (Rossmaessler) (Bargues, *et al*., 2001). Some aspects of the ecology of *L. peregra* are understood (Dussart, 1976, 1979, Dussart & Kay,1980) but it is generally under-researched.

The Stage 2 and Stage 4 filter towers allow comparisons of performance. The Stage 2 towers had a healthy biofilm, had a relatively low population of mature snails and were achieving their design criteria. However, with a huge population of smaller snails, the Stage 4 towers were not achieving their criteria. The thin slime film on the concrete surfaces of the Stage 3 clarifiers suggesting that the snails were badly affecting the partially cleaned Stage 3 effluent. Indeed, it appeared that the sewage was deteriorating as it progressed through the works. The total snail biomass appeared to have two effects on the efficiency of the filter.

Firstly, there was the effect of the normal ecological activity of live snails. Gut content analysis suggested that the snails were feeding on the predominantly *Zoogloea* spp. which coats the plastic medium. The resulting lack of film led to an almost complete lack of normal biological functions for the filter beds. The relatively small snail size suggests that either the snails were short of food or that the population was in a relatively continuous log phase of growth, possibly as a result of snails being continuously lost from the bottom of the towers. Alternatively, this may have been a relatively recently hatched cohort which was growing to mature size. In either case, the high reproductive rate would mean that hatchlings (about 1mm) were escaping to all parts of the works. Also, this snail population was consuming oxygen, and producing carbon dioxide, faeces and mucus, the latter causing problems of foaming in the later stages of the works.

Secondly, there was the effect of dead snails. Moribund snails were being washed into dead spaces in the plastic medium, or down to the bottom of the towers and thence into the next stage of the process. As the snail biomass increased, the problem increased, especially when the snails died and decomposed. Decomposing snails produce a particularly noxious and clinging smell and taste in the water.

Despite that fact that molluscs cause many serious problems to industrial users of water, the niche requirements of freshwater molluscs have been relatively little studied. In future, it might be possible to alter the chemical composition of the effluent so that there is a change in the niche. For example, the balance of calcium and magnesium is known to be important in the distribution of some freshwater snails (Meier-Brook, *et al.,* 1987), and aspects such as this might be manipulated. However, the multiple regression analysis is limited in that it does not include biological aspects such as food availability and predators.

An initial attempt to kill the snails with sodium hydroxide failed, probably because *L. peregra* can thrive in alkaline conditions. This procedure has apparently been successful at works in the USA but the American species of snail may have been less tolerant to such conditions.

Because this problem involves indigenous snails, it is possible to take an ecological view. The Berkhamsted STW appears to have created an almost perfect ecological niche for *L. peregra,* partly defined by the water chemical parameters shown in Table 2 and summarised in the multiple regression equation. In the Stage 4 filters, environmental conditions were favourable, there was a good food supply and there were no significant predators. The long-term trends in Fig. 3 show that the works is generally performing better than it did before the refurbishment. Nevertheless, to solve the problem in the long term, it will be necessary to change the conditions in the works so that the *L.peregra* niche is constrained. This might involve some re-structuring of the treatment processes. It is most likely that the infiltration water dilutes the influent to the works such that it does not control snail populations as it might have done in the past. This would appear to be one of the first issues to address for long-term control. Pores in the previous clinker might have created microhabitats for parasites and pathogens of the snails. Also, these rugose surfaces would have retained biofilm so that it could not be reached by the snails, and purification could have continued unabated in these local areas. However, a smooth plastic medium offers no such refuges from the action of the snail radula. Further research is needed into the biology of aquatic snails which are living in these humid, air-filled voids inside the filters, and also into their relationships with the smoothness of the surface on which they live.

REFERENCES

Askew M (1966). Plastics in waste treatment *Process Biochemistry* December 3-10.

Bargues M; Vigo M; Horak P; Dvorak J; Patzner R; Pointier J-P; Jackiewicz M; Meier-Brook C; Mas-Coma S (2001) European Lymnaeidae (Mollusca Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-sequences. *Infection, Genetics and Evolution* **1**, 85-107.

Bruce A S (1969). Percolating filters *Process Biochemistry* April 1-5.

Dussart G B J (1976). The ecology of freshwater molluscs in North West England in relation to water chemistry. *Journal of Molluscan Studies* **42,** 181-198.

Dussart G B J (1979). Life cycles and distributions of the aquatic gastropod molluscs *Bithynia tentaculata* (L.), *Gyraulus albus* (Müller), *Planorbis planorbis* (L.), *and Lymnaea peregra* (Müller) *in relation to water chemistry. Hydrobiologia* **67,** 223-239.

Dussart G B J; Kay R (1980). Relationships between water chemistry and respiration rate in several populations of *Lymnaea peregra* (Müller), *(Gastropoda, Mollusca). Hydrobiologia* **69,** 57-65.

Gloer P; Meier Brook C (2003). *Susswassermollusken,* Deutscher Jugendbund fur Naturbeobachtung, Hamburg.

Hubendick B (1951). *Recent Lymnaeidae, their variation, morphology, taxonomy, nomenclature and distribution.* K. Svenska. Vekensk. Handl. 4th series. Stockholm, Sweden.

Meier-Brook C; Haas D; Winter G; Zeller T (1987). Hydrochemical factors limiting the distribution of *Bulinus truncatus* (Pulmonata: Planorbidae). *American Malacological Bulletin* **5**, 85-90.

Ministry of Technology (1968). The use of plastic filter media for biological filtration. *Notes on Water Pollution* **40**, 1-4.

Pearce P; Foster D (1999). Optimising nitrification on biological filters. *Water and Environmental Management* **13**, 406-412.

Pearce P; Williams S (1999). A nitrification model for mineral -media trickling filters. *Water and Environmental Management* **13**, 84-92.

Biological control of slugs in vegetable crops in Croatia

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ABSTRACT

In order to evaluate the efficacy of some non-chemical methods of slug control, experiments in beans, lettuces and Swiss chard were carried out in the field in 2002. The slug-repellent or molluscicidal effects of some botanical treatments (based on lavender and rosemary), physical barriers (saw-dust), a product based on bicarbonate of soda, as alternative techniques of slug control, and methiocarb, metaldehyde or iron phosphate pellets as chemical molluscicides, were tested. The most abundant slug pests were *Arion rufus* and *Deroceras reticulatum*. In the experiments carried out in lettuce and beans, phytotoxic effects of the lavender mixture and the product based on bicarbonate of soda were noticed. These results will eliminate these treatments from further investigation. The same treatments did not cause phytotoxic effects in Swiss chard. The small effect of some treatments may be due to high soil moisture content or to high populations of slugs (up to 47 *A. Rufus* / 2.2 m^2). A product based on the slug-parasitic nematode, *Phasmarhabditis hermaphrodita*, was tested in cabbage in a plastic tunnel in 2003, where it demonstrated good results even 30 days after application.

INTRODUCTION

Slug pests, of which the most abundant species are *Arion rufus* (L.) and *Deroceras reticulatum* (Müller), are among the most important problems in vegetable production in Croatia. Producers complain of the low efficacy of chemical molluscicides and progressive damage in lettuce, red long-rooted radish, Swiss chard, beans, red pepper, tomato and many other vegetable crops. In agricultural magazines, producers can read about alternative methods of slug control, but with no exact results of their efficacy, or warning about possible phytotoxicity. In the 1980s, the efficacy of some insecticides for slug control in combination with fermented wheat baits alone or with metaldehyde was investigated (Igrc, *et al.*, 1983). In some Croatian publications, authors were writing about different methods of slug control (Oštrec, 1998; Oštrec, 2002) but since, 1983 no investigation had been conducted in Croatia. In order to evaluate the efficacy of some non-chemical slug control methods, experiments were carried in the field in 2002 and 2003, in lettuce, Swiss chard and beans and in cabbage in a plastic tunnel.

In these experiments, we investigated the efficacy of some botanical treatments (based on lavender and rosemary), physical barriers (saw-dust), a product based on bicarbonate of soda, and methiocarb, metaldehyde and iron phosphate pellets as chemical molluscicides. The efficacy of the rhabditide nematode, *Phasmarhabditis hermaphrodita* (Schneider) for slug control was investigated in cabbage in spring 2003.

MATERIALS AND METHODS

In 2002, three experiments were conducted in the field in beans, lettuce and Swiss chard. The experiment in beans (Experiment 1) was conducted in May 2002 and consisted of seven treatments and four replicates (each plot 1.4 m^2) per treatment. The treatments were metaldehyde (Pužomor), methiocarb (*Mesurol*), iron phosphate (*Feramol*), a product based on bicarbonate of soda (*Biopuž*), lavender mixture and saw-dust. All commercial products were used at recommended field rates. The lavender mixture consisted of 1 Litre water: 0.125 Litre apple vinegar: 5 g lavender oil and was applied by spraying, as also was the product based on bicarbonate of soda. Sawdust was sprinkled around the plants. This experiment lasted for 6 days.

The experiment in lettuce (Experiment 2) was conducted in June 2002 and consisted of six treatments and four replicates (each plot 1.4 m^2) per treatment. The treatments were metaldehyde, iron phosphate, the product based on bicarbonate of soda, lavender mixture and rosemary mixture (boiling water poured on 100 g of dry rosemary and left for 24 h in a covered pot). The former treatments were applied as described for Experiment 1, while the rosemary mixture was applied by watering each plant. Experiment 2 lasted for eight days.

The experiment in Swiss chard (Experiment 3) was conducted in June 2002 and consisted of five treatments and four replicates (each plot 2.2 m^2) per treatment. The treatments were methiocarb, iron phosphate, lavender mixture and the product based on bicarbonate of soda. All treatments were applied as described for Experiment 1 and Experiment 2. This experiment lasted for ten days.

In April 2003, the experiment (Experiment 4) in cabbage in a plastic tunnel was conducted. This consisted of only two treatments (methiocarb and *Phasmarhabditis hermaphrodita*, (Phasmarhabditis-System)), and four replicates (each plot 6 m^2) per treatment. The experiment lasted for one month. The soil temperature was measured at a depth of 10 cm and ranged from 10 °C to 21 °C.

At each assessment day in all experiments the percentage of leaf area consumed by slugs was estimated. All data were subjected to ANOVA and Duncan's test ($P = 0.05$).

RESULTS

In Experiment 1, in beans, the leaf area consumed by slugs was assessed 2, 4 and 6 days after the application of the treatments (Figure 1). On the 2^{nd} day, there were no significant differences between treatments. On the 4^{th} day, metaldehyde and iron phosphate treatments reduced slug feeding significantly, in comparison to the untreated control. On the same day, phytotoxic effects of the bicarbonate-of-soda-based product and the lavender mixture were observed. On the 6^{th} day after application, only metaldehyde baits showed a significant reduction in damage. Iron phosphate was the next best treatment. Because of their phytotoxic effects, the bicarbonate of soda treatment and lavender treatment were not satisfactory. The lavender mixture also resulted in even greater leaf damage than on control plots. In this experiment, because of high soil moisture, the sawdust treatment also did not give a satisfactory result. The most abundant slug species was *A. rufus*.

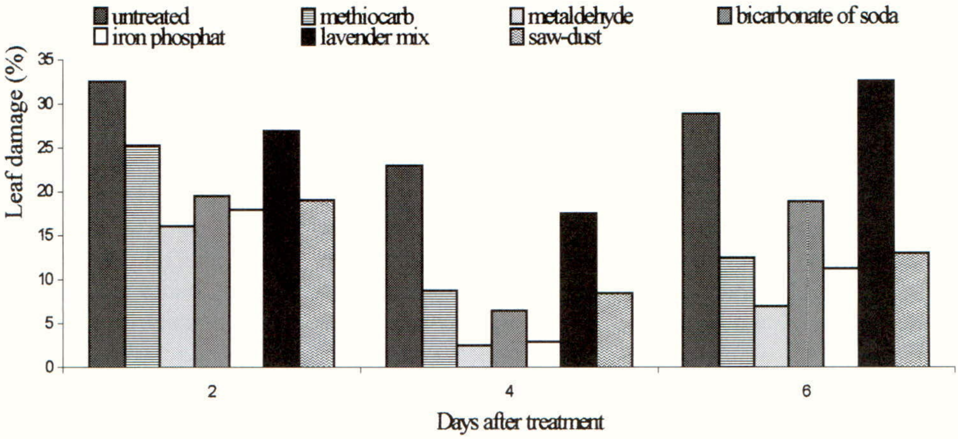

Figure 1. The results of Experiment 1 conducted in beans in 2002.

In Experiment 2 in lettuce, the leaf area consumed by slugs was assessed 2, 4, 6 and 8 days after the application of the treatments (Figure 2). On the 2nd day after application, there were no significant differences between the treatments. On the 4th day, phytotoxic effects of the lavender mixture and bicarbonate of soda treatment were evident. The phytotoxicity was even greater in the following days. Therefore, these treatments wre eliminated from further investigations in lettuce. Metaldehyde showed significantly less damage than other treatments on the 4th day, but in the following assessments there was no significant difference between the treatments. The most abundant species in lettuce was *D. reticulatum.*

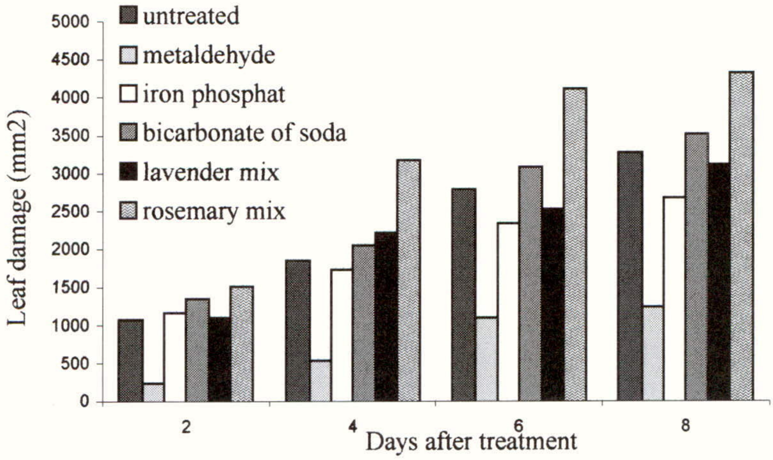

Figure 2. The results of Experiment 2 conducted in lettuce in

In Experiment 3 in Swiss chard, the results were assessed 2, 4, 6, 8 and 10 days after application (Figure 3). Because of the great leaf mass which provided good shelter for a large population of *A. rufus* (up to 47 slugs per plot), it was difficult to provide good protection, even with chemical molluscicides. In this experiment no phytotoxic effect of the lavender mixture or bicarbonate of soda treatments was evident. Although slightly better results were noted on the methiocarb treatment compared with others, no significant difference between the treatments was established

In April 2003 in Experiment 4 in cabbage grown in a plastic tunnel, the goal was to find out if the rhabditid nematode *P. hermaphrodita* could protect cabbage until picking. The results of this experiment were assessed 3, 6, 8, 10, 13, 17, 24 and 30 days after application (Figure 4). On the 3rd day after treatment *P. hermaphrodita* demonstrated significantly better results than methiocarb. On the 6th and 8th day, both these treatments were equally good in terms of slug

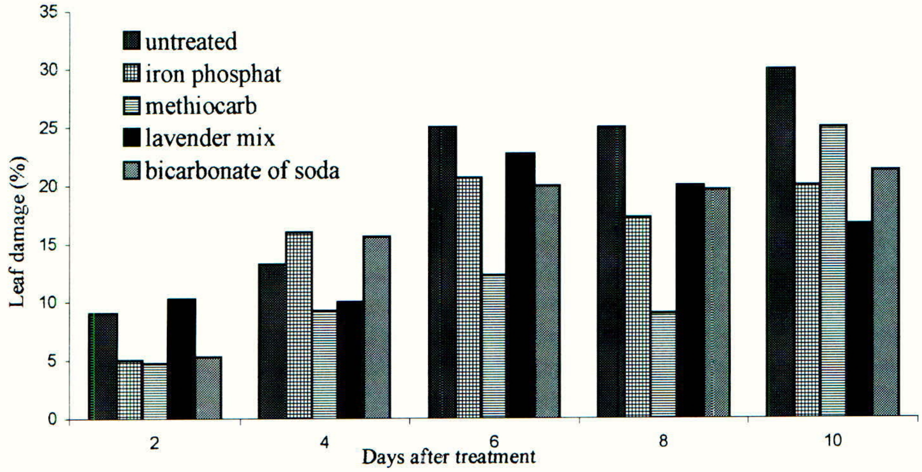

Figure 3. The results of Experiment 3 conducted in Swiss chard

control, but from the 10th to the 30th day *P. hermaphrodita* showed a significantly better results in preventing leaf damage caused by slugs than methiocarb. After the 30th day, the leaf damage on *P. hermaphrodita* treated plots started to increase, but by that time picking of cabbage had started. The soil temperatures ranged from 10°C to 21°C, suitable for *P. hermaphrodita* survival in the soil and for efficient parasitism of slugs. In this experiment the most abundant slug species was *D. reticulatum*.

DISCUSSION

The field experiments in beans, lettuce and Swiss chard (Experiments 1, 2 and 3) reported here had two goals: to establish the efficacy of molluscicidal baits, which were reported to be not as effective expected, and to investigate the efficacy of some non-chemical treatments, as alternative methods for slug control in vegetable crops. Chemical baits based on metaldehyde, methiocarb and iron phosphate (which still does not have permission for use in Croatia) showed significantly better efficacy in preventing slug damage than non-chemical methods. In

Experiment 1 in beans, the best protection was provided by metaldehyde, followed by iron phosphate baits, until the last day of assessment. In Experiment 2 in lettuce, the best treatment was metaldehyde again, but it provided significant protection for only 4 days after treatment. After the 6th day, metaldehyde was not significantly better than any other treatment in the trial. In Experiment 3 in Swiss chard, no chemical or non-chemical treatment showed significant slug control. The main result was a failure of slug control and significant damage to Swiss chard.

In all field experiments, failure in slug control, at least after the 6th day of assessment, was evident. This result points to an existing problem in vegetable crop production, as reported by producers, even when molluscicides were applied in recommended rates. This situation is mainly caused by high populations of slugs in our fields, and often by unfavourable weather conditions.

The second goal, an investigation of possible alternative methods of slug control, did not show satisfactory results. The product based on bicarbonate of soda showed a phytotoxic effect in beans and lettuce, which was not mentioned in any instructions for use. The lavender mixture, recommended for use in vegetables by some popular agricultural magazines, also caused phytotoxic symptoms on beans and lettuce.

Rosemary mixture should be adapted and investigated in further experiments. The ability of sawdust to reduce slug damage was probably dependent on weather conditions, which were unsuitable for this material in Experiment 1.

Experiment 4, conducted in cabbage in 2003, showed promising results, but the product based on *P. hermaphrodita* was expensive and will probably not be brought to the Croatian market for years. Nevertheless, its use in vegetable crop protection in Croatia will probably continue to be investigated. As established in Experiment 4, this product protected cabbage from slug damage until picking time, with only one treatment.

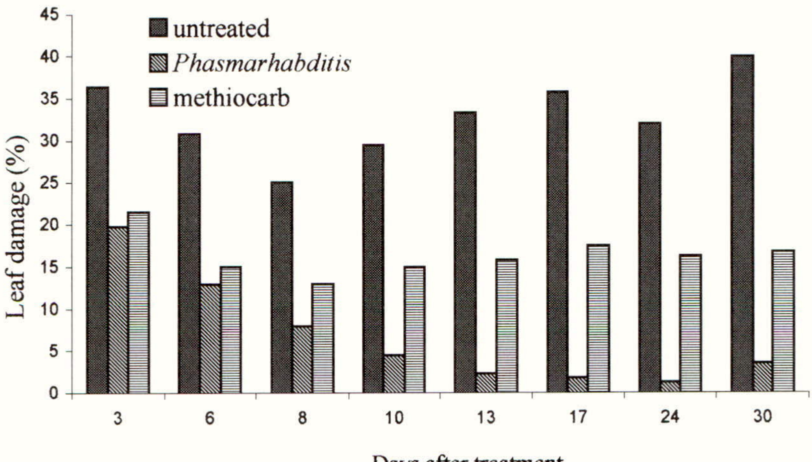

Fig. 4. The results of Experiment 4 conducted in cabbage in 2003.

ACKNOWLEDGMENTS

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REFERENCES

Igrc J; Rucner Z; Maceljski M(1983). Rezultati proučavanja problema puževa i njihovo suzbijanje na povrću.*Zaštita bilja* **34**, 303-310

Oštrec Lj (2002). Koljeno Mollusca- Mekušci. In: *Poljoprivredna entomologija* ed. M Maceljski, pp.479-480.Zrinski: Čakovec.

Oštrec Lj (1998). Gastropoda-puževi. *Zoologija, štetne i korisne životinje u poljoprivredi*, pp.76-82.Zrinski: Čakovec.

Impacts of transgenic crop technology upon the field slug *Deroceras reticulatum* (Müller)

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ABSTRACT

The potential for transgenic oilseed rape (OSR) plants expressing the cysteine protease inhibitor oryzacystatin-1 (OC-1) to affect the field slug *Deroceras reticulatum* was investigated. Characterization of proteolytic digestive enzymes from the slug's digestive gland demonstrated that all age classes of *D. reticulatum* utilise the OC-1 sensitive cysteine protease, cathepsin-L.

Vegetative tissues of transgenic OSR plants were shown to express OC-1 at levels of 0.025-0.1% of total soluble protein. Bio-assays were carried out over a 6 week period to determine the effects of OC-1-expressing OSR on the growth, development and survival of *D. reticulatum* juveniles; effects on plant damage were also monitored. Despite OC-1 being both a potent inhibitor of digestive proteolysis *in vitro* and reducing endogenous proteolysis *in vivo*, slugs in the experimental group performed significantly better in terms of survival and development; furthermore, plant damage was also significantly greater in this group.

INTRODUCTION

Recombinant DNA technology has provided an important tool in the production of crops with increased levels of resistance to pests and diseases. Use of such crops is one means to reduce the reliance of agriculture upon pesticides and the damaging environmental impacts of these chemicals. Two of the most successful examples of this technology in agriculture are herbicide tolerant crops, resistant to the broad spectrum herbicide glyphosate, and insect resistant crops. To date, the only insect resistant transgenic crops which are currently commercially available express δ-endotoxins from the soil bacterium *Bacillus thuringiensis* (*Bt*). Currently 10.2 million hectares of *Bt*-expressing genetically modified crops are grown worldwide.

In order to prevent over-reliance upon *Bt*-expressing crops and to delay the build up of insect resistance to these *Bt* Cry proteins, it is vital that other strategies for GM pest control are developed. The expression of foreign protease inhibitors (PIs) in crops is one such route.

Naturally occurring proteinaceous protease inhibitors from plants were first reported nearly 60 years ago. Their role in defence against herbivores has long been considered, partly due to their high levels in plant tissues (5-15% of total proteins), these far exceed those required to control intracellular proteolysis. There are several different families of PIs including those that inhibit serine, cysteine, aspartate and metallocarboxypeptidase classes of proteolytic enzymes.

Protease inhibitors have been shown not only to have an adverse effect upon insect growth and development, but also upon survival. They are thought to primarily act by directly inhibiting protein digestion, thus resulting in a deficiency of essential amino acids. It has also been demonstrated that some species of insects are able to compensate either by an over-production of sensitive proteases, or synthesis of novel insensitive enzymes; however, this compensatory response is carried out at the expense of other essential protein production.

The cysteine PI from rice, oryzacystatin-1 (OC-1) was first shown to be an effective *in vitro* inhibitor of insect midgut cysteine protease activity by Liang, *et al.* (1991); subsequently transgenic plants expressing this inhibitor have been shown to confer partial resistance to insect pests which rely on cysteine proteases for proteolytic digestion. Since the major digestive protease present in field slugs is the cysteine protease cathepsin L (Walker, 1996), it is possible that transgene expression of this PI may provide a means of controlling slug populations in the field. The present study reports the effects of this inhibitor on juvenile *D. reticulatum* when delivered via transgenic plants.

MATERIALS AND METHODS

Materials: Adult *D. reticulatum* were field collected from the University's Close House field station, Northumberland. Slugs were maintained at 15^0C in moist conditions and fed a diet of Chinese cabbage (*Brassica chinensis*) and carrot (*Daucus carota.)*. Eggs produced were collected, rinsed with water and incubated at 20^0C in Petri dishes lined with moist filter paper. For all bio-assays, juveniles of between 1-2 months were used; mature slugs were used for enzyme studies.

Transgenic oil seed rape (OSR) plants expressing OC-1 were obtained from Laboratoire de Biologie Cellulaire, INRA France. The highest-expressing line harbouring a single copy of the OC-1 sequence was selfed, and the homozygous T_4 progeny used in all subsequent assays carried out in this study; the non-transformed line Drakkar was used as a control. Plants were grown under controlled conditions (20°C±1.5°C). OC-1 expression was quantified by immuno-assay using Western blot analysis.

Bio-assay: A single bioassay of six weeks duration was carried out to determine the effects of OC-1 expression on development and survival of *D. reticulatum*. Juvenile slugs were placed individually into 500 ml sealable plastic food boxes lined with moist tissue paper. A single detached leaf, either from experimental or control plants, was placed into the box, its stem sealed in a water filled 1.5 ml microcentrifuge tube. Slug weight change was measured every three days, fresh leaf material was provided every six days and the amount of leaf tissue consumed was determined every six days using a 2 mm^2 grid. Fifty replicates were carried out for both control and experimental tests.

Enzyme assays: Mature *D. reticulatum* digestive glands were dissected out over ice, flash frozen and homogenised in 1mM DL-Dithiothreitol (DTT) to obtain a crude enzyme preparation. Proteolytic activity was determined using the synthetic substrate carbobenzoxy-1-Phenylalanyl-1-Arginine-p-nitroanalide (Z-phe-arg-pNA) at a final concentration of 0.5 mM; activity was determined over a pH range 4.0-8.0. using an over-lapping pH buffer system in order to determine the optimum. The diagnostic chemical inhibitor benzyloxycarbonyl-L-Phenylalanyl-L-Phenylalanyl-Diazomethylketone (Z-phe-phe-CHN_2) was used to characterise this enzyme activity (concentration ranges of 1-0- 0.125 mM).

Endogenous proteolytic activity was determined using the protein substrate BODIPY-FL Casein at a final concentration of 0.5mM. Effects of the cysteine protease inhibitor oryzacystatin (OC-1) were determined at a concentration of 0.5µM; all assays were carried out at pH 6.5.

RESULTS and DISCUSSION

Characterisation of digestive gland proteases

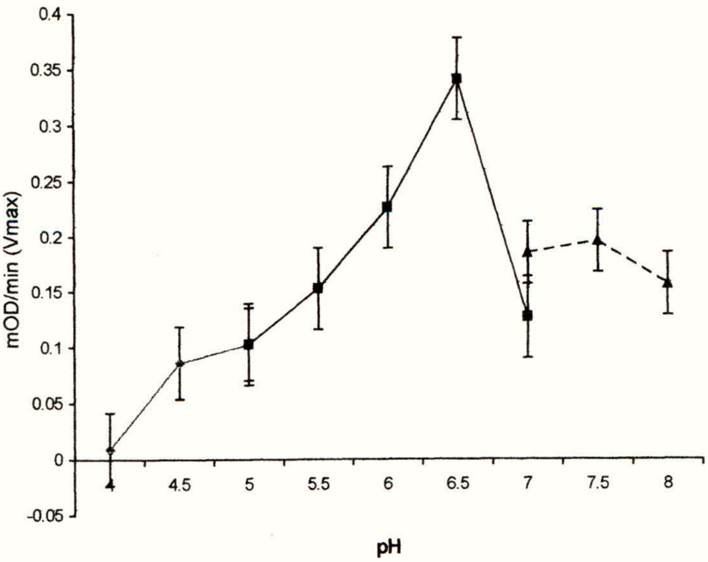

Figure 1. Variation of proteolytic activity (Z-Phe-Arg-pNA substrate) of *D. reticulatum* digestive gland extracts with pH.

The pH optimum of digestive gland proteolysis using Z-Phe-Arg-pNA as a substrate was 6.5 (Figure1). Activity within the range pH 5.0-7.0 indicates cysteine proteinases. Diagnostic chemical inhibitors were used to further characterise enzyme activity. The inhibitor Z-Phe-Phe-CHN_2.(0.005mM) was shown to inhibit activity by 84.4%, thus confirming that the major digestive gland proteolytic activity in *D. reticulatum* is Cathepsin L. These results confirm earlier studies carried out by Walker, *et al.* (1999).

Effects of OSR plants expressing OC-1 on development and survival

Effects on survival and weight gain

Significant differences were observed between slug survival in the control and experimental group over the course of the bioassay. At the end of the six week assay period, survival in the experimental group was approximately twice that of the control group (46% and 22%).

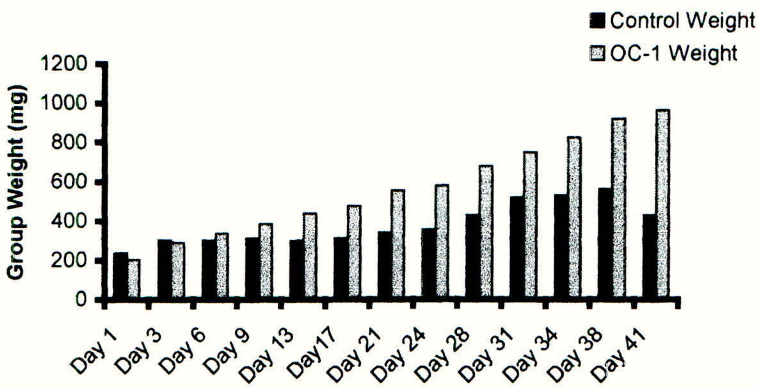

Figure 2. Increase in the total weight of *D. reticulatum* groups over the six-week bioassay.

Although there were small differences in slug weight at the start of the bio-assay, there was no significant difference in the total weight of slugs in each treatment (Two-sample t test on log transformed weight data $P>0.05$) However, by the end of the six week assay period there was a significant difference in the final weights of slugs in the two treatments ($P>0.001$), those feeding on the OC-1 expressing OSR being significantly heavier compared to the control group (Figure 2). Both data sets were corrected to allow for mortality during the bioassay.

Effects on leaf damage

Significant differences were seen throughout the bioassay period in the amount of leaf material consumed by slugs from the two treatments. By the end of the trial there was a significantly ($P< 0.05$) greater amount of leaf material consumed in the experimental group compared to the control group (Figure 3).

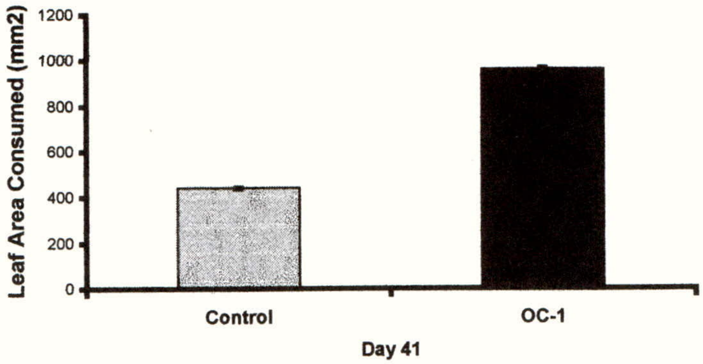

Figure 3. Differences in leaf damage caused by juvenile *D reticulatum* on the final day of the bioassay (Day 41).

Effects of OC-1 expression on endogenous proteolytic activity

In order to determine the impact of the treatments upon the endogenous proteolytic enzyme activity of the digestive gland, samples from the glands of individual slugs from the above bioassays were removed and proteolytic activity was measured using the protein substrate BODIPY-FL Casein. The results revealed a 26.6% decrease in the overall level of general proteolysis in OC-1 treated *D. reticulatum* (Figure 4).

CONCLUSIONS

Initial studies carried out upon the digestive enzymes of the field slug *D. reticulatum* confirm that cysteine proteolytic enzymes are potential targets for phytocystatin oryzacystatin-1 (OC-1).

Observations based upon the results of a six week bioassay suggest that OC-1 expression in transgenic oilseed rape plants had no deleterious effects on either slug survival or weight gain. Interestingly, despite OC-1 being a potent inhibitor *in vitro*, slugs fed OC-1 expressing plants appeared to perform better and consumed more leaf material compared to control fed slugs. These results suggest a limited use for OC-1 in potential slug control. However, in contrast to slug survival and weight gain, analysis at the physiological and biochemical level revealed detrimental effects of the PI upon the field slug. OC-1 treated individuals showed an altered form, size and structure of their digestive gland (data not shown) and a significant reduction in the levels of proteases produced by the gland. Two potential explanations for these results are: over production of susceptible forms of protease in an attempt to overcome the impact of the