Project No:	ON-00549
Contract No:	PO4500011485
AWI Project Manager:	Bridget Peachey
Contractor Name:	University of Queensland
Project Leader:	Peter James
Project Collaborators:	Michael Yu, Neena Mitter
Publication Date:	May 2022

Nanotechnology for sheep flystrike control

Published by Australian Wool Innovation Limited, Level 6, 68 Harrington Street, THE ROCKS, NSW, 2000

AWI invests in research, development, innovation and marketing activities along the global supply chain for Australian wool. AWI is grateful for its funding which is primarily provided by Australian woolgrowers through a wool levy and by the Australian Government which provides a matching contribution for eligible R&D activities

Contents Table

Table of Figures

Abstract

With ever-growing requirement for cost of production efficiencies and low labour inputs, flystrike protection requires control methods that can give extended periods of protection. New chemical formulations for flystrike control are required to reduce the wool industry's reliance on mulesing, and because of the development of resistance which reduces periods of protection provided by the most widely used flystrike control compounds. This project designed, fabricated and tested unique silica nanoparticle formulations with spikes on the particle surface to aid adherence and purpose-designed release characteristics to give prolonged periods of protection against flystrike with minimal residues and off-target effects. When tested in laboratory assays against sheep blowfly larvae following exposure to artificial UV degradation, rainfall and environmental weathering, the rough surface nanoparticles containing ivermectin and cyromazine showed extended periods of protection in comparison to conventional formulations and silica nanoparticles without the surface spikes. Proof of concept is provided for the ability of the silica nanoparticles to provide extended periods of protection in laboratory assays and studies are now required to assess periods of protection against flystrike when the formulations are applied to sheep.

Executive Summary

Background

Flystrike and lice are amongst the most costly diseases affecting the sheep industry and are repeatedly ranked as high priority for research in producer surveys. With ongoing requirements to increase production efficiency and constraints on the availability of labour, achieving effective fly control often relies on having chemical protection in place when a flystrike wave begins. For this reason, methods that can provide prolonged protection are strongly favoured by wool producers, and flystrike control has historically relied on two major methods, mulesing and the application of chemicals, although breeding more resistant sheep is an increasingly important element in most flystrike control programs. Mulesing is increasingly contentious because of sensitivity to animal welfare concerns in major wool markets and chemical controls are increasingly compromised by the development of resistance or restricted due to OH&S, residue and environmental concerns.

Traditional formulations of pesticides depend, for prolonged action, on a single initial high-level treatment so that control is maintained until concentrations decay below effective levels. This necessitates the application of relatively high levels of chemicals which can increase the risk of residues, off target effects and safety impacts. In addition, there is often an extended 'decay tail' of pesticides during which pests are exposed to sub-lethal amounts of chemical and resistant pest genotypes can be selected.

State-of-the-art nanotechnology provides an ideal solution to address the issues with current sheep parasite control formulations. Nanoparticle formulations can be designed to administer active ingredient at steady active levels delivered over a prolonged period or designed to release only at times and sites where they are needed Nano-encapsulated formulations also have the important attribute that they can generally be applied using existing application equipment. Nanotechnology offers a means of providing extended protection of sheep against flystrike and of potentially making environmentally 'softer' or 'natural' chemicals, which generally have short protection periods, a practical option.

Silica nanoparticle properties

The nano-capsules described in this project have a core design of a large hollow cavity and porous silica shell with numerous spikes on the surface. The silica shell protects the internal active payload against degradation, while pores in the shell allow easy loading of chemical actives into the hollow cavity and sustained release of the active compound. Silica spikes (or whiskers) cover the outer surface of the 'rough'

type of particles and aid the retention of the particles on surfaces such as wool fibres and the exocuticle of insects. Silica is well recognized as inert and abundant in the environment with good bio-compatibility and is approved by Food and Drug Administration (FDA) for oral delivery of human pharmaceuticals and bio-active compounds. Moreover, the UQ patented technology provides a relatively simple approach to the fabrication of nanoparticles, employing cheap industrial chemicals, and is amenable to scale up for commercial production.

Six nanoparticle designs were fabricated and tested. Initially rough and smooth nanoparticle formulations (RNP and SNP) and the RNP particles with the surface modified to make them more hydrophobic (RNP-C18) containing lipophilic ivermectin and water soluble cyromazine were tested and later, two new particle types (FSN60 and FNS60-hydrophobic) containing cyromazine were fabricated and evaluated. The FNS60 formulations tested in this project were loaded with 23% insecticide to enable direct comparison with the earlier particle designs. However, higher chemical loading, up to approximately 50%, is possible with these particles which it is expected, depending on release characteristics, can be used to further improve longevity.

The silica nanoparticles adhered to wool and furthermore, the rough surface hydrophobic nanoparticles (RNP-C18) adhered better to wool than rough nanoparticles without the C-18 modification and much better than smooth nanoparticles. In addition, more of the C18 and RNP particles than SNP particles remained attached following water washing. This pattern was also observed using fluorescein (FITC)-labelled particles. The adhesion properties of the 'spiky' silica nanoparticles appeared to result in reduced leaching of the chemical from the fleece and increased resistance to photo-degradation of insecticide.

The studies with both blowfly larvae and sheep lice showed high density of the labelled particles (RNP) in the insect gut following exposure in wool assays. Cuticular adhesion was also noted with both blowfly larvae and lice, but the level of fluorescence was much lower than seen in the gut. This suggests that oral ingestion is the primary route of uptake by sheep blowfly larvae and lice. The mode of feeding by both insects, whereby they use scarifying mouthparts to scrape at the food surface, would seem to favour accumulation of these particles from wool and skin surfaces. This could be an important consideration when designing optimal application strategies for rough-surface nanoparticle formulations.

Efficacy of the silica nanoparticles following environmental exposure

All of our studies have indicated significantly prolonged persistence of the rough topography nanoparticle formulations (RNP, FSN60, RNP-C18 and FSN60-hydrophobic) in comparison with the current commercial formulations and compared to smooth silica nanoparticles when exposed in artificial weathering tests. There appeared to be only small differences in effectiveness between the different designs of rough surface topography formulations with small and inconsistent differences between the RNP and the RNP-C18 in assays where there was a direct comparison. Overall, the FSN formulations appeared to give somewhat better protection than the RNP formulations and there was indication of a slight advantage for the FSN60-hydrophobic formulation in comparison with FSN60 non-hydrophobic formulation in some assays.

Two main environmental effects are thought to contribute to loss of insecticidal effectiveness over time in the field, photo-degradation from the effects of sunlight and leaching from the fleece by rainfall. Dilution of insecticide by diffusion into new wool grease, or movement of chemical away from the skin as the wool fibre grows can also be important contributing factors.

Both ivermectin and cyromazine are known to be subject to photo-degradation and the studies reported here confirmed the ability of the rough surface silica nanoparticles to protect against photo-degradation of these two chemicals. Further, the results indicate that the particles with rough surface topography (RNP, RNP-C18, FSN-60 and FSN-60 hydrophobic) provided significantly higher levels of protection against breakdown by sunlight than offered by the smooth particles. A clear advantage from reduction in photo-degradation was also seen in our previous studies with similar silica nanoparticles containing spinosad applied to cattle skin in a cattle skin assay.

Leaching from the fleece during heavy rainfall has also been suggested to reduce periods of protection from cyromazine, which is considered to be particularly susceptible to leaching due to its high-water solubility. Reduction in the persistence of ivermectin from the effects of leaching was also indicated in artificial wetting studies in this project and was thought to probably be facilitated by the detergent action of suint compounds in the wool grease. In this study, encapsulation in rough-surface nanoparticles was shown to reduce both cyromazine and ivermectin leaching resulting in longer persistence of effect following wetting by both immersion in water and exposure to simulated rainfall.

It has been suggested that rainfall can also play a part in extending periods of protection from cyromazine by washing the water-soluble chemical down into the proximal wool and onto the skin, where strikes develop. The silica nanoparticles were shown to provide a slow-release mechanism of chemical release of cyromazine and it is possible that the spiky-surface particles could help prolong this effect by 'anchoring' the particles on wool fibres, and maintaining a 'depot' of chemical in the fleece. The rough surface particles, could both reduce photo-degradation of chemical as well as release cyromazine into the wool over subsequent wettings, potentially extending periods of protection by this means. Although ivermectin is poorly water soluble, in this study, it did appear to be removed from the fleece by wetting and whether similar movement in the fleece could result from the effects of rainfall and the detergent action of the suint requires investigation.

Periods of protection in the field are also strongly influenced by application method. For example, periods of protection from spray-on and backline formulations of cyromazine are shorter than when sheep are treated by hand jetting, and hand jetting gives longer periods of protection than jetting races which generally deposit most chemical near the top of the fleece. Although part of the reason for shorter periods of protection with backline and spray on application relates directly to chemical placement, the half-life of chemical in the fleece is known to be markedly shorter when chemical is applied as a backline or spray treatment compared to hand jetting, which suggests that photo-degradation is also a major factor. The use of the nanoparticle types described here could be of particular benefit in improving the longevity of effect from off-shears and spray on formulations, favoured by sheep producers because of their labour-saving attributes and could also help in increasing the longevity of protection from jetting race treatments.

This project has clearly demonstrated that encapsulating both lipophilic and water-soluble insecticides in different designs of silica nanoparticles with rough surface topography extends the period of protection compared to conventional formulations when exposed to artificial and environmental degradation. However, most of these studies were conducted with treated wool samples exposed to sheep blowfly larvae in *in vitro* systems. Wool fibres were exposed laid horizontally on racks so that the full length of the staple was exposed, whereas on sheep it is mostly the tip wool that is exposed and most of the rest of the fleece is protected from sunlight by adjacent fibres. When applied to sheep a number of other factors, such as the degree to which insecticide is translocated disto-proximally along wool fibres in the wool yolk and laterally across the skin surface are important to the duration of protection provided. That the preliminary studies with conventional and nanoparticle formulations applied to sheep also showed clear advantage for the rough-surface nanoparticle formulations is extremely encouraging and suggests that silica nanoparticle formulations will be able to extend protection against flystrike in field situations. Investigations should now progress to studies with live sheep, with the animals run in the field over different periods and tested in pen studies by on-sheep larval challenge.

Conclusions

The results of this project clearly demonstrate that encapsulating ivermectin and cyromazine in silica nanoparticles can provide extended protection in comparison to 'conventional' commercial formulations when compared in *in-vitro* systems. The advantage is greatest with more labile or volatile chemicals that generally have impractically short protection periods but are more environmentally 'attractive' because they break down quickly in the environment. Further these results suggest a significant advantage for the 'rough surface formulations in comparison with smooth surface nanoparticles. Although the differences in effectiveness between the different rough surface particles were small, the FSN60 and FSN60

hydrophobic formulations generally gave the best effect. Furthermore, the FSN particles have a considerably higher chemical loading capacity than used in this project which offers the flexibility of higher insecticide loading than with the other particle types and would be expected to further extend the protection period possible.

Although the studies reported here indicate clear advantage for the spiky surface nanoparticles over conventional formulation types, the effects of sheep factors, fleece dynamics and differences in the level of environmental exposure when flystrike prevention formulations are applied to sheep make it difficult to relate the advantages in protection efficiency demonstrated in the *in vitro* assays to effectiveness and duration of protection under field conditions. Also, there is likely to be interaction between the method of application and the relative advantage realised from different nanoparticle formulations and the advantage in field protection could be either much larger, or alternatively less, than suggested by the results reported here. Clearly the next stage in this work is pen studies with these formulations applied to sheep. It is suggested that that FSN60 particles, with a higher chemical loading, should be tested in these studies.

1. Introduction

1.1 Background

Flystrike and lice are amongst the most-costly diseases affecting the sheep industry and are repeatedly ranked as high priority for research in producer surveys (MLA 2015, Reeve and Walkden Brown 2014). Flystrike is also a significant animal welfare concern (Phillips 2009). Effective and efficient flystrike control still relies heavily on two major methods, mulesing and the application of insecticides, although significant advances have been made in breeding more resistant sheep. Mulesing, the most effective and cost-efficient means of preventing breech strike is increasingly contentious because of sensitivity to animal welfare concerns in major wool markets. Effective chemical products are critical for the prevention of breech strike, particularly in unmulesed flocks, as well as for the control of bodystrike and for the treatment of struck sheep. However, they are increasingly compromised by the development of resistance. Effective chemicals or formulations are also central to sheep lice control. Although well planned biosecurity programs and careful flock management can reduce the introduction and spread of lice, ultimately eradication of lice from flocks and the maintenance of a low industry prevalence of lice relies on efficient chemical methods.

With ongoing requirements to increase production efficiency and constraints on the availability of labour, achieving effective fly control often relies on having chemical protection in place when a flystrike wave begins. Prediction of flystrike is difficult and fly waves often coincide with periods of other high labour requirement (for example during harvest). For this reason, methods that can provide prolonged protection are strongly favoured by wool producers.

Traditional formulations of pesticide depend, for prolonged action, on a single initial high-level treatment so that control is maintained until concentrations decay below effective levels. This necessitates the application of relatively high levels of chemicals which can result in residues, off target effects and safety impacts. In addition, there is often an extended 'decay tail' of pesticide during which pests are exposed to sub-lethal amounts of chemical and resistant pest genotypes can be selected. Both sheep blowflies and lice have a long history of development of resistance to control chemicals, resulting in reduction in protection times or complete loss of effectiveness. Sheep blowflies have developed resistance to nearly all chemical groups introduced for their control including organochlorine pesticides, organophosphates, carbamates, benzoyl phenyl ureas, cyromazine and dicyclanil. The recent detection of resistance to cyromazine and dicyclanil in both Australia and New Zealand (Levot 2012, Levot et al. 2014 Waghorn et al. 2013) is a particularly alarming development as these two chemicals are currently used for flystrike control by 90% of Australian wool producers (dicyclanil 54%, cyromazine 36%) (Colvin et al. 2020). Larval

implant studies indicate a potential reduction in protection periods with cyromazine from 14 weeks to less than 8 weeks and with dicyclanil from 18-24 weeks, to less than 11 weeks (Levot et al. 2014, Sales et al. 2020). More recent studies have suggested that this resistance is widespread in New South Wales and Victoria and increasing in Western Australia and South Australia, although it should be noted that these results were derived from voluntarily submitted flocks rather than a random survey.

Some chemicals have also been lost from use for other reasons. Organochlorines were withdrawn from use against ectoparasites because of residue issues and potential market impacts, and organophosphates, most recently diazinon applied by dipping or jetting, were withdrawn because of safety and occupational exposure concerns. The trend to 'ecolabelling' and potential for some chemicals contained in sheep ectoparasite treatments to cause residues in wool and enter wool scouring effluent have also led to reduction in the use of some control chemicals.

1.2 Controlled release and nanotechnology

State-of-the-art nanotechnology provides an ideal solution to address the issues with current sheep parasite control formulations and recent years have seen significant advances in the area of controlled release technology to meet the requirement for prolonged periods of protection. A number of long-acting injectable formulations for internal and blood feeding parasite control are now registered (Iezzi et al. 2017), and controlled release devices such as rumen capsules for helminth control, polymer matrix ear tags for buffalo flies in cattle and flea collars for parasite control on cats and dogs, have become major methods for providing extended protection against animal parasites (Witchey-Lakshmanan 1999, Swiger and Payne 2017). Some of these technologies have also shown potential for ectoparasite control in sheep (Anderson et al. 1989, Rugg et al. 1998, James et al. 1989, 1990, 1994). In addition, a starch xanthate encapsulated diazinon formulation designed to release insecticide in the presence of moisture provided protection against poll strike in rams for 30 weeks whereas protection from jetting broke down after 12 weeks. However, development of this methodology was not pursued because of difficulties in designing an acceptable application methodology for the relatively large particles (James et al. 1994).

Since that time remarkable innovations in the area of nanotechnology have led to the development of a variety of nanoparticle-based pesticide formulations, including polymeric/cellulose nanocrystals and lipid nanoparticles. By encapsulating active ingredients into nanoparticles, pesticides can be administered at steady active levels delivered over a prolonged period or designed to release only at times and sites where they are needed. Nanoparticle formulations also have the important attribute that they can generally be applied using existing application equipment.

Controlled release systems, and in particular nanoparticle formulations, can be used to avoid resistance-selecting "decay tails", for example by maintaining high levels of insecticide through the fly season and then decaying during the winter when no flies are present, or by delivering less persistent insecticides which are released only at the times of high strike risk and then degrade rapidly after release.

Nanoparticle formulations also provide advantages in addition to extended periods of release. Encapsulation can reduce breakdown of active constituents by protecting against effects such as UV degradation (Yu et al. 2014) and the UQ nanoparticles can improve efficacy by reducing loss from the fleece and increasing uptake by insects. Compared with traditional formulations which require repeated and high-dose application for persistence of effect, the nanoparticle formulations require lower doses and less frequent application, which reduces the chance of tissue absorption, residues or subclinical toxic effects on animals or workers. Nanoparticle technology could also facilitate the use of 'softer' chemistries and natural compounds which otherwise have limited persistence, contributing to the 'pure and natural' image of wool.

1.3 University of Queensland silica nanoparticles

The University of Queensland has developed a patented technology to fabricate novel hollow silica (SiO_2) nanoparticles that can be loaded with active molecules to enable superior protection against insect pests)

(Song et al. 2016; Zhang 2020). These nanoparticles have a large hollow cavity and porous silica shell with numerous spikes on the surface (PCT/AU2016/050283). The silica shell protects the internal active payload against degradation, while pores in the shell allow easy active loading into the hollow cavity and sustained release of the active compound. Silica spikes (or whiskers) cover the entire nanoparticle outer surface evenly, showing a pollen-like topology, and aid retention of the capsules on different surfaces. Preliminary results employing ivermectin as a model pesticide show efficient loading of active ingredient into the nanoparticles and sustained release of ivermectin. Compared to the pure ivermectin (white crystals in appearance), which degrades into yellow powders under UV irradiation, the ivermectin-UQ-nanoparticle formulation remained stable with little degradation.

To demonstrate the adhesion performance, Merino wool with and without wool grease present were immersed in an aqueous solution of UQ nanoparticles (with 'whiskers'), and smooth nanoparticles followed by a draining process to identify the adhesion properties. UQ nanoparticles exhibited three times higher amount of retention on the wool surface than conventional nanoparticles with a smooth surface. Large amounts of nanoparticles can be directly observed on the wool surface under scanning electron microscopy. This enhanced adhesion provides resistance to leaching and extended protection periods of pesticide formulation under field conditions formulations employing the UQ-nanoparticles have already shown enhanced toxicity against plant and urban insect pests in comparison with the unencapsulated compound (data not shown). It is envisaged that the adhesion of nanoparticles to the insect body would also result in sustained release of active directly onto the cuticle, or into the insect gut, with increased effectiveness and efficiency against sheep ectoparasites.

The UQ nanoparticles also possess advantages compared to other types of nanoparticles for translation to a viable commercial product. Polymer or lipid nanoparticles are often expensive or unstable under field conditions, whereas silica has been well recognized as inert and abundant in the environment with good bio-compatibility and is approved by the Food and Drug Administration (FDA) for oral delivery. Moreover, the UQ patented technology provides a relatively simple approach to the fabrication of nanoparticles, employing cheap industrial chemicals, which is ideal for large scale commercial oriented production.

This project designed and tested nanoparticle formulations of flystrike control chemicals. Three potential approaches to achieve prolonged protection were investigated:

- Persistence of insecticide-containing nano-capsules that are protected from breakdown or leaching from the fleece and which are ingested by blowfly maggots at times of infestation.
- Slow release of active pesticide on the fleece and skin surface over extended periods of time.
- Strategic release at time of flystrike susceptibility or strike commencement. (It is envisaged that the capsules would remain inert in the fleece during periods of low strike risk and then release chemical in the presence of moisture from predisposing causes - urine stain, faeces staining, serum from irritated or ruptured skin).

The project iteratively designed and produced formulations with different loading, adherence and release characteristics and tested them for efficacy and persistence of effect against sheep blowflies and lice. Initially formulations containing two chemicals, ivermectin and cyromazine, were developed, but the intent was to determine an optimal formulation technology that could be used with a range of chemicals to provide extended, low residue and safe protection against breech, body and poll flystrike.

2. Objectives

The primary goal of this project was to develop nanoparticle formulations that can provide prolonged, safe and residue-free protection against sheep flystrike and slow the development of resistance.

The specific objectives were to adapt nanoparticle technology to:

- Provide patentable controlled release formulations that provide extended periods of persistence in the fleece and prevent environmental degradation of flystrike control larvicides.
- To develop 'smart' formulations that release only when conditions are suitable for flystrike (both breech and body strike), thereby extending periods of protection and avoiding resistance-selecting levels of chemical in the fleece at other times.
- Through the development of nanoparticle formulations, to increase the number of chemical actives that can provide practically significant periods of protection against strike, thus providing more chemical options and reducing pressure for resistance development.
- To increase the practical feasibility of using 'softer' and 'natural' compounds, such as plant essential oils by increasing their longevity of activity.

3. Methodology

3.1 Formulations of ivermectin

3.1.1 Fabrication of silica nanoparticle with smooth surface (SNP), rough surface (RNP), and rough surface modified with C18 (RNP-C18)

The fabrication strategy is based on the technology described in The University of Queensland patent (Composition, particulate materials and methods for making particulate materials, PCT/AU2016/050283). Fabrication, described in broad terms consists of adding resorcinol and formaldehyde into an ethanol-ammonia aqueous solution to facilitate polymerization. This forms a RF resin nano-core, on which the silica and RF precursor condensate forms a silica-polymer interpenetrating layer. The synthesized composites were calcined at 550°C for 5 hours in air to burn out the polymer composition, leaving silica nanoparticles with a hollow cavity and spiky surface, noted as RNP. Smooth silica nanoparticles were fabricated following a similar protocol with only silica precursor coated on the RF core to the dense silica shell, denoted as SNP. Hydrophobic modification of RNP was conducted through post modification of C18 chain-linked silane in toluene at 110°C, grafting the silica surface with C18 functional groups, denoted as RNP-C18.

To load ivermectin, 30 mg amounts of silica nanoparticles were dispersed into 5 mL of methanol, and 9 mg of ivermectin was dissolved in 5 mL of methanol. The silica nanoparticle and ivermectin solutions were then mixed and transferred to a Rotavap at 40°C until all solvent was evaporated. The ivermectin loaded silica nanoparticles were collected as dry powders.

3.1.2 Synthesis of RNP with different particle sizes

Different size nanoparticles were prepared according to the synthesis protocol described above, but by altering the resorcinol and formaldehyde amounts added, different size nano-cores were formed. For instance, with a small amount of RF precursor added, a RF-core of around 100 nm was formed, resulting in a RNP particle size of around 180 nm. With larger amounts of RF precursor added, RF-cores of around 220, 350 or 600 nm were formed, with RNP particle sizes of 330 to 500 and 800 nm.

In a preliminary experiment RNP particles of 180 and 800 nm were compared for larval toxicity without weathering whereas in a later experiment, particles of 180, 300, 500 and 800 nm were synthesised and compared for relative efficacy and persistence after exposure to environmental weathering.

3.2 Formulations of cyromazine

3.2.1 Smooth and rough surface nanoparticles loaded with cyromazine

The fabrication strategy for these three particle types was based on the methodology described for formulation of ivermectin, but with different active chemicals loaded into these nanoparticles, SNP, RNP, RNP-C18.

To incorporate the more water soluble cyromazine, the chemical was dissolved in ethanol with the respective nanocarrier in an ultrasonic bath and then rotary evaporation used to remove all of the ethanol solvent. A loading rate of 23% cyromazine was used to standardise with the rates used for other actives. Technical cyromazine solubilised in water was used as a control for most range finding assays whereas a commercial cyromazine flystrike formulation (Venus®, Norbrook Pharmaceuticals), which includes wetting agents, was used at similar concentration to the nanoparticle formulations for comparison in the weathering assays

3.2.2 Release of cyromazine from silica nanoparticles in water

For the release test, 3.33 mg of RNP-C (containing 1.0 mg cyromazine) was dispersed in 50 ml of MilliQ water solution. The mixtures were kept at 25°C in dark and static conditions for 30 days and 2 ml of the water supernatant was collected at the same time each day. To maintain the water volume, 2 ml of additional MilliQ water was added to the extraction solution following each sampling. The supernatants were centrifuged, filtered with a 220 nm filter and the supernatant then freeze dried, re-dissolved using 0.5 ml of acetonitrile, and kept in the dark. The concentration of the supernatant was diluted to 100 pM for quantitative measurement of cyromazine by HPLC to assess the amount of cyromazine released.

3.3 FSN-60 (fractal silica nanoparticles) and FSN-60 hydrophobic particles

The FSN formulation was a different configuration of silica nanoparticles and different to the rough surface particles previously prepared. To prepare the FSN-60 particles aqueous-alcoholic solution was prepared by mixing ethanol and distilled water at a volume ratio of 4:1 while stirring at 60°C. Ammonium hydroxide and ethylene-diamine solution were then added to create the basic conditions for polymerization. Formaldehyde solution, 3-aminophenol, and TEOS were then added to this solution and the mixture was stirred vigorously for 5h, allowing the resin and silica to co-polymerize. The synthesized composites were collected by centrifugation, followed by ethanol washing and drying. Finally, the monodispersed mesoporous FSN were harvested after calcination in air, and the formulation recorded as FSN-60. Hydrophobic FSN was prepared following the same protocol as described in 2.1.1, where FSN was modified with hydrophobic C18 groups through the slow deposition of octadecyltrimethoxysilane in toluene at 110 °C, followed by centrifugation and washing with toluene and ethanol, denoted as FSN60 Hydrophobic.

A similar chemical loading procedure was used as was used for the loading of the RNP-cyromazine formulations. Briefly, FSN-60 particles (100 mg) were dispersed in 10 mL of methanol and 30 mg of cyromazine was dissolved in 5 mL of methanol. The silica nanoparticle –cyromazine mixture, was then transferred to a Rotavap vacuum evaporator set at 40^{o}C to remove the solvent. With gradual evaporation of the solvent, the cyromazine was concentrated and adsorbed into the pores of FSN-60 by a capillary effect. A loading rate of 23% cyromazine was used to facilitate comparison with the other particles. Following evaporation of all of the solvent, the cyromazine-loaded silica nanoparticles were collected as dry powders. The morphology of the resultant particles was characterised by TEM and SEM. It should be noted that although 23% cyromazine was used to facilitate comparison with the early nanoparticle types, the FSN particles have a higher pore volume that allows a much higher drug loading capacity (up to ~50% weight) than the earlier RNP formulations.

3.4 Characterisation of particles

The morphologies of RNP, SNP, RNP-C18 and FSN60, FSN Hyrophobic particles were observed using a transmission electron microscope (TEM). The samples were prepared by dispersing and drying the powder samples-with ethanol dispersion on carbon film on a copper grid. The morphologies of the nano-carriers before and after the loading of cyromazine were also observed using scanning electron microscopy (SEM) at 1.0 kV. For SEM measurements of pure silica, the samples were prepared by dispersing the powder samples in ethanol, after which droplets were applied to aluminium foil pieces and attached to conductive carbon film on SEM mounts. For SEM measurements of nanoparticles encapsulated with chemicals through rotary evaporation, the samples were directly attached to the conductive carbon film on SEM mounts.

To characterise the pore parameters, nitrogen adsorption-desorption isotherms were measured at -196 °C using a Micrometrics Tristar II system, before which the samples were degassed at 200 °C overnight on a vacuum line. The total pore volume was calculated from the amount of nitrogen adsorbed at a maximum relative pressure (P/P_0) of 0.99. The Barrett–Joyner– Halanda (BJH) method was utilized to calculate the pore size from the adsorption branches of the isotherms; and the Brunauer–Emmett–Teller (BET) method was utilized to calculate the specific surface areas. Dynamic light scattering (DLS) experiments and zeta potential of the silica nano-carriers were measured by using a Zetasizer Nano instrument after dispersing particles into water under ultrasonication for 5 min, and then measured at least 3 times. Fourier transform infrared (FTIR) spectra were collected on a ThermoNicolet Nexus 6700 FTIR spectrometer equipped with a Diamond ATR (attenuated total reflection) Crystal. For each spectrum, 128 scans were collected at resolution of 4 cm^{-1} over the range 500-4000 cm^{-1}. A Mettler Toledo GC200 thermogravimetric analysis (TGA) station was used for the loading amount and differential scanning calorimetry (DSC) study at a heating rate of 2 °C min^{-1} in air.

3.5 Encapsulation of labile actives

To test the effect of nanoencapsulation of activity of label organic materials, a highly active alkaloid-enriched fraction from *the African native plant, C. anisata* was tested either as a raw methanol extract or encapsulated in rough nanoparticles. The methanol extract, and encapsulated formulations were applied to the chromatography paper at equal serially diluted concentrations, dried for 24h and then tested for larvicidal action using the chromatography paper–serum assay described in section 2.7. The dose range tested was chosen on the basis of previous tests with the compound.

3.6 Fleece and pest interactions

Designing optimal formulations relies on an understanding of how the formulations react with the surface to which they will be applied as well as an understanding of how they will be absorbed by the pest and knowledge of how these factors affect longevity and efficacy of effect. The three formulations that we are working with (SNP, RNP, RNP-C18) have different surface topologies that can be ‘tuned’ to provide different optimal effect. As such, determining how well they adhere to wool fibres and their longevity and persistence on wool is critical. In addition, how they interact with the insect cuticle and the mode/efficiency of adsorption/absorption, across the cuticle, orally or possibly through the spiracles in the vapour phase is critical to determining optimal design. In the case of the RNP and FSN formulations, spikes (or whiskers) cover the entire nanoparticle outer surface evenly, showing a pollen-like topology which can aid retention of the capsules on different surfaces and provide a potential advantage in comparison to other smooth-surface designs of nanoparticles. Stronger adhesion to hydrophobic leaf surfaces has been shown to increase resistance to leaching, which is likely to also be the case with wool, while encapsulation can reduce photo-degradation and potentially susceptibility to bacterial breakdown. The RNP-C18 has added hydrophobicity which can further aid their adhesion to hydrophobic surfaces and rain fastness.

3.6.1 Adherence of nanoparticles to wool fibres

Adherence to wool fibres was determined by immersing unscoured wool and clean wool with the grease (wax and suint) removed by methanol washing in aqueous nanoparticle suspensions (1mg/ml) for one minute. To test water fastness, the treated wool was rinsed with water 3 times, residual water was allowed to drain off and the wool allowed to dry overnight before examination by electron microscopy to assess likely rain fastness. Residual effectiveness was assessed by bioassay with *L. cuprina* as described in section 2.7.

3.6.2 Evaluation of ingestion and cuticular penetration in parasites

The distribution and cuticular adherence of the different silica nanoparticle types on *L. cuprina* larvae were determined using fluorescent microscopy after exposure to fluorescein-5-isothiocyanate (FITC)-labelled particles. Blowfly larvae were exposed to the labelled nanoparticles using the larval wool assay described below. The exposed pests were immobilised or cold-killed by placing them in the freezer for 15 to 30 minutes and then immediately observed under a fluorescence microscope.

3.7 Testing against different stages of *L. cuprina*

In most instances flystrike insecticides act through their effects on newly hatched larvae and the early larval instars of *L. cuprina* and generally the effectiveness against this stage will be of primary concern. Few flystrike insecticides have direct ovitoxic effects, although newly eclosed 1st instar larvae may be killed by exposure during hatching. In the case of ivermectin the same concentration is used for both protective applications and for treatment of strikes, which will likely contain a mix of eggs and first, second and third instar larvae. Adult flies can potentially also come in contact with pesticides during preparation for egg laying and oviposition. Most flystrike compounds do not act as adulticides, although one compound (alpha cypermethrin) provides protection of sheep by disrupting oviposition by the adult females. Therefore, it was of interest to examine effects of the nanoparticle formulations against other stages of *L. cuprina* to gain some indication of possible toxic effects.

3.7.1 Assays against 1st instar larvae

A number of assay systems were used for efficacy testing. These included a method adapted from that of Hughes and Levot (1987), using 1st instar larvae with test compound applied to chromatography paper, dried and then sheep serum added, either directly onto the paper or in the base of the tube. This assay was adapted for testing treated wool by replacing the chromatography paper with 100 mg of wool. A larval dipping assay, designed specifically to test topical toxicity was also used in which the larvae were immersed in different concentrations of the test solution for 60 secs. This minimises the opportunity for uptake by ingestion and tests topical exposure.

For the basic chromatography paper assay 1 mL serial dilutions of the test solution or dispersion was used to treat 120 X 30 mm strips of chromatography paper which were dried overnight before use in assays. The paper was then folded concertina style and inserted into 16 by 50 mm round bottom glass tubes. 1ml amounts of sheep serum containing 2 % yeast extract and 0.5 % KH_2Po_4 were then added to the bottom of each tube. Twenty newly hatched larvae counted into each vial and incubated at 28°C and 70 % RH for 24 or 48 hours, depending on the assay. For the paper assays, strips of chromatography paper, 120 X 30 mm, were folded concertina style and then inserted into 50 x 16 mm vials and the particles are mixed in the serum. For assays using wool, staples of wool (groups of wool fibres) weighing 100mg, harvested from sheep known to be pesticide free and measuring 3cm in length were used and each was immersed in 1 ml of the test solutions, then air dried. There were 3 replicates of 20 larvae for each concentration.

To prepare the nanoparticles for the tests they were dispersed in deionized water or serum by ultrasonication for 1 hour. The parent solution was then serially diluted in water, acetone or fortified sheep serum (2 % Yeast Extract; 0.5 % KH_2P04), depending on the assay. Usually, a range finding assay is conducted followed by a more restricted dose range chosen to provide a good range of concentrations from 0 to 100% kill and enable analyses for LD toxicity statistics calculated using PoloPlus software.

3.7.2 Larval assays for effect of cyromazine formulations

Cyromazine differs from ivermectin in its mode of action in that it is a growth regulator compound that acts by disrupting moulting whereas ivermectin acts primarily as a neurotoxin. This has implications for assay design in that whereas neurotoxins are generally relatively quick acting, cyromazine can take an extended period to kill all larvae. Thus, depending on the age of the larvae entering the assay, the assays must run for at least 48 hours to realise full effect of the pesticide, and mortality rates are generally higher when assessed at 48h than at 24h.

Two different assay methods were used. For initial range finding assays and preliminary toxicity tests a standard method using 1st instar larvae with test compound applied to chromatography paper was used. For the basic chromatography paper assay, 1 mL serial dilutions of the test solution or dispersion was used to treat 120 X 30 mm strips of chromatography paper which were then dried overnight. The paper strips were folded concertina style and inserted into 16 by 50 mm round bottom glass tubes. 1ml amounts of fortified sheep serum (2 % Yeast Extract; 0.5 % KH_2P04) were then added. Twenty newly hatched larvae were counted into each vial and incubated at 28°C and 70% RH for 24 or 48 hours, depending on the assay.

For comparison of different formulations and tests of the effects of weathering a similar assay with the test formulations applied to staples of wool harvested from Merino sheep known to be pesticide free were used. The staples were cut into 3 cm lengths and 100 mg amounts of wool treated with the test formulations and air-dried overnight were used. There were 3 replicates of 20 larvae for each concentration.

To prepare the nanoparticles for the tests they were dispersed in the carrier compound by ultrasonication for 1 hour. For cyromazine the test formulations were then serially diluted in hexane or fortified sheep serum (2% Yeast Extract; 0.5% KH_2P04), depending on the assay. Range finding assays were conducted followed by a more restricted dose range chosen to provide a good range of concentrations from 0 to 100% kill for each assay. For the cyromazine assays all assays were assessed at 24 and 48 h and surviving larvae given a “stunting score” of 0 to 3 based on degree of stunting and morphological abnormalities in comparison to controls. As moulting is frequently delayed in some larvae under the effects of the growth regulator compound, but the larvae eventually die when kept longer, affected larvae were scored as dead for analysis.

3.7.3 Egg Assays

Ovitoxicity was tested by wrapping lots of 20 eggs in fine gauze material and immersing them for 60 s in a concentration series of acetonic solutions of the test formulations up to 0.256 ppm, with three replicates per concentration and controls immersed in acetone. Eggs were then allowed to air dry and removed to moistened filter paper in sealed Petri dishes and observed under a binocular microscope for hatching up to 48h.

3.7.4 Assays against third instar larvae.

As many flystrike preventative compounds are also used to treat struck sheep, and to be acceptable for this use a quick kill of all stages of blowfly strike is required, the topical toxicity of an RNP nanoparticle formulation against 3rd stage larvae was assessed. Third instar larvae were immersed for 60s in dispersions of RNP or equivalent concentrations of a commercial formulation of ivermectin formulated with surfactants to aid wetting (Paramax®, Coopers Australia Ltd) and the mortality of larvae was assessed at 24h.

3.7.5 Assays against adult L. cuprina

Measurement of topical toxicity against adult flies was attempted in two ways. Topical application using micro-dosing onto the thorax and abdomen was tested, but it was not possible to get sufficient retention without use of a solvent, which it was considered could compromise the encapsulation of ivermectin. Therefore, adult flies were immersed in water dispersions of RSNP for 60 s as for the L3 larvae.

3.8 'Weathering' treated wool samples to test the effects of solar radiation and wetting

3.8.1 Ultraviolet degradation

Ivermectin-loaded nanoparticle samples, unencapsulated ivermectin or treated wool samples were exposed under a UV lamp (Emax=365 nm) for 3h and samples with and without UV exposure were used for bioassay analysis. For analysis of the ivermectin content, exposed and unexposed samples were extracted using acetone, filtered through a 220 nm syringe filter and measured by HPLC.

3.8.2 Rainfastness

As there was relatively little rainfall during natural weathering, rainfastness of the nanoformulations in comparison to the commercial product was measured in the laboratory. Wool staples 3cm in length and weighing 100 mg were held in place and the desired amount of 'artificial rain' applied from a plastic spray bottle from a height of 50 cm above the sample. The spray bottle was calibrated to deliver 10 cm of 'rainfall' per 180 strokes. The weight of the water hitting a paper surface and draining was determined by weighing to ensure that equivalent amounts of water was being applied each time. The wool fibre was allowed to dry for at least 24 hours at room temperature after the rain application before testing for insecticidal activity in larval assays.

3.8.3 Environmental weathering

Treated and control wool samples were stapled to cardboard attached to metal trays and secured in place by a metal grid in full sunlight on the roof (6^{th} floor) of Ecosciences Precinct in Brisbane (Figure 1). The samples were in place on the roof from 8am to 4pm each day and exposed to full sunlight, ambient temperature and rainfall during the daylight hours. Complete weather details including temperature, solar radiation, humidity and rainfall during the period of exposure were collected from a directly adjacent weather station.

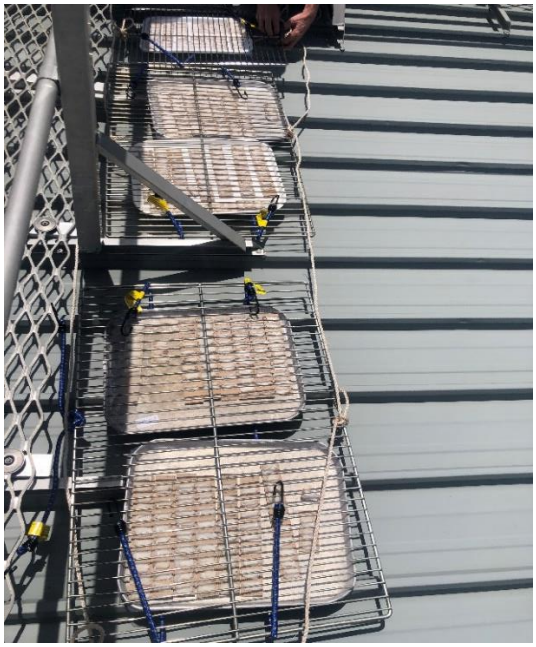


Figure 1. Wool samples secured in full sun on the roof of the EcoSciences Precinct for artificial weathering

At different intervals of time, depending on the experiment (nominally 1 day, 1 week, 6 weeks and 12 weeks and 20 weeks), weathered wool samples were removed from the trays and tested in larvicidal activity assays. Control samples were stored at -25^{o}C and a full set of unweathered samples were tested in larval assays alongside samples from each time point.

3.9 Sheep studies

When searching for a new blowfly insecticide, although larvicidal activity is clearly a key parameter consideration of dynamics of the insecticide in the fleece is also of critical importance. Harrison and Rundle (1983) note that the ability of an insecticide to translocate in the fleece is a key parameter in determining period of field protection and that it is essential to assess the possible translocation of ability of potential larvicides on sheep before labour intensive protection trials are undertaken.

The first part of this study assessed both the persistence of activity when different formulations were applied to live sheep with >6mths wool and the extent of movement of candidate nanoparticle formulations along wool fibres and laterally in the fleece. A commercial formulation of ivermectin (Paramax®) applied at label directed rates acted as a positive control, and two rough surface nanoparticle types RNP and the RNP-C18 particles mixed to provide equivalent ivermectin concentration and at half concentration were tested. The nanoparticle formulations were sprayed onto the wool surface in defined areas. Wool samples and skin washings were collected at 2 weeks after treatment both within the application strip and at 5 - 10 cm laterally from the application strip. Wool samples were divided to proximal, medial and distal thirds and the concentration of nanoparticles assessed. Skin washings were also collected at the sampling sites and nanoparticle concentration measured at the application site 5 - 10 cm laterally to assess lateral formulation movement.

The sheep in the second part of the study were 4 newly shorn sheep, shorn by QASP staff, held, free standing in a weighing crate for the application of treatments and collection of samples. Tests were conducted with a commercial formulation of ivermectin applied to the skin surface from a pipette and the four nanoparticle treatments indicated for the first part of the study. Nanoparticle and control formulations (5ml) were applied to the clipped wool/skin in 4cm diameter areas on the loin and midback of each sheep by holding a 4cm open cylinder against the sheep skin and applying the required volume from a hand held pipette. Washings were taken from skin surface/wool stubble at 24h and 2 weeks after treatment in 3 cm areas, 5 - 10 cm in four directions (’north, south, east and west’) from the point of application. Washings were collected by agitating Tween20 inside 3cm diameter plastic tubes and then collecting the washings from the skin surface with a pipette. Wool stubble in the washed area was also collected by clipping with small animal clippers.

4. Results

4.1 Characterisation of particles

4.1.1 Ivermectin particles

As shown in Figure 2, the TEM images had distinct smooth (SNP, Figure 2a) and rough surface (RNP, Figure 2b) of nanoparticles with a large hollow interior. The shells of the RNPs were covered with nanosized silica spikes to form the rough surface morphology, mimicking the surface topology of pollen grains. Hydrophobic modification of RNP did not alter the rough morphology as shown in Figure 2c. Nitrogen adsorption-desorption isotherms of these three particles as shown in Figure 2d indicate the highly porous nature of the rough nanoparticles, having a pore size around 10 nm attributed to the void space between the surface spikes.

Field emission scanning electron microscope (FE-SEM) image of conventional ivermectin crystals (Figure 3a) showed a large size around 10-50 µm. However, after loading ivermectin into RNP and RNP-C18 through rotatory evaporation method, no large ivermectin crystal can be observed (Figure 3c and d), because these chemicals were adsorbed and confined on the nanosized pores and particles. Small sized nanoparticles with rough surface can be observed in the inset images. SNP with no nanosized porous structure may not be able to adsorb such a large content of ivermectin on the particle surface, thus

allowing the ivermectin to re-crystalise into large particles as shown in Figure 3b. The loading capacity of ivermectin in these nanoparticle forumulations were characterised using TGA and DSC (Figure 4), where pure ivermectin showed a dramatic weight loss during 200-600 ^{o}C due to decomposition in air under heat. The weight loss for nanoparticle formulations are similar to around 23 wt%, indicating a uniform ivermectin loading ratio as 23 wt% in these formulations as silica are inert under heat showing no weigh loss. DSC analysis of these formulations (Figure 4b) showed an obvious endothermic peak at around 160 ^{o}C for pure ivermectin, ivermectin-nanoparticle physical mixture and ivermectin loaded in SNP, due to the existence of large ivermectin crystals which requires extra heat to break the large crystals. In contrast, when loading ivermectin into the highly porous RNP and RNP-C18, no endothermic peak was observed, indicating there is no such large ivermectin crystals in these formulations, confirming the observations from SEM images.

The rough silica nanoparticles had uniform size of ~300 nm and negative surface charge of ~ -20 mV, although particles of 180 nm, 300 nm, 500mm and 800mm were also synthesised (Section 2.1.2, Figure 5) showing distinct rough surface and porous structure with a pore size around 10-16 nm.

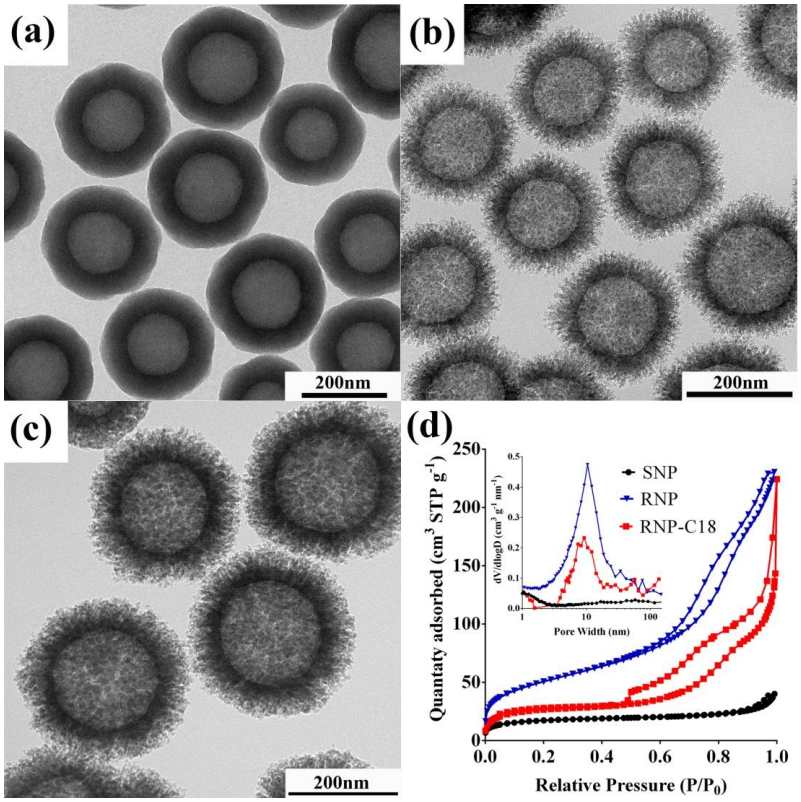


Figure 2. TEM images of smooth nanoparticles (SNP, a), rough nanoparticles (RNP, b), rough nanoparticles after C18 modification (RNPC18, c), and corresponding nitrogen sorption isotherms and pore size distributions derived from BJH desorption branch (d)

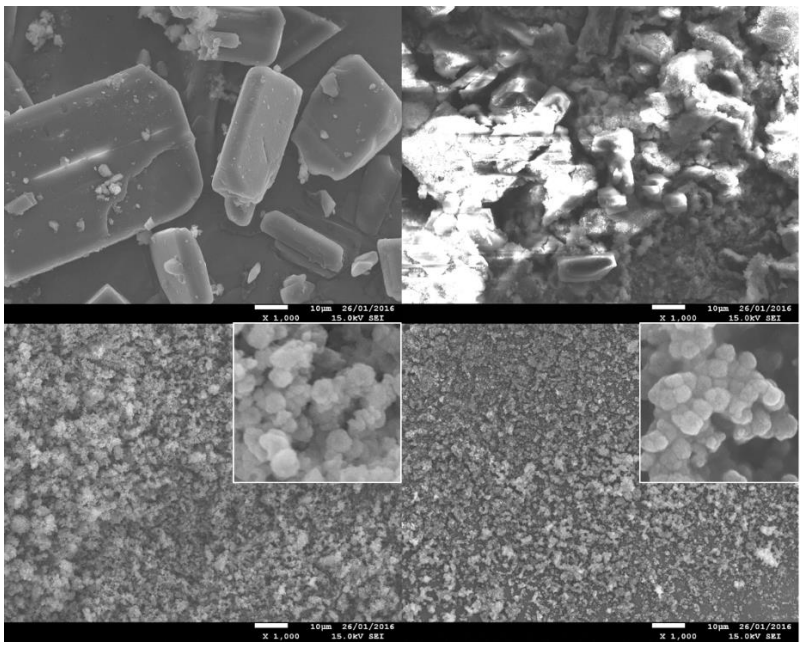


Figure 3. SEM images of ivermectin crystals (a) and ivermectin loaded in SNP (b), RNP (c, high magnification inset) and RNP (d, high magnification inset)

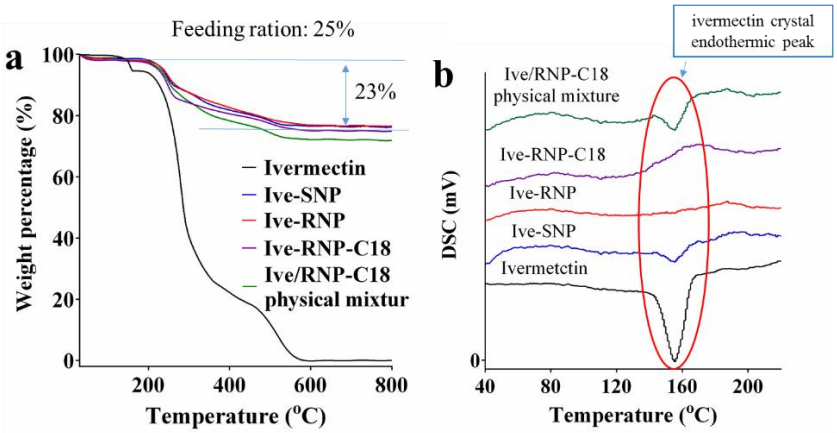


Figure 4. TG (a) and DSC (b) curves of ivermectin, ivermectin loaded silica nanoparticles and ivermectin with silica nanoparticle physical mixture

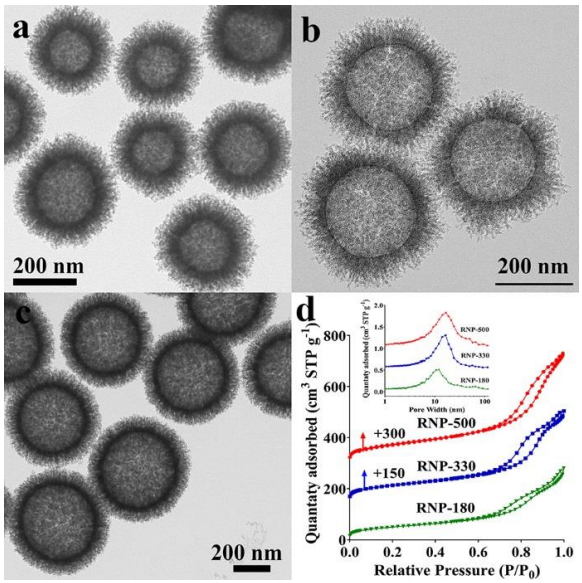


Figure 5. Transmission electron microscope (TEM) images of RNP with particle size of 180 nm (a), 330 nm (b), 500 nm (c) and corresponding nitrogen sorption analysis (d)

4.1.2 Cyromazine particles

Whereas ivermectin tends to be more lipophilic and has low water solubility c. 0.001 g/L) cyromazine is more hydrophilic with a solubility of (c. 13 g/L). As flystrike always occurs in the presence of moisture, the notion was of a strategic release formulation that would remain relatively inert in the fleece with release only in the presence of moisture or in the insect gut following ingestion. That is, to produce a formulation with a longer presence in the fleece and designed to release only at times and in sites where control is needed. Cyromazine was tested here as model for encapsulation of other water-soluble actives, because of its relative Diptera specificity and also because of its growth regulator mode of action.

The methods used to synthesise mesoporous silica RNP and SNP nanospheres with hollow cavity described for ivermectin were employed, with slight modification, to also be suitable for cyromazine. The cyromazine particles were of uniform size of ~380 nm with a negative surface charge of ~ -20 mV (Figures 6, Table 1). The shells of the RNPs were covered with nanosized silica spikes to form the rough surface morphology, mimicking the surface topology of pollen grains. The RNP and SNP in this study were also noted as mesoporous silica hollow spheres-rough surface (MSHS-RS) and mesoporous silica hollow spheres-smooth surface (MSHS-SS) as show in the following figures. Both SNP and RNP showed uniform and similar particles sizes in water as determined by DLS (Figure 7). Nitrogen sorption analysis indicated that the RNPs have 14 nm mesopores (Figure 8). SNPs containing cyromazine with similar particle size and surface charge properties to the RNPs were also synthesized for comparison with the rough surface topology cyromazine nanoparticles (Figures 6; 7; 8 Table 1).

Without using nano-carriers, pure cyromazine forms micron-sized crystals (Figure 9B). Such crystals were not observed in FE-SEM images of cyromazine loaded RNP (RNP-C) and cyromazine loaded SNP (SNP-C) (Figures 9E, 4H) indicating the complete encapsulation of cyromazine at the submicronmetre scale.

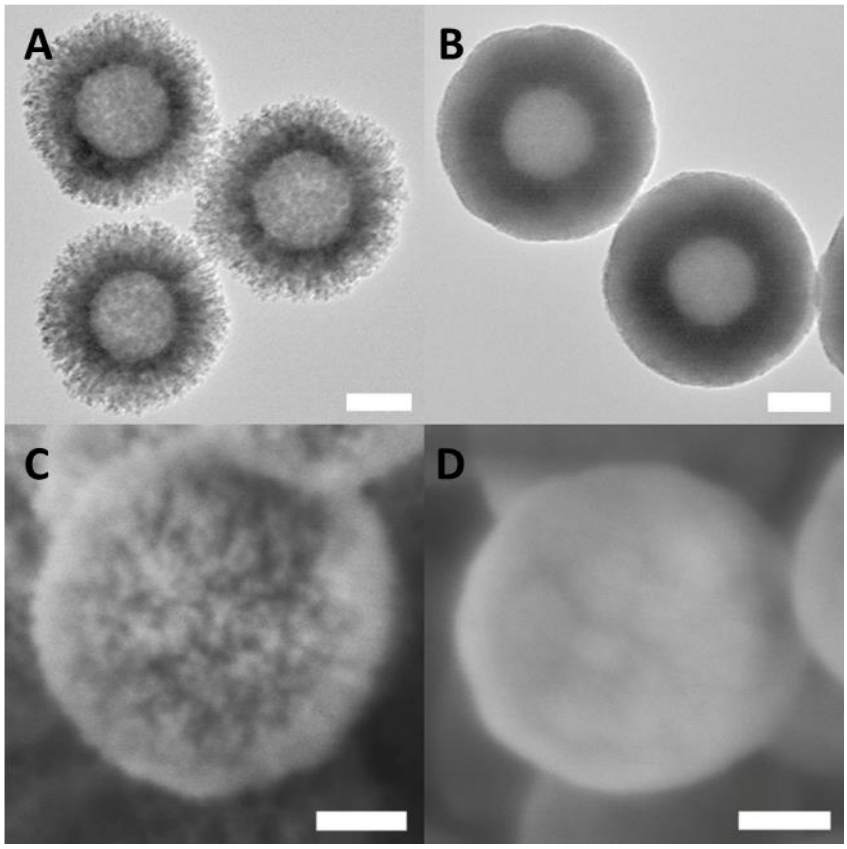


Figure 6. TEM images of RNP (A) and SNP (B), FE-SEM images of RNP (C), SNP (D), and Scale bar: 100nm

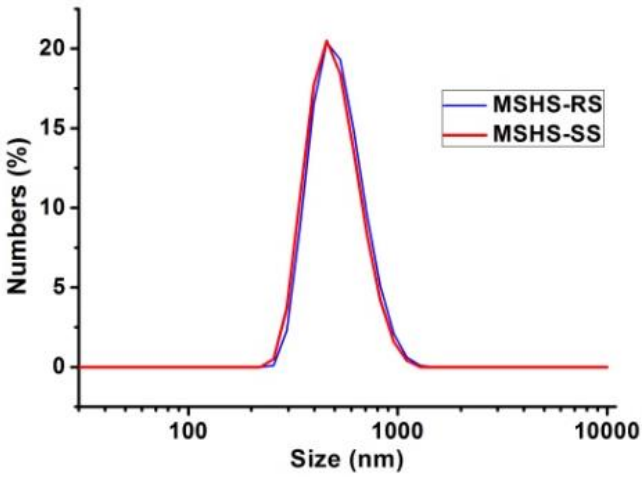


Figure 7. Particle size distribution for the RNP (=MSHS-RS) and SNP (=MSHS-SS) nanoparticles as measured by dynamic light scattering experiments

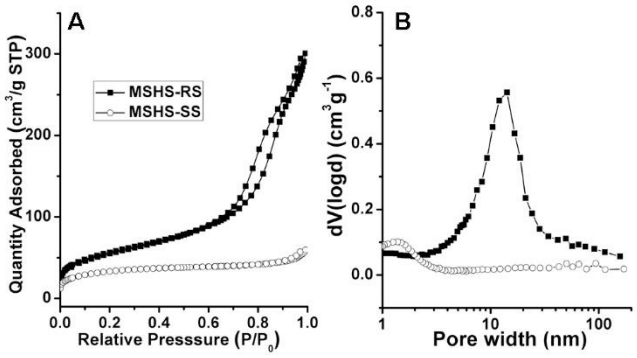


Figure 8. (A) Nitrogen sorption isotherms and (B) BJH pore size distribution curves for silica RNPs and SNPs

Table 1. Structural information of silica nanoparticles.

Sample Name	Size (nm)	Zeta potential (mV)	Pore size from adsorption branch (nm)	V_p ($cm^3\ g^{-1}$)	S_{BET} ($m^2\ g^{-1}$)
RNP-C	380	-23.5	14	0.50	169
SNP-C	380	-19.6	-	0.10	112

Note: V_p: total pore volume; S_{BET}: BET surface area.

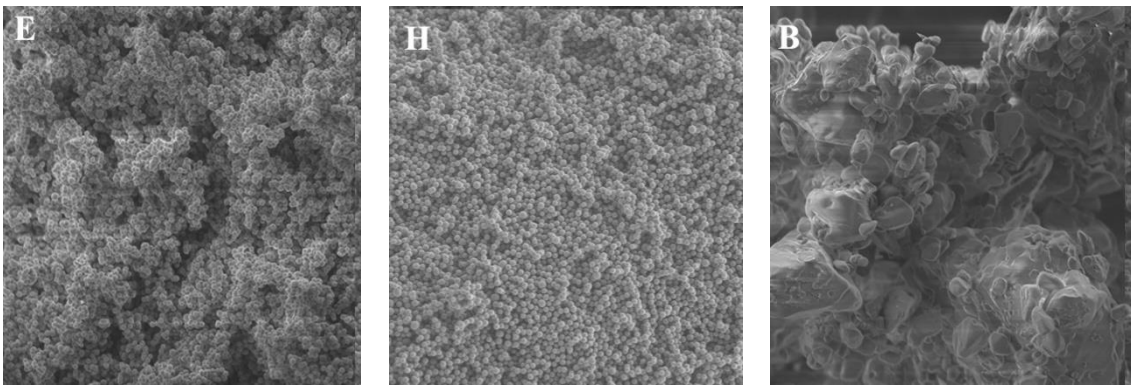


Figure 9. Characterization of nano-cyromazine: FE-SEM images of cyromazine (B), RNP-C (E) and SNP-C (H);

The loading amount of cyromazine was determined by thermogravimetric analysis (Figure 10A). Unencapsulated cyromazine shows complete weight loss of 99.9 % at 900 °C whereas silica nano-carriers show negligible weight loss of <1.0 %. The actual loading amounts of cyromazine were calculated from the weight loss of nano-pesticide and were ~23% (Figure 10A). The results match very well with the cyromazine:silica feeding ratio, suggesting the complete encapsulation (~ 100%) of pesticide actives in the silica carriers. The presence of nano-cyromazine after loading was also confirmed by differential scanning calorimetry (Figure 10B). Pure cyromazine displays an endothermic peak at 255 °C, indicating the melting point of crystalline cyromazine (Bottom line in Figure 10B). Similar to pure silica, RNP-C shows no obvious peaks in the range of 25-200 °C, indicating an amorphous state of cyromazine in the NP formulations.

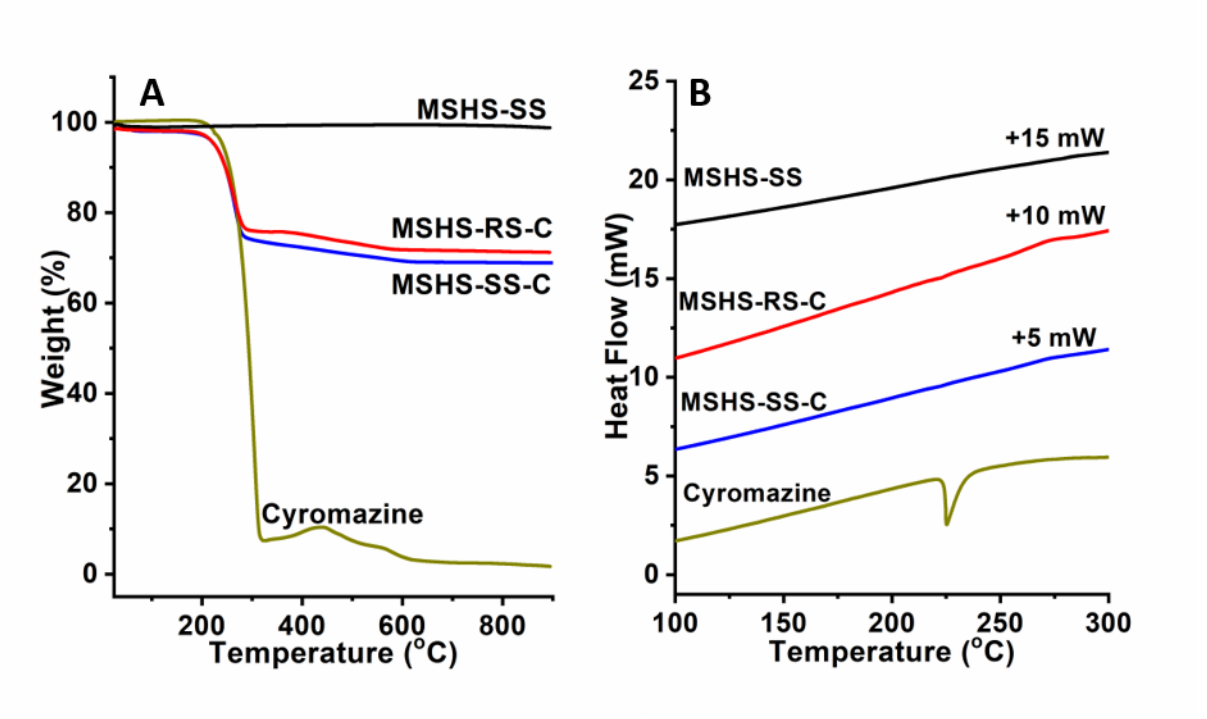


Figure 10. (A) Thermogravimetric analysis and (B) Differential scanning calorimetry profiles of SNP, (=MSHS-SS) SNP-C (=MSHS-SS-C), RNP (=MSHS-RS) and RNP-C (=MSHS-RS-C) particles, and cyromazine

The Fourier transform infrared spectrum of pure cyromazine show a series of characteristic peaks in the range of 500-1750 cm^{-1} (Figure 6). In the spectrum of RNP-C, the characteristic peaks in the range of 1300-1750 cm^{-1} are still present, indicating successful loading of cyromazine. The other peaks from cyromazine in the range of 800-1200 cm^{-1} overlap with the peaks for silica.

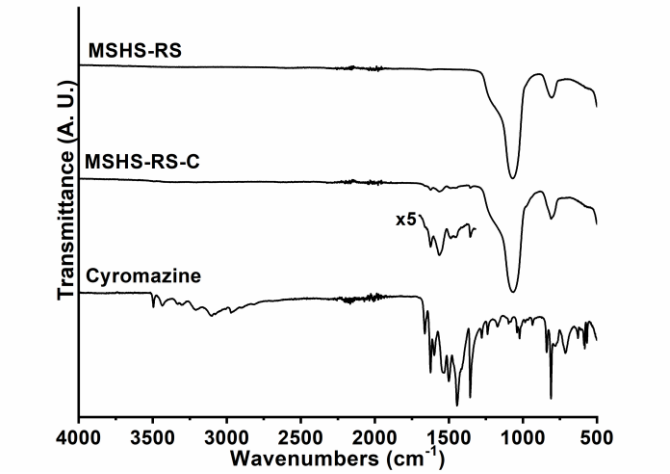


Figure 11. FTIR spectrum of RNP-C, SNP-C, and pure cyromazine

The release behaviour of cyromazine from SNP-C and RNP-C in water were tested in a 12-day immersion test. Cyromazine release/dissolution from the nanoparticles in the water reached a maximum within 6 days (Figure 12) whereas cyromazine had dissolved in water within 1 day. Cyromazine formulated in the RNP formulation, showed similar gradual discharge behaviour when immersed in water with release over 5 days (results not shown).

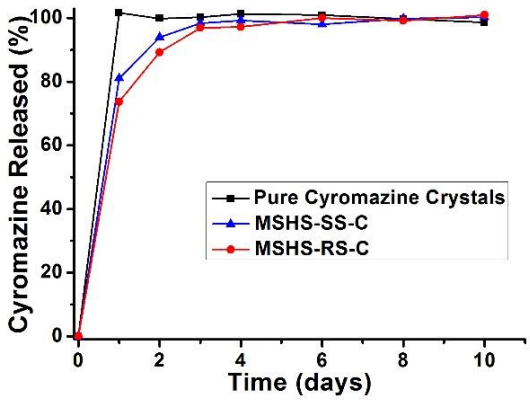


Figure 12. Release behaviour of pure cyromazine crystals and nano-formulated cyromazine in water

Figure 13 shows water suspensions of 3 RNP formulations (left 3 tubes and pure chemical active (right 3 tubes). Pure cyromazine can be completely dissolved in water due to its high-water solubility. The nanoparticle formulations form suspensions following simple hand shaking. Use of an ultrasound probe increased the stability of the formulation and resulted in the particles staying in suspension for a number of days.

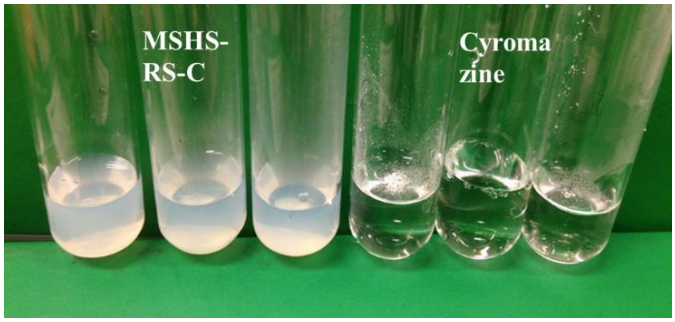


Figure 13. Digital photos of water suspensions of RNP formulations (left 3 tubes) and pure pesticide active, formed by hand shaking

4.1.3 Characterisation of FN60 formulations

The FSN-60 particles have a rich porous nature (thicker shell) and the appearance of the formulation is clearly different to that used previously (Figure 14). They have a similar pore size to the previously synthesised RNP nanoparticles, but a much higher surface area of 378 m^2/g (RNP of 164 m^2/g), a pore volume of 0.97 cm^3/g (RNP of 0.33 cm^3/g) as determined by nitrogen sorption analysis (Figure 15), a smaller particle size (180 nm) and no hollow central core. Notably, the higher pore volume of FSN-60 allows a much higher drug loading capacity than the previous RNPs (up to ~50% compared to ~23% for the RNP). However, the FSN particles used for these studies, which showed similar rough surface as FSN were loaded with 23% cyromazine to enable a direct comparison of the two particle types in studies of insecticidal effect. Hydrophobic FSN particles were fabricated following the hydrophobic modification protocol for RNP-C18, and similarly loaded with cyromazine at 23% weight ratio. The particles show similar rough surface to the FSN particles.

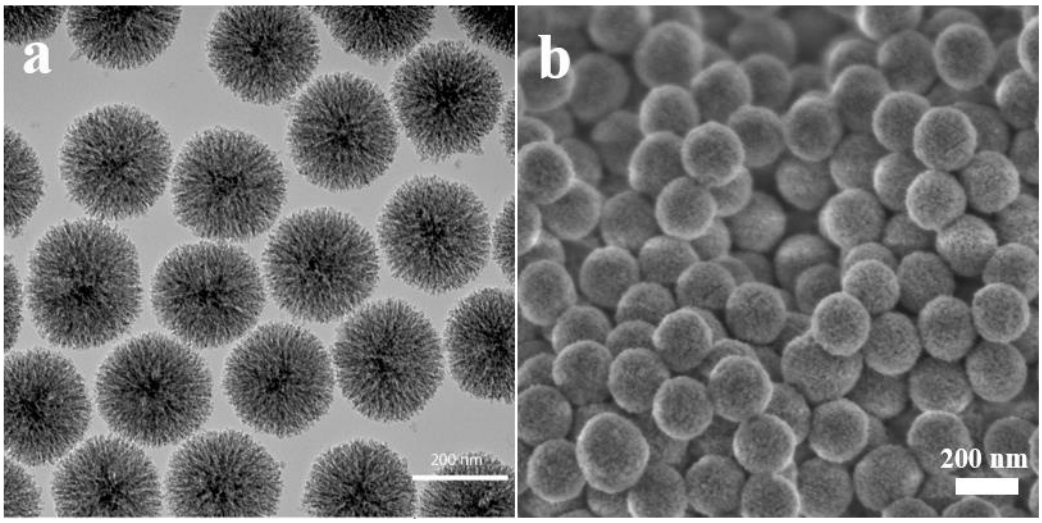


Figure 14. TEM (a) and SEM (b) images of FSN-60

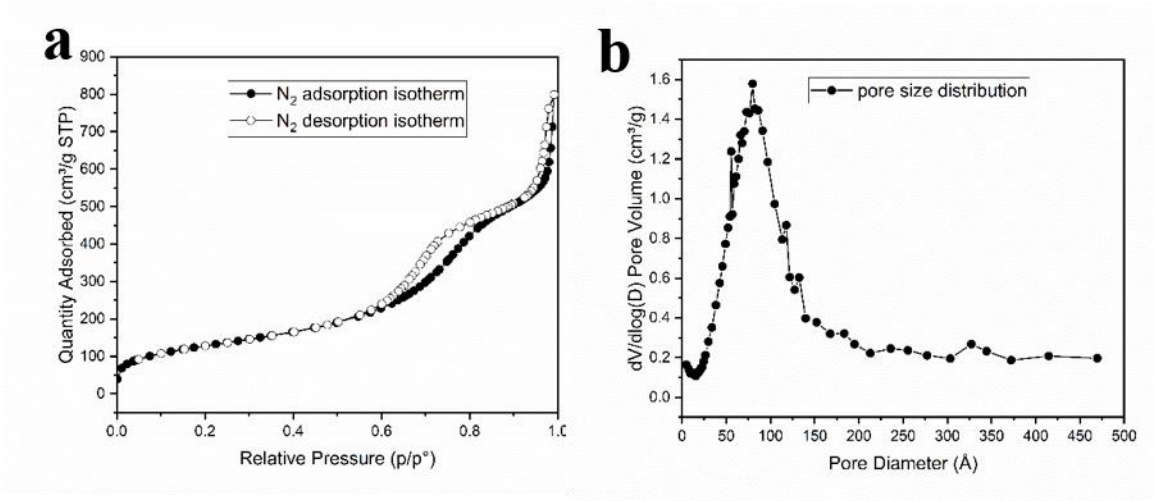


Figure 15. Nitrogen sorption isotherm (a) and pore size distribution (b) of FSN-60

Thermal gravimetric analysis of cyromazine loading FSN-60, similar to that shown in Figure 10, confirmed the loading content of 23% (designed for direct comparison with the RNP and SNP). However, as noted above, from measurement of the surface area and pore volume it is expected that a maximum loading capacity of twice this amount will be possible with the FSN particles.

4.2 Adherence of nanoparticles to wool fibres

4.2.1 Unscoured ('greasy') wool

The electron micrographs are shown below.

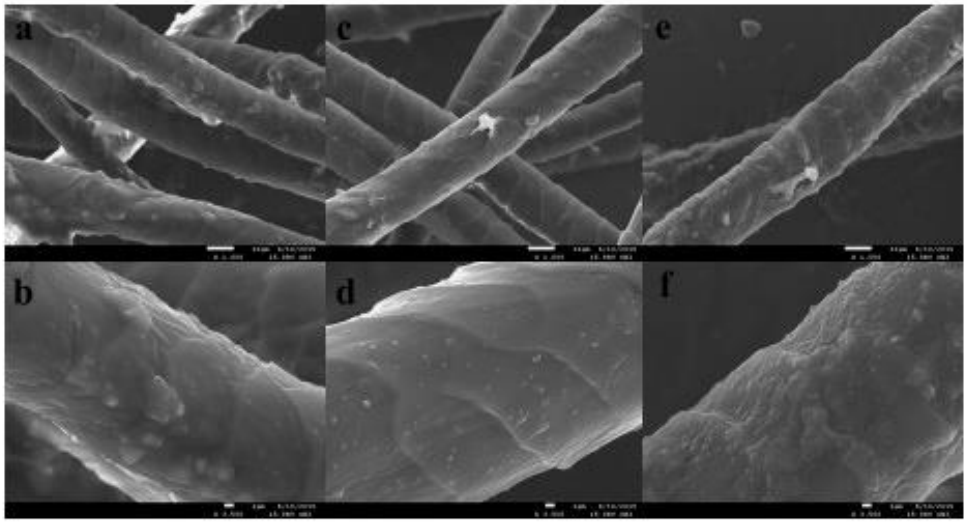


Figure 16. Scanning electron micrographs of unscoured wool treated with smooth nanoparticles at different magnifications (a, b); rough nanoparticles (c,d) and RNP-C18 nanoparticles (e, f)

In the 'greasy wool' before methanol washing (Figure 16) it is difficult to make out the nanoparticles which appear to be imbedded in the lipid material covering the wool fibre conferring a 'rough; look to the lipid'. It is known that potassium and other salts (mainly in the sudoriferous secretions) mixed with sebaceous secretions covering the wool fibre partially emulsifies the wool wax in the presence of water and assists in leaching some of it from the fleece. In Figure 17 which shows the greasy wool after water washing and presumably removal of some wool grease, the presence of nanoparticles on the fibre and in the remaining grease is more evident. This effect appears to be most marked with the C18 nanoparticles (Figure 17c) with the remaining particles more evident than with the smooth and rough particles.

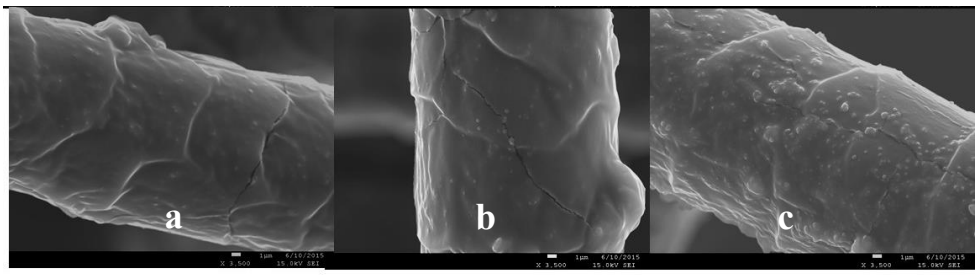


Figure 17. Electron microscope images of nanoparticle-treated unscoured wool after water rinsing; Smooth nanoparticles (a) rough nanoparticles and (b) C18 nanoparticles (c)

4.2.2 Scoured wool

Adhesion of the particles is more evident in the tests with the scoured wool where there is less 'wool grease' (lipid and sudoriforous secretions which coat the wool fibre) present to obscure the attached nanoparticles (Figure 18). The rough particles with the C18 modification showed strong interaction with wool surface and there was clear difference in the level of adherence by different particles with most RNP-C18 particles and least SNP adhering following treatment. It also appears that more of the C18 particles and RNP remained attached following water washing (Figure 19). There were clearly less of the SNP attached both before, and particularly after, water washing. This pattern was also observed using FITC labelled (fluorescent) particles (Figure 20).

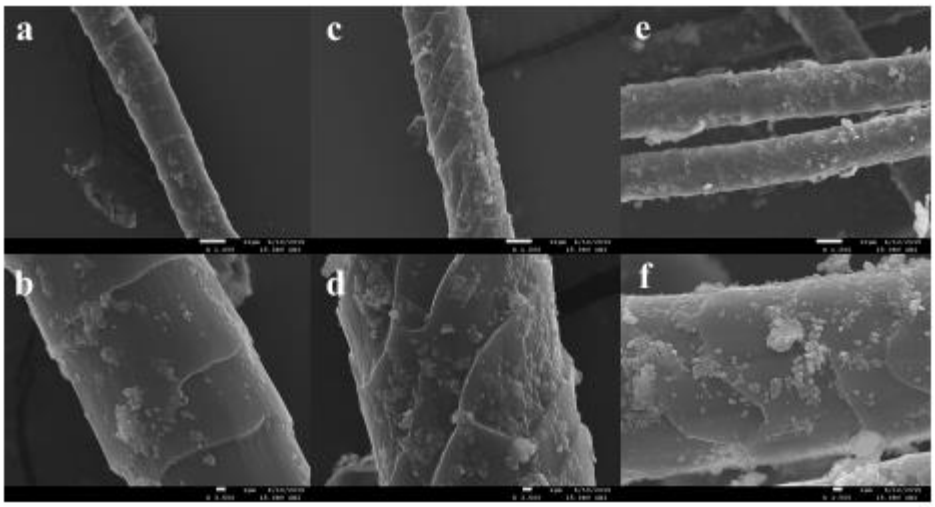


Figure 18. Electron micrograph images of nanoparticles on scoured wool (wool washed with methanol to remove wool grease before treatment with smooth nanoparticles (a, b) rough nanoparticles (c,d) and C18 nanoparticles (e, f).

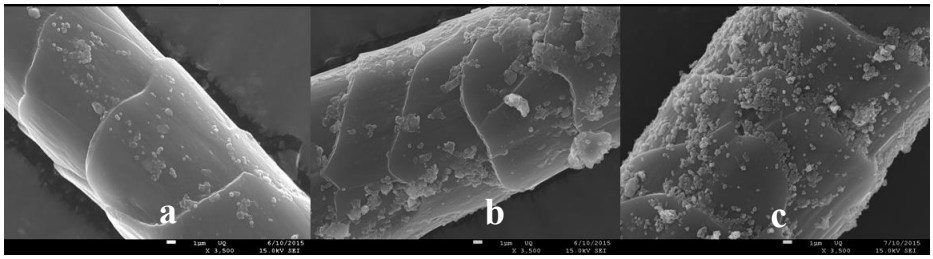


Figure 19. Electron microscope images of nanoparticles adhered to scoured wool after water rinsing; smooth nanoparticles (a) rough nanoparticles (b) and RNP-C 18 nanoparticles (c)

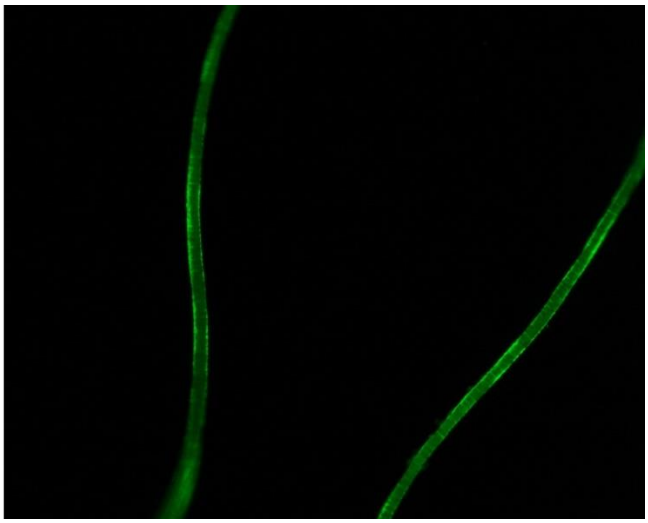


Figure 20. Wool fibre exposed to the C18 nanoparticle after 2 water washes (10X magnification). Fluorescent labelling indicates persistent adherence of nanoparticles along the full length of the fibre

4.3 Cuticular adhesion and ingestion of nanoparticles by larvae

Studies with blowfly larvae indicate high density of the FITC- labelled particles (RNP) in the insect gut (Figure 21). Assays with sheep lice (*Bovicola ovis*) also indicated accumulation of the fluorescent particles in the gut (Figure 22). This indicates that both insects are ingesting significant amounts of the labelled particles. Whether the particles are transient, possibly attaching to gut lining or peritrophic membrane, or whether both insects are actively accumulating the particles during feeding is unclear at this stage. The feeding habits of both insects would seem to favour active accumulation of particles. Blowfly larvae 'scrape' at the food surface and with the assay design used here where the particles were applied to the

wool, would be expected to ingest significant numbers of particles. Sheep lice are chewing lice which also use their mandibles to 'scrape' at the skin or wool surface and feed on lipid, skin debris and skin cells from the superficial epidermis, again favouring the ingestion of particulate matter. Whether there is a difference between the different types or designs or possibly sizes of particles in the degree to which they are ingested or accumulate awaits further studies.

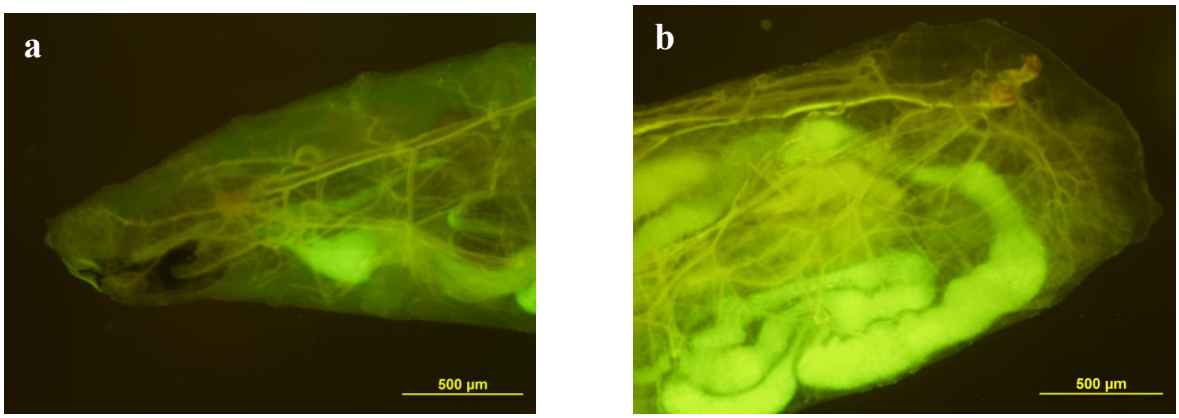


Figure 21. Concentrations of fluorescent nanoparticles in the anterior (a) and posterior (b) gut of blowfly larvae, confirming oral ingestion of nanoparticles

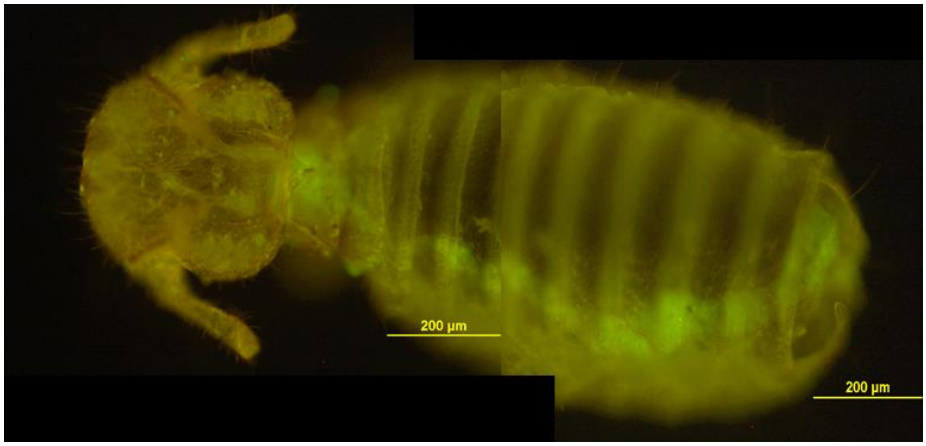


Figure 22. Sheep louse fed on louse diet treated with fluorescent nanoparticles showing concentration of nanoparticles in the gut, but also with pale green colouration of the cuticle, suggesting that there was also adherence of particles to the integument

Cuticular adhesion was also noted with both blowfly larvae and lice but fluorescence was lower than in the gut. This is not unexpected as ingestion of particles occurs actively during feeding whereas the particles on the cuticle would be acquired passively. The cuticular EMs for both blowflies and lice suggest that the C18 and rough nanoparticles adhere more strongly to cuticle than the smooth particles, Figures 23-25) and in the case of the fly larvae at least, the C18 particles adhere more strongly than the rough particles. This is seen most clearly with the blowfly larvae in Figures 23 and 24. In both of these Figures the larvae exposed to the C18 particles have a full body coverage of fluorescent particles, despite that these larvae were rinsed after exposure to the labelled particles, suggesting strong adherence. With the SNP and RNP particles the fluorescent staining is mainly in the posterior portion of the larvae. This corresponds to the convoluted part of the intestine, which fills most of the haemocoel in the posterior half of the larvae. Thus, that only the back part of the larvae shows significant fluorescence with the SNP and RNP because fluorescence in this part of the larvae is due mainly to ingested particles rather than particles adherent to the cuticle. With the louse cuticle, again the C18 and rough particles show most adherence, whereas there is only a weak indication of attached nanoparticles in the case of SNP (Figure 25).

These results suggest that best effect is likely to be achieved from the nanoparticles against both blowfly larvae and lice when they are administered with the objective of oral toxicity. However, the rough or C18 particles could also be expected to add to the toxic dose delivered, particularly with purpose designed chemical payload and release characteristics. This could be an important consideration when designing application strategies.

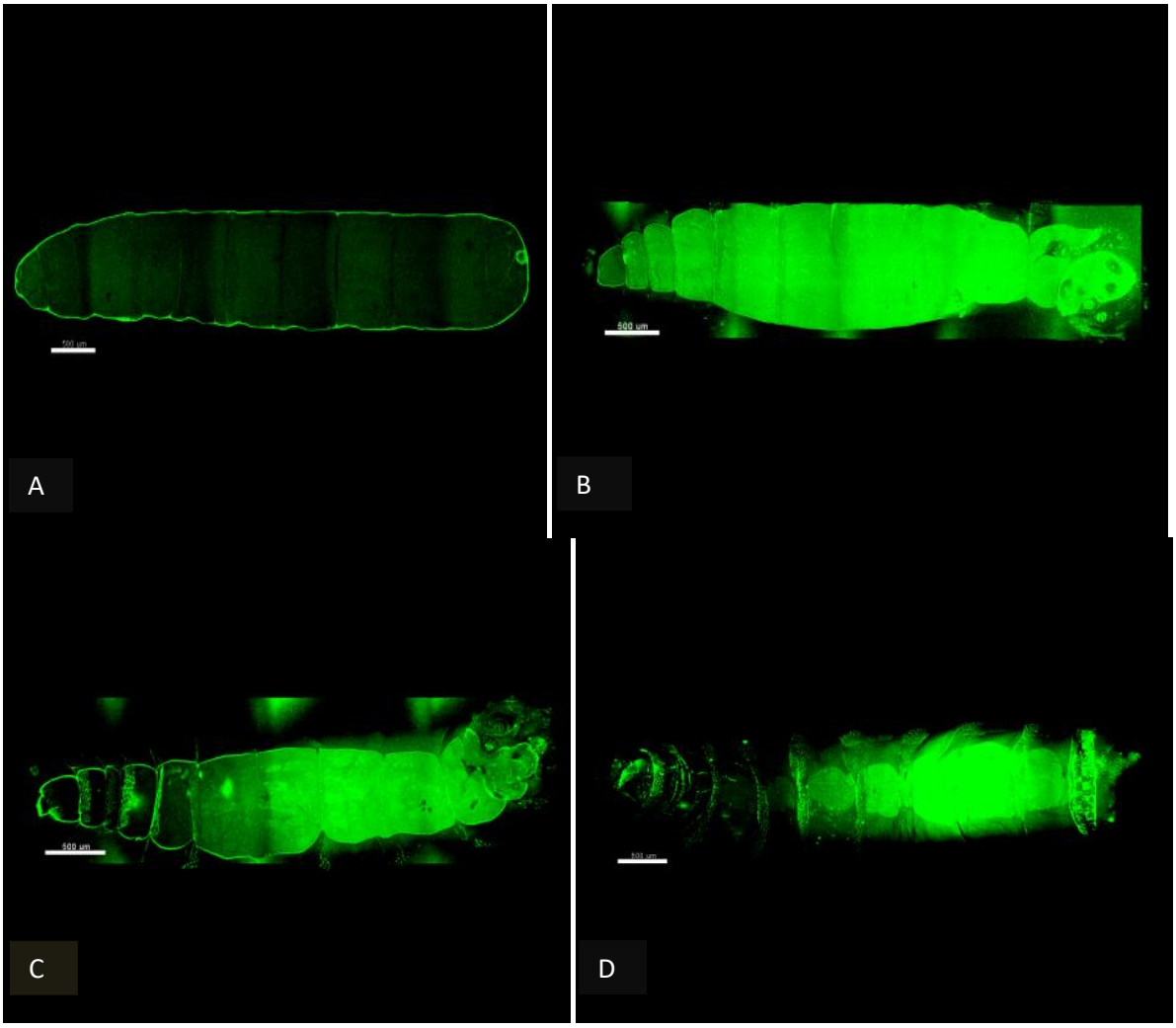


Figure 23. Full volume view images of L. cuprina larvae after a 24h exposure to serum containing green FITC- labelled nanoparticles: control – sheep serum only (A); C18 nanoparticles, (B): Smooth nanoparticles (C) and rough nanoparticles (D)

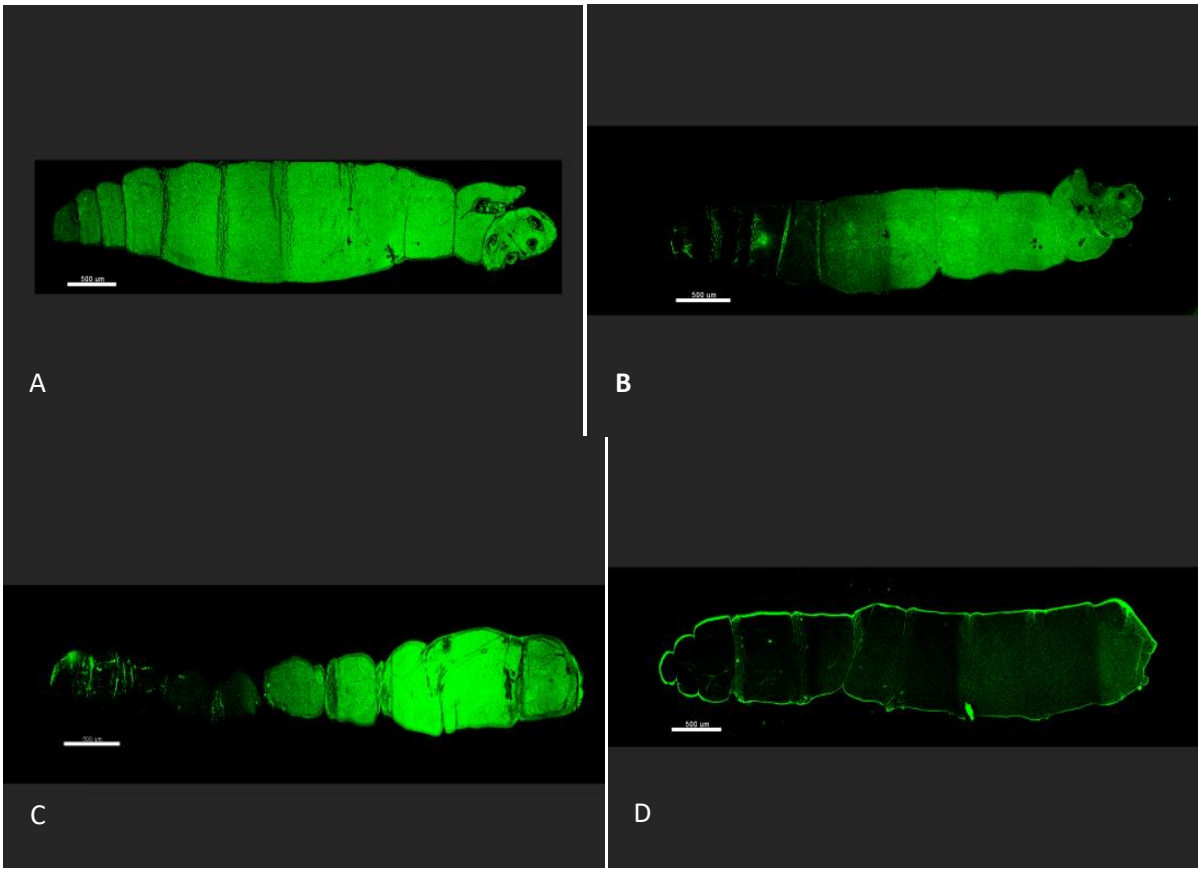


Figure 24. Surface slice view images *L. cuprina* larvae after exposure to serum containing C18-modified Nanoparticle (A) Smooth nanoparticles (B) rough nanoparticles (C), and sheep serum only (D)

This could be an important consideration when designing application strategies.

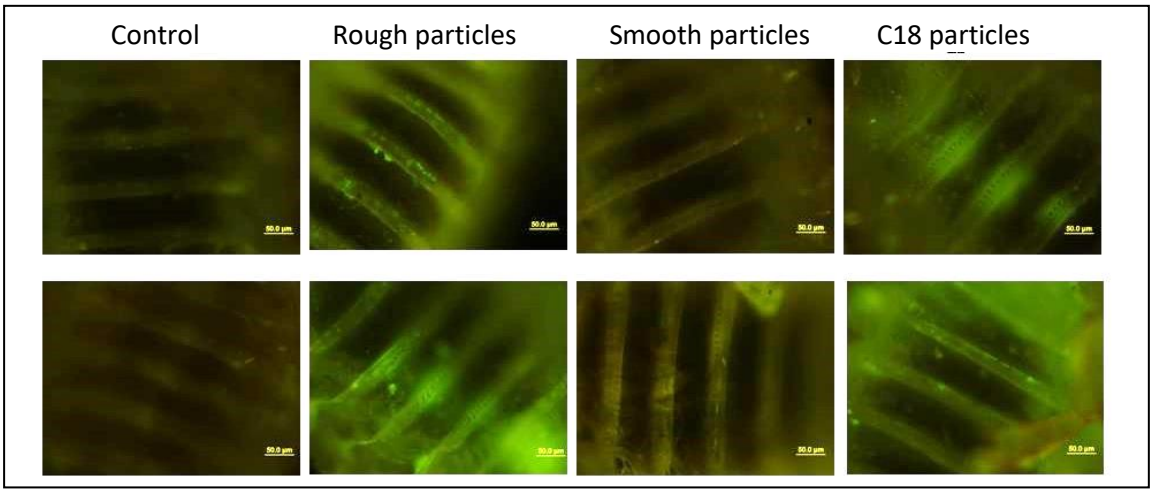


Figure 25. Sections of sheep louse cuticle following exposure to nanoparticles on wool not treated (a) or treated with rough particles (b) smooth particles or (c) and C18 particles (d)

4.4 Assays with unweathered ivermectin particles

4.4.1 Paper and serum assays

In these comparisons, nanoparticles were mixed at the required concentrations and either applied to the chromatography paper or mixed at similar concentrations in serum provided in the base of 50 x 16 mm tubes. Typical results are shown in the graphs below (Figure 26).

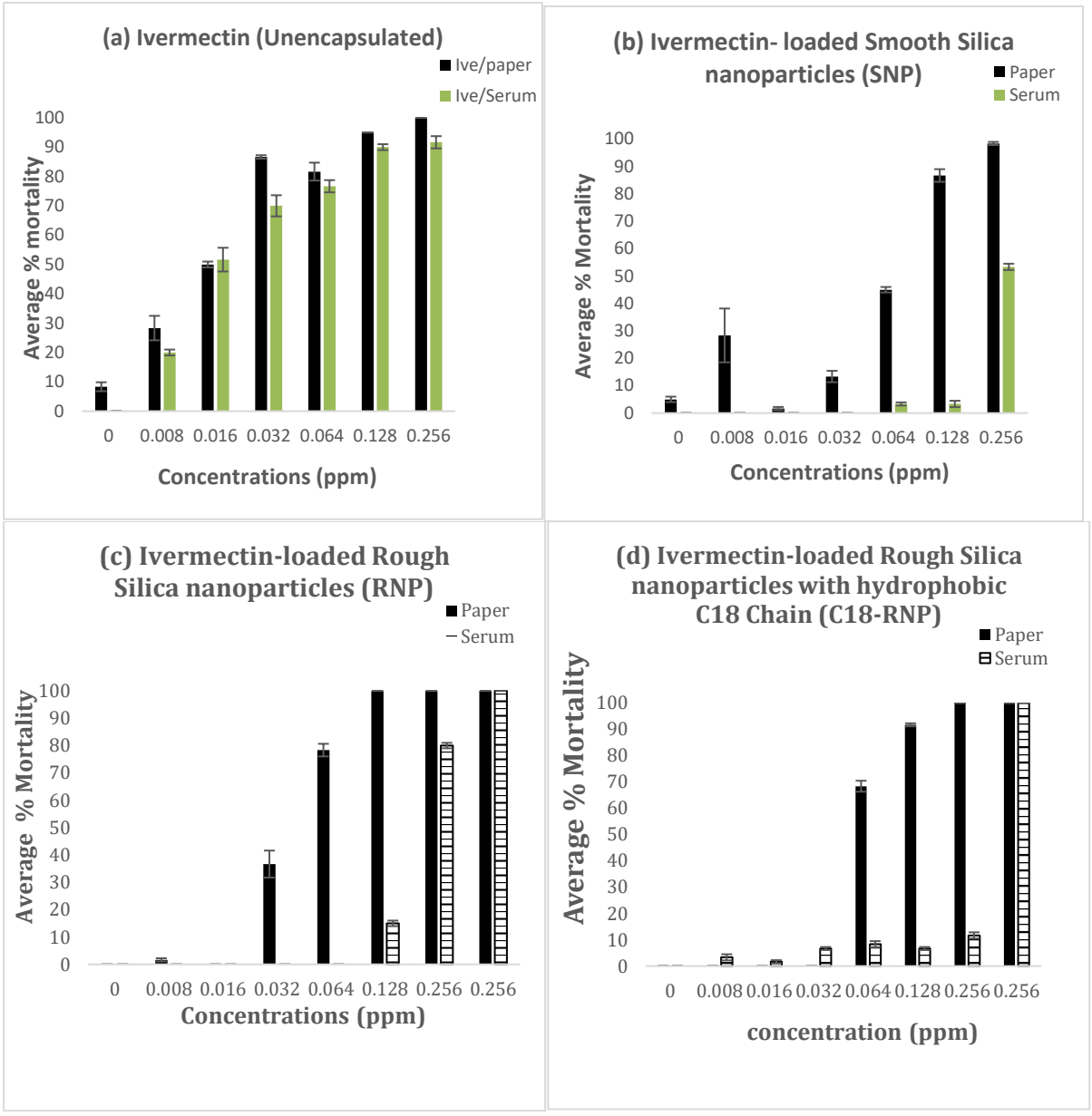


Figure 26. Relative toxicity against 1st instar sheep blowfly larvae of un-encapsulated ivermectin (a), ivermectin encapsulated in smooth silica nanoparticles (SNP) (b), rough silica nanoparticles (RNP) (c) and rough silica nanoparticles with the C18 modification (C18-RNP) (d). (Treatments applied directly on the paper (paper); or diluted in sheep serum (serum)

There was a marked difference in the results from the assays with the nanoparticle formulations and those with unencapsulated ivermectin. With the unencapsulated ivermectin, results did not differ significantly between tests with the chemical applied to the paper or mixed in the serum. However, with the three NP formulations, much better effect was shown in the assays where the formulation was applied to the paper than when mixed in the serum. This may be to do with feeding behaviour. The fly larvae tend to 'scarify' the surface of the paper when feeding. With the particles spread evenly over the treated surface, the chance of different larvae accumulating an even lethal dose while feeding would be maximised. An alternative could involve the distribution of the NP in the serum which may become clumped because of the hydrophobic nature of the particles. With an uneven distribution in the serum

some larvae would be expected to ingest a very high dose whereas many others may ingest only a few particles or a sub-lethal amount of ivermectin. This may account for the low mortality seen at some concentrations in the test with the RNP-C18 formulation in the test shown (Figure 26d). At most concentrations the effect from the RNP and C18NP particles was as good as the unencapsulated ivermectin, although the ivermectin appeared to give slightly better effect at lower concentrations. This is a good result as this is in a test under conditions where the advantages of the nanoparticles are not expected to be demonstrated. That is, the nanoparticles would be expected to show best effect under conditions where chemical is subject to loss from the fleece by leaching, photo-degradation, or other breakdown. Results presented later in this report show a clear advantage for the nanoparticle formulations under these conditions. These results also demonstrate a clear advantage for the RNP and RNP C18 formulations.

4.4.2 Wool and serum assays

Similar effects were seen with the wool-serum assays, with both encapsulated and non-encapsulated formulations giving better effect when applied to the wool than in the serum at similar concentration (Figure 27). In addition, both encapsulated and unencapsulated ivermectin appeared to give better effect at lower concentrations in the wool assay than the paper assay, although this was not a direct comparison. This may be due to a proportion of the ivermectin or particles being absorbed by the chromatography paper where it is not accessible to the larvae during feeding in the paper assays, but absorbed into the lipid covering of the wool fibres and more assessable to ingestion by the larvae in the wool trials. These results suggested that that the wool-serum assay system provided the most suitable method for testing formulation modifications and for examining the effect of UV weathering, rainfall and sheep wool growth on the longevity of protection from different formulations and this system has been used in most tests reported in this report.

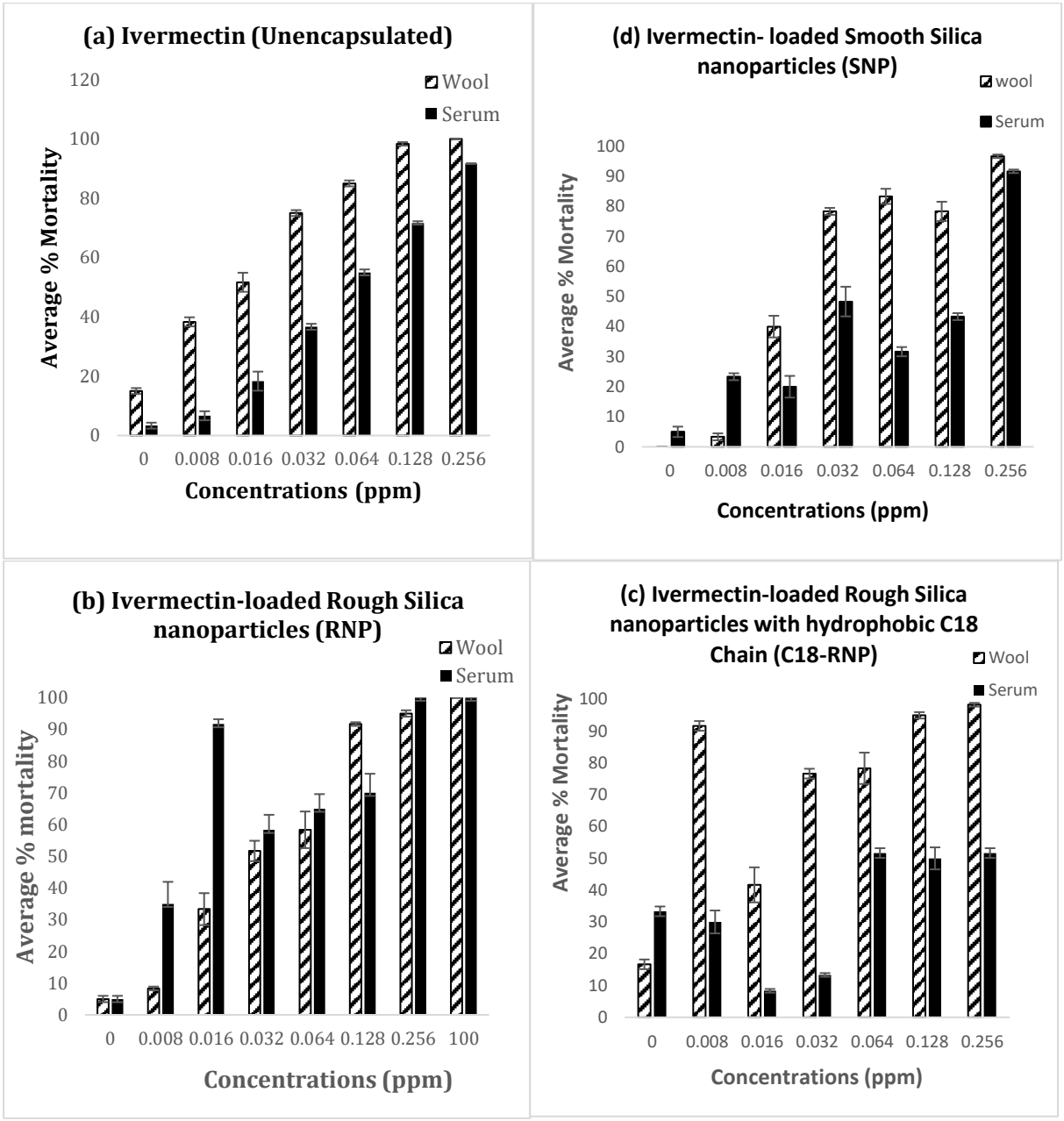


Figure 27. Relative Toxicity of un-encapsulated ivermectin (a), encapsulated ivermectin in rough silica nanoparticles (RNP) (b), rough silica nanoparticles with hydrophobic C18 chain (C18-RNP) (C), and smooth silica nanoparticles (SNP)(d) against 1st instar larvae of sheep blowfly, *Lucilia cuprina* using wool-serum assay*after 48h * Treatments applied directly on the wool (wool); treatments were diluted in sheep serum (serum)

4.5 Effect of particle size on larvicidal action

Silica nanoparticles can be 'tuned' for a number of characteristics including particle size, which can be changed by controlling the amount of resorcinol and formaldehyde added at the beginning of the fabrication process. With a small amount of resorcinol-formaldehyde (RF) precursor added, a RF-core of around 100 nm will be formed, resulting in a RNP particle size of around 180 nm, whereas, with larger amounts of RF precursor added, a larger RF-core will be formed, resulting in a RNP particle size of around 330 - 800 nm. Size of the particles is an important factor, as it potentially influences parameters such as chemical payload, distribution on, or movement across, surfaces following application and the likelihood of absorption across the host skin in the case of ectoparasiticides.

4.5.1 Effect of particle size in unweathered ivermectin particles

In preliminary tests 180 and 800 nm capsules were fabricated, loaded with 23% ivermectin and compared for toxic effect in paper and serum assays. In this study there is some indication that the larger particles were more toxic in the paper assays than the smaller ones at the 0.128 ppm concentration, but overall there was no significant difference between the two formulations (Figure 28). In the serum assays there was no clear pattern of difference. This is consistent with the 1st instar larvae accumulating greater amounts of ivermectin, at least at the higher concentrations when they 'scraped' it off the surface during feeding. The smaller particles appeared to provide a more characteristic mortality by concentration curve than the larger particles, which could result from a more even distribution of particles in the assay system than in the case of the much larger particles.

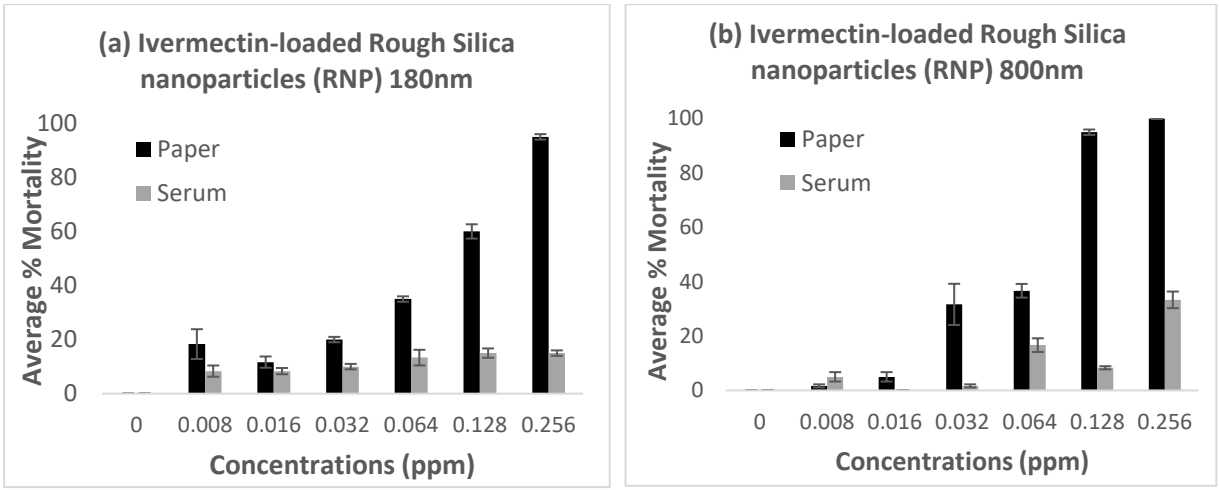
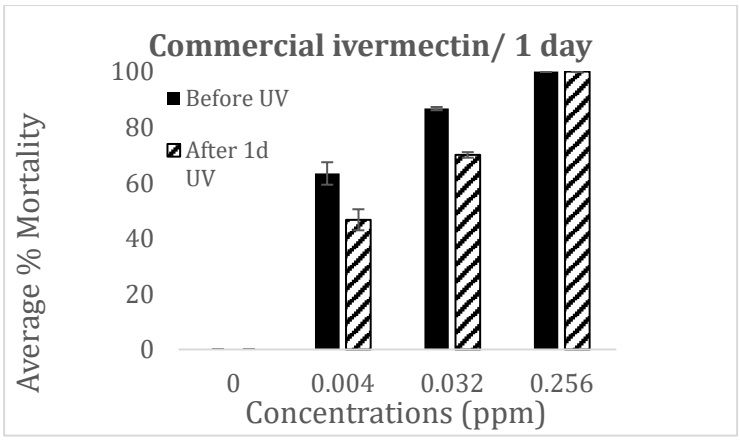


Figure 28. Relative Toxicity of encapsulated ivermectin in different size of rough silica nanoparticles (RNP): 180 nm (a) and 800 nm (b) against 1st instar larvae of sheep blowfly, *Lucilia cuprina* using Paper-serum assay* after 48 h. Treatments applied directly on the wool (wool); treatments were diluted in sheep serum (serum)

4.5.2 Effect of particle size in weathered ivermectin formulations

To examine the effects of particle size, RNP-ivermectin particles were synthesised in four different size ranges (180, 300, 500 and 800 nm) and compared for relative efficacy and persistence following 1 day, 6 weeks or 18 weeks of environmental weathering. In these studies, the different sized nanoparticle formulations generally gave longer-lived protection than the commercial (unencapsulated) formulation. There was some suggestion that the 180 nm RNP performed better than the 300, 500 and 800 RNP nanoparticles at some time points, although the differences weren't consistent. There was also some evidence that the 800 nm particles were not as good as smaller particles at some time points, but again, the results were inconsistent, and differences were usually not significant (Figures 29 to 31).



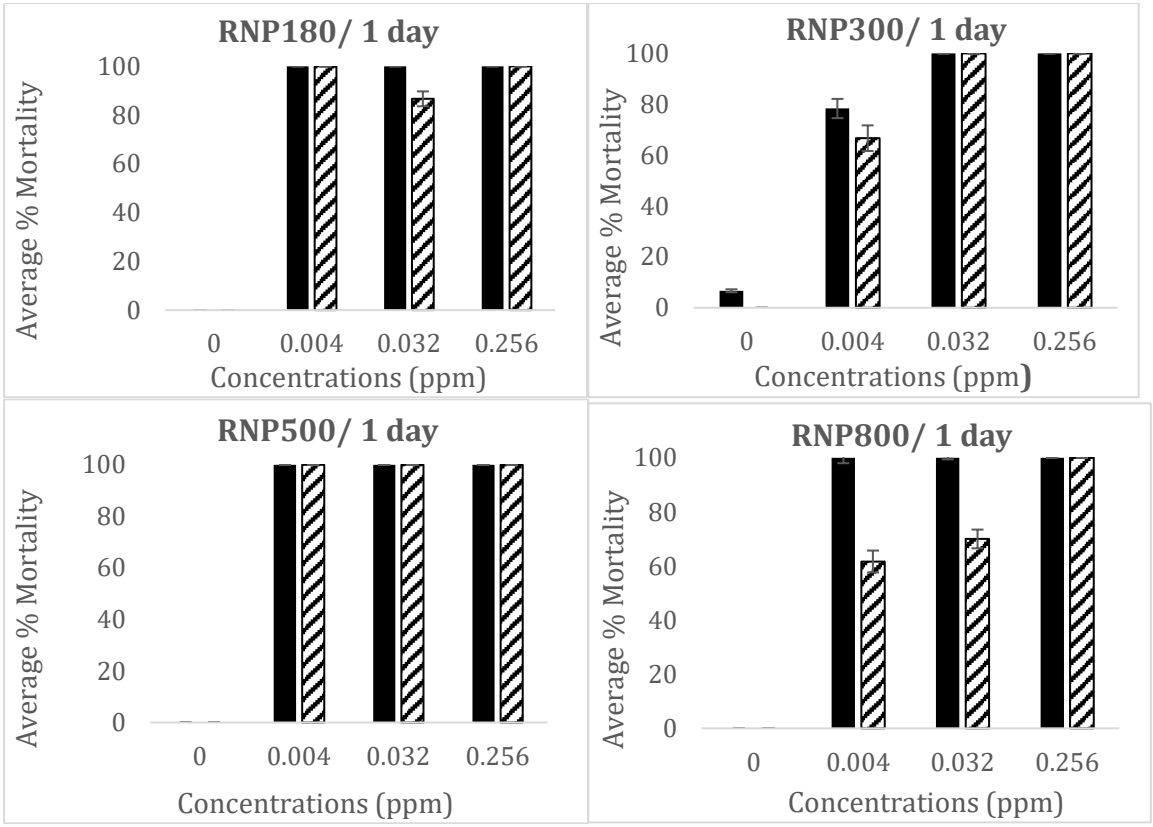


Figure 29. Larval toxicity with different size RNP particle formulations and concentrations of ivermectin following 1 day exposure to the day light ultra-violet radiation

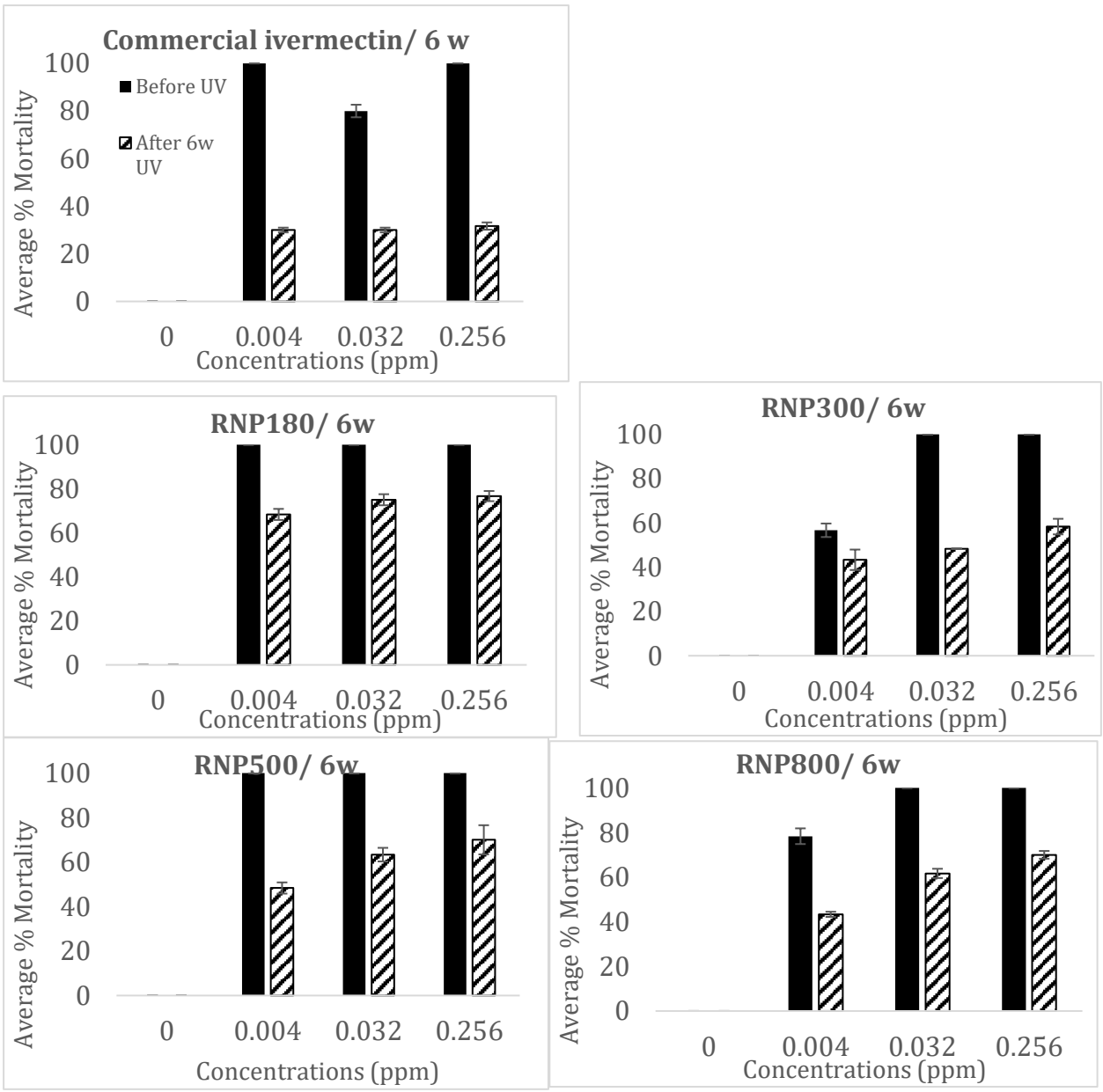


Figure 30. Larval toxicity with different size RNP particle formulations and concentrations of ivermectin following 6 weeks exposure to the day light ultra-violet radiation

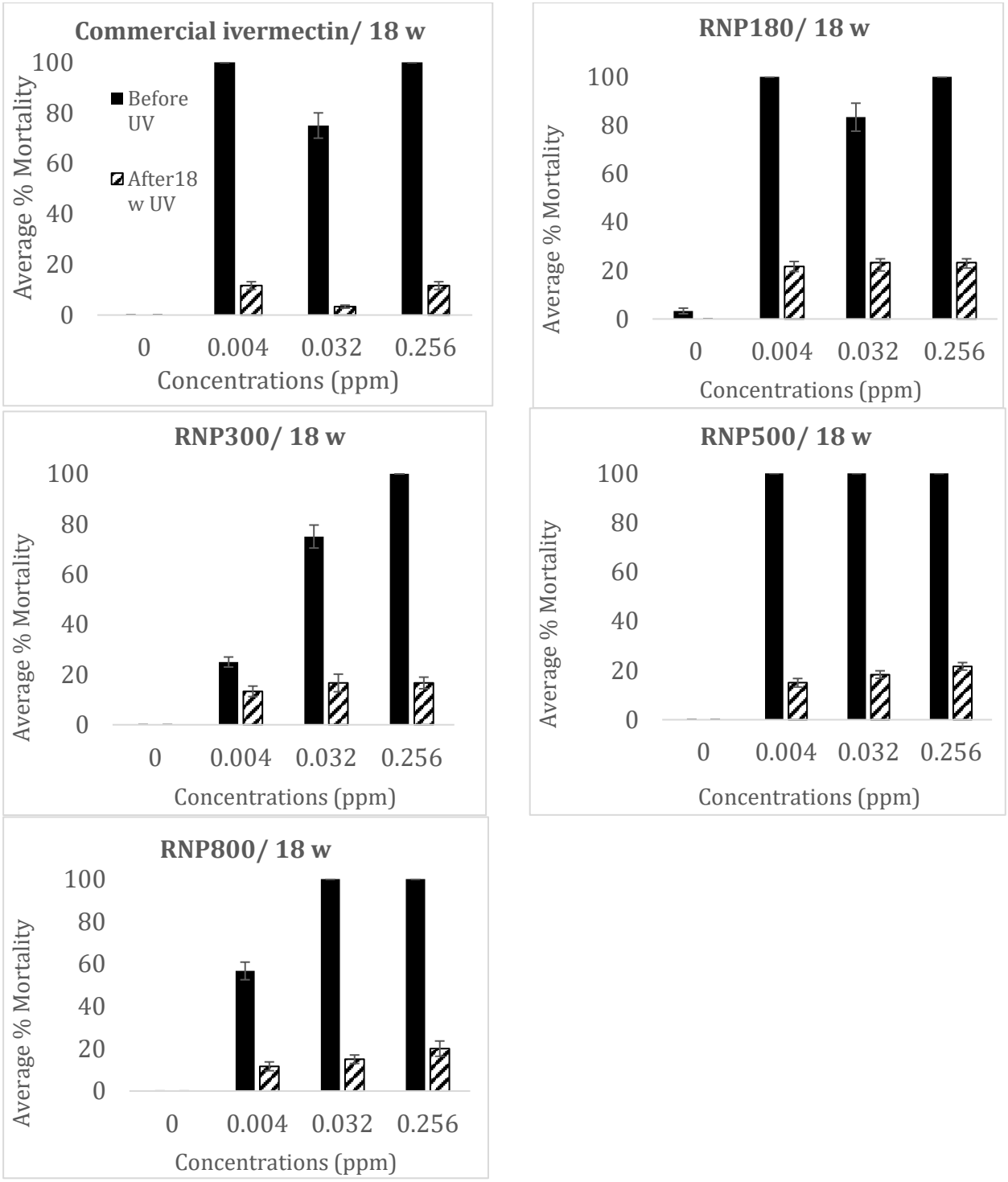


Figure 31. Larval toxicity with different size RNP particle formulations and concentrations of ivermectin following 18 weeks exposure to the day light ultra-violet radiation

4.6 Effect against other stages of sheep blowflies

4.6.1 Eggs

A number of egg dipping assays were conducted, with relatively high mortality of controls in each, even with relatively short immersion times. However, there was no significant additional mortality induced from immersion in any of the ivermectin or nanoparticle formulations, even at the highest concentration. Few insecticidal actives registered for flystrike control are known to have topical action against eggs, the benzoyl phenyl ureas, diflubenzuron and triflumuron, being notable exceptions. For most insecticides it appears that the egg chorion is an effective barrier to penetration. Particularly with larger nanoparticles,

it is unlikely that any penetration to the egg occurred. We did not investigate whether there was adherence of any of the particle types to the egg surface but even if this was so, it would seem to provide little advantage. Eggs only take 12-24h to hatch and even short lived insecticidal actives can usually persist for this period of time with potential effect against hatching larvae.

4.6.2 Third instar larvae

Only a short time of immersion was used in these assays to minimise the likelihood of any significant oral ingestion. The results show that at the concentrations tested, even Paramax was not very effective in killing late-stage blowfly larvae. The difficulty of killing late-stage larvae even with extended immersion times has been previously indicated (Levot et al. 1999) so the low mortality even with the commercial product was not unexpected. However, the almost complete lack of activity of the RNP capsules in this assay may suggest low topical effect and similar low mortality was observed with nanoparticle formulations in other larval dipping tests (data not shown). However, in the practical situation, longer exposure times may lead to much greater cuticular adherence and significant ingestion of particles by third stage larvae would likely increase levels of mortality, particularly if the particles are adherent in the gut as suggested by the fluorescent labelling studies discussed earlier.

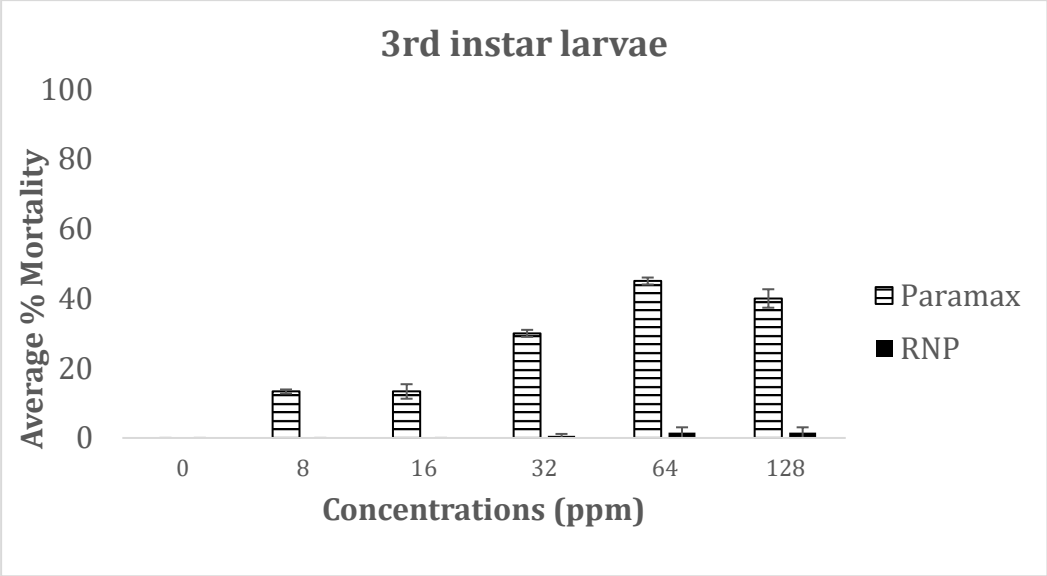


Figure 32. Mortality from immersion of 3rd instar larvae in different concentrations of commercially formulated ivermectin (Paramax®) or administered in water dispersion of rough nanoparticles

4.6.3 Adult L. cuprina

Topical toxicity was assessed against adult flies using micro-dosing onto the thorax and abdomen, but it was not possible to get sufficient retention without use of a solvent, which could compromise the encapsulation of ivermectin. Therefore, adult flies were immersed in water dispersions of RSNP for 60 s as for the L3 larvae. The results are given in Figure 33. No studies were undertaken to test adherence of the two formulations to the flies' cuticle, but it is possible that the level of mortality could increase with further time as the flies ingest adherent particles during fly 'grooming' behaviour.

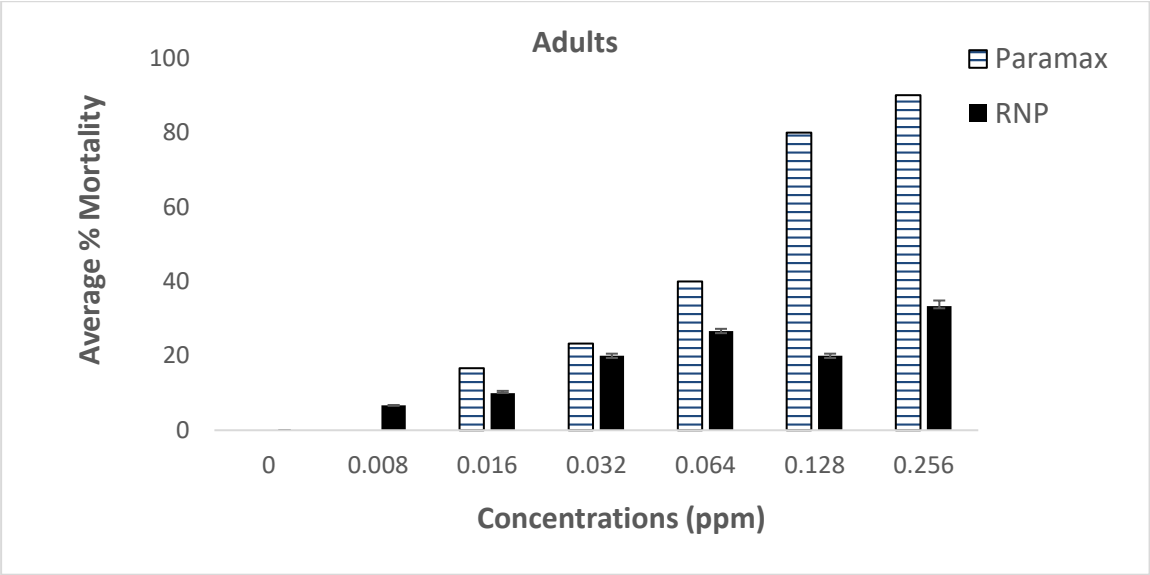


Figure 33. Effect of a commercial (Paramax ®) and RNP formulation of ivermectin following immersion of adult flies in different concentrations for one minute. Mortality assessed at 48h

4.7 Effectiveness of cyromazine particles

4.7.1 Range finding studies with cyromazine formulations

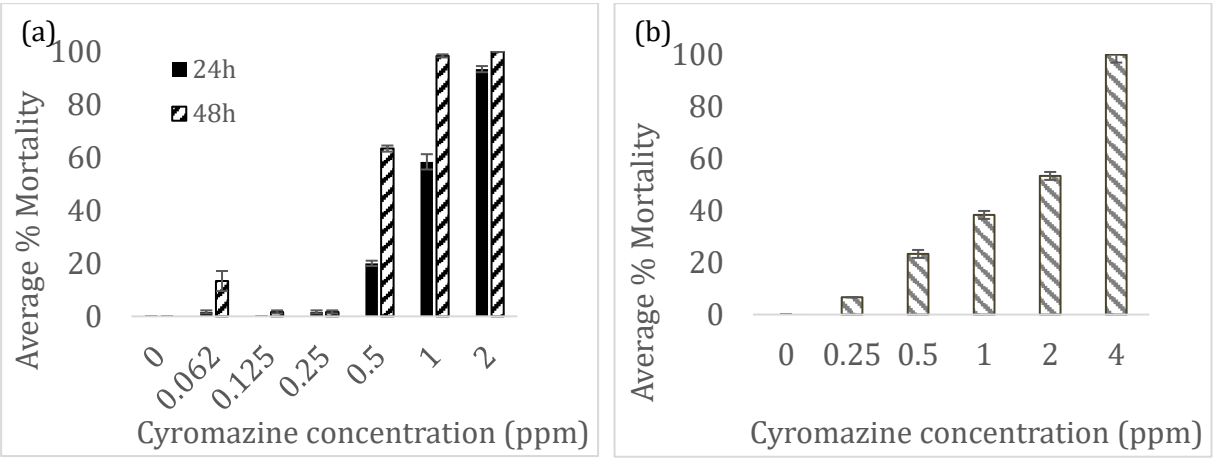


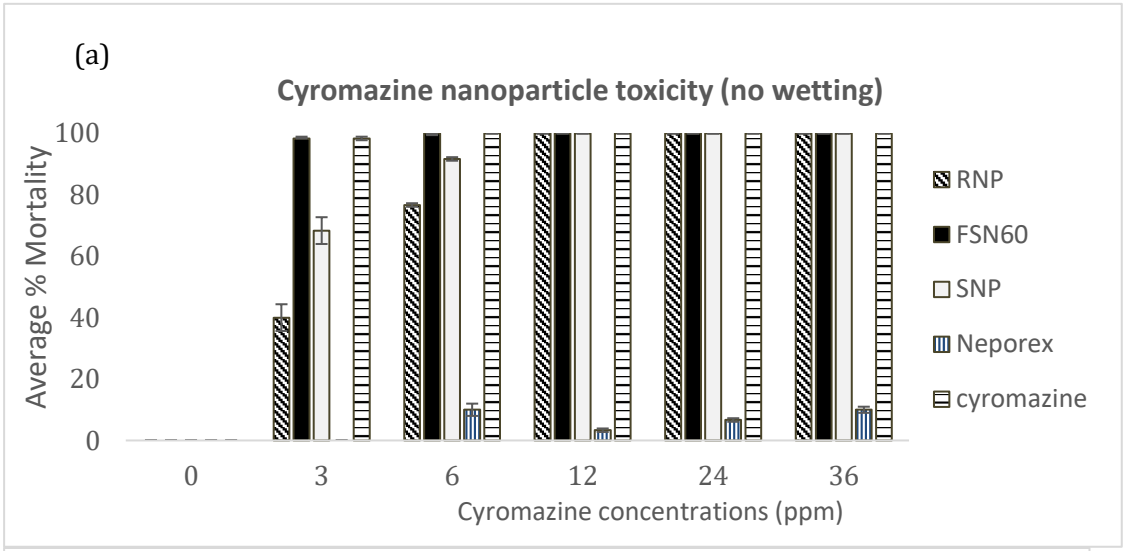
Figure 34. Range finding assays with: (a) Technical cyromazine applied to chromatography paper (24 and 48 h mortalities) and (b) Wool treated with a commercial flystrike formulation (Venus®, 48h)

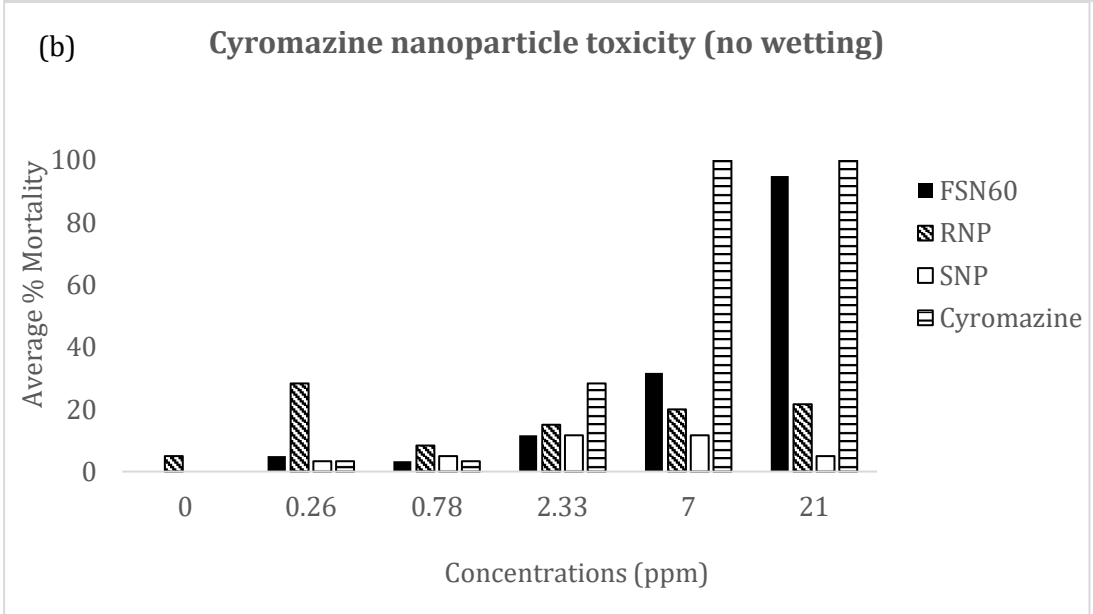
Results of the range finding assays with unformulated cyromazine are presented and indicate two features of studies with cyromazine formulations. Cyromazine has a different mode of action compared to neurotoxins such as ivermectin and acts to kill larvae only when they moult between stages. A side effect is that it often also extends the length of the larval stages so that live larvae may still be seen for an extended period, but they may be stunted in comparison to comparably aged untreated larvae or sometimes show cuticular malformations. The slower kill with cyromazine can be seen in Figure 34(a) where mortality of larvae is much higher at 48h than 24h. Some larvae were alive at the higher concentration at 48h but all of these were considerably smaller than in the control groups, and these larvae eventually die without moulting. As an example, with the commercial formulation at 4ppm (Figure 34b) 72% of the larvae were still alive at 48h. However, all of the surviving larvae were severely stunted, not feeding and would have later died. This effect could be further exacerbated with the nanoparticle formulations where, because of the slow-release effect of the nanoparticles it may take longer for larvae to accumulate a toxic dose.

This difference in initial toxicity is indicated in Figure 35, which shows different range finding assays run to assess the relative toxicity of nanoparticle and unformulated cyromazine. In these assays, toxicity of conventionally formulated and aqueous formulations of cyromazine was often equivalent to or higher than the nanoparticle formulations. Part of the rationale for encapsulation is to protect against degradation or leaching, it is not unexpected that the nanoformulations show little advantage over unencapsulated cyromazine in the absence of environmental exposure.

Neporex® a wettable granular formulation of cyromazine for controlling houseflies in poultry manure that can be formulated in water for use as a spray was used in the first assay (Figure 35a) as we did not have a commercial flystrike formulation available at the time this assay was conducted. However, this product was clearly not suitable for use on wool and gave very low toxicities at all concentrations, perhaps underlining the importance of appropriate formulation to insecticidal effectiveness.

Earlier studies in this project suggested that particles accumulate in the gut of larvae and that an oral toxicity route is likely to be the major means of chemical absorption. In this situation it may take a longer period for accumulation and release of toxic amounts of chemical compared to compounds and formulations where transcuticular absorption plays a larger role. This together with the mode of action of the chemical may be manifest as initially lower toxicity.





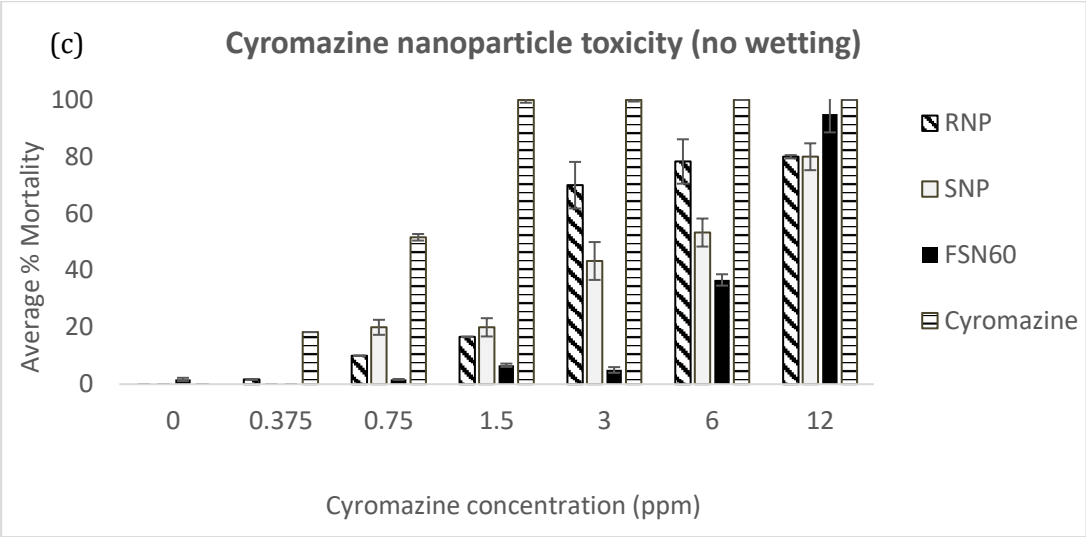
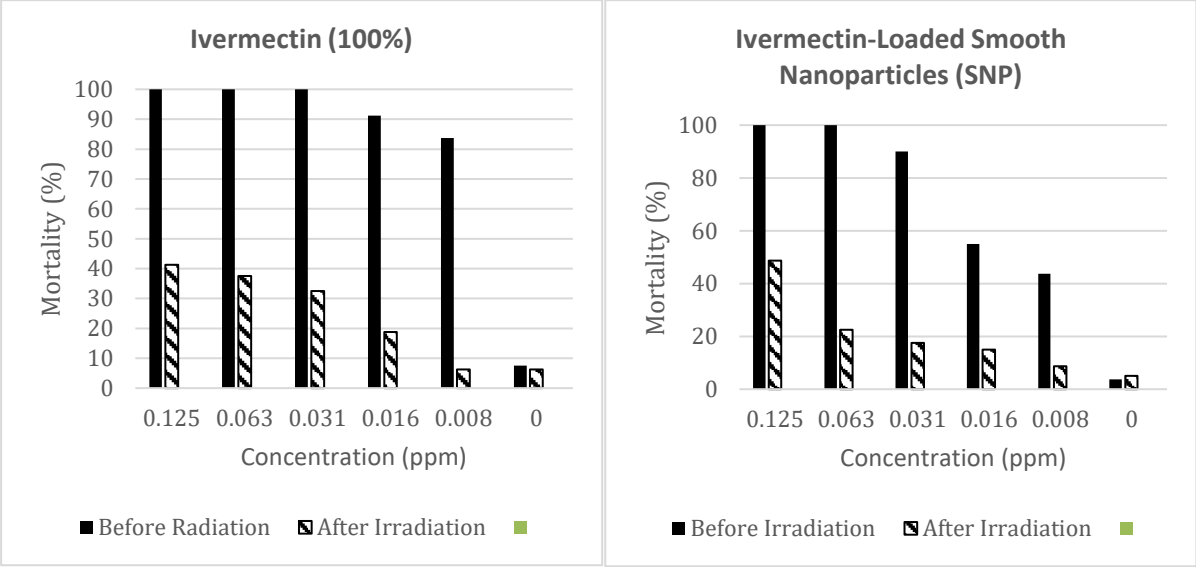


Figure 35. Range finding assays conducted with *L. cuprina* in wool assays showing relative toxicity in the absence of weathering

4.8 Effectiveness following artificial weathering – ivermectin particles

4.8.1 Ultra-violet exposure

There was a clear reduction in larvicidal efficacy following the exposure to UV weathering with both unformulated ivermectin and ivermectin formulated in smooth silica nanoparticles, whereas there was no reduction in efficacy with the RNP or RNP-C18 formulations of ivermectin (Figure 36). These results suggested that there may actually have been an increase in effectiveness with the two RNP formulations following irradiation. Whether this was an experimental artefact, or a real effect is uncertain. However, there was indication of a similar effect in some other studies and the possibility that this was a real effect may warrant further investigation.



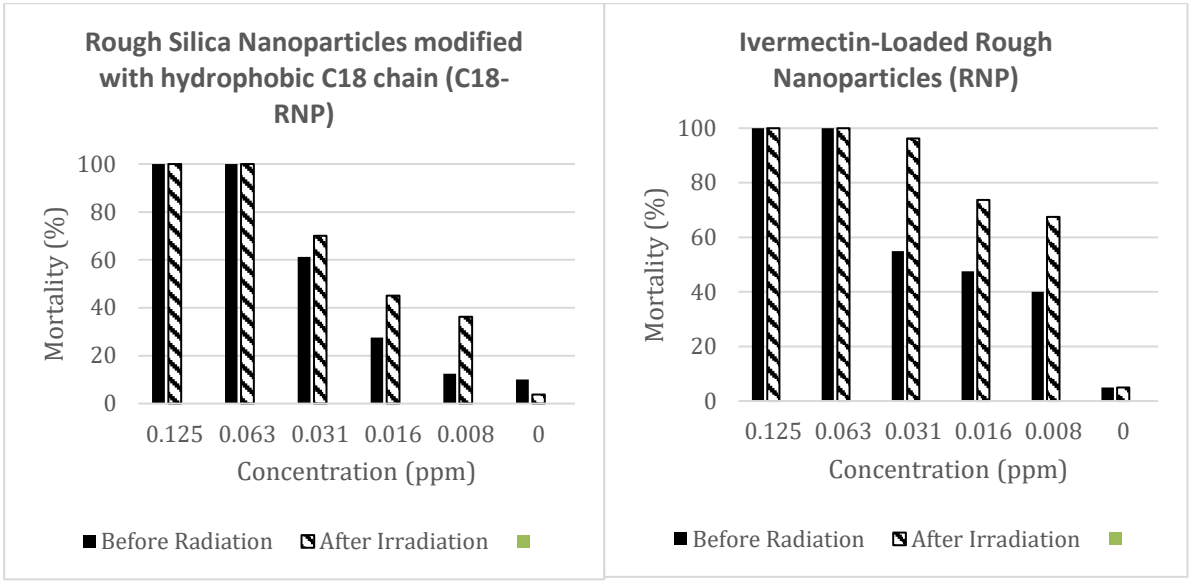


Figure 36. Mortality induced by formulations of ivermectin (a) unencapsulated ivermectin; (b) smooth nanoparticles; (c) rough nanoparticles; and (d) C18 rough particles before (solid bars) and after (striped bars) after artificial UV weathering

3.7.2 *Artificial wetting*

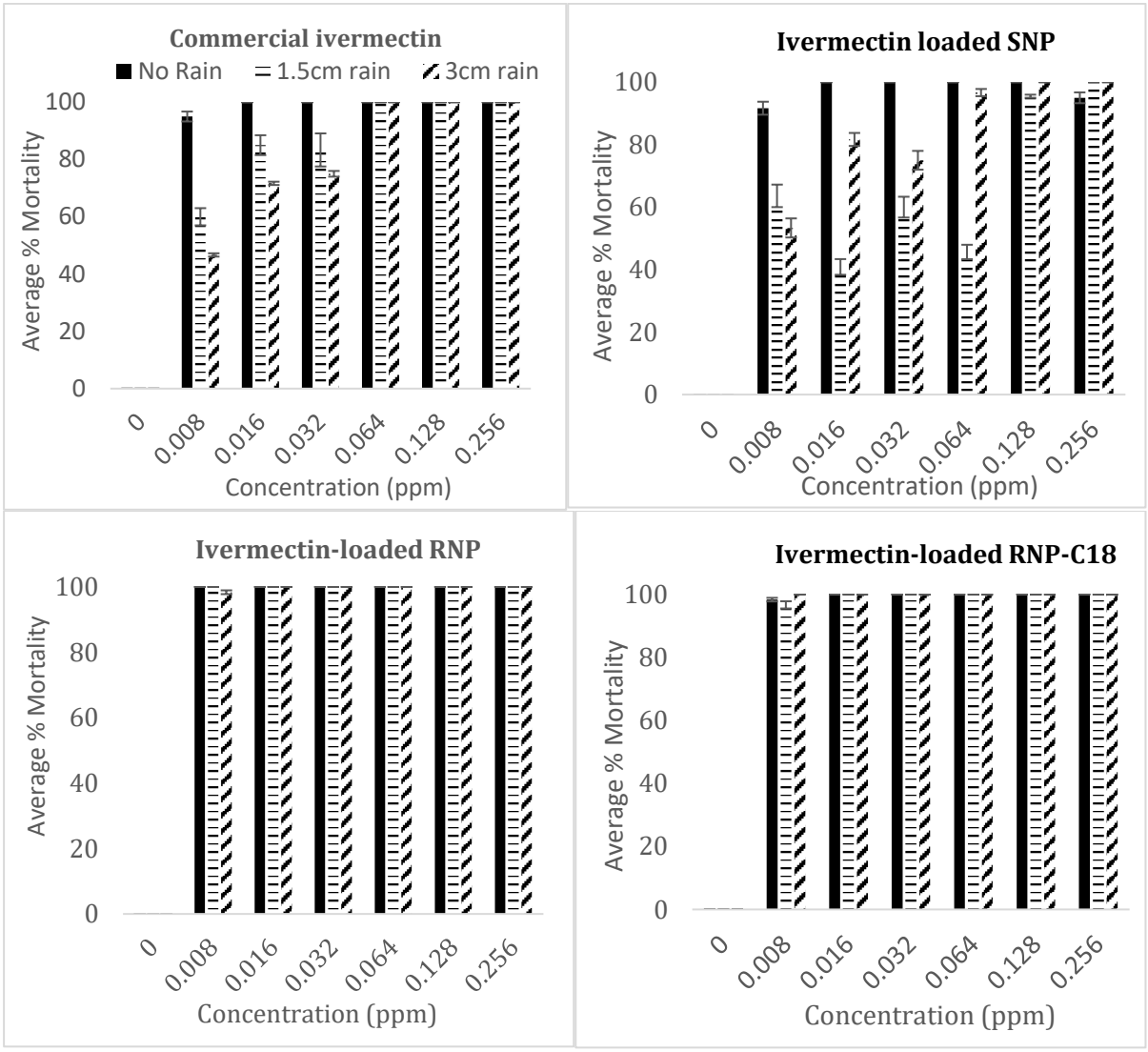


Figure 37. Larval toxicity with different formulations and concentrations of ivermectin following artificial wetting tests

When the larvicidal efficacy of RNP and RNP-C18 nanoparticle formulations of ivermectin were compared with the SNP formulation and commercial formulation following the application of 1.5 and 3.0 cm of artificial rain, there was a clear advantage for the RNP formulations. With both the commercial formulation and smooth nanoparticle formulations both rainfall amounts gave a significant reduction in protection, as compared to wool with no water applied at 0.0008, 0.016 and 0.032 ppm concentrations. With the SNP formulation there was also a significant reduction at 0.064 ppm with 1.5cm 'rain' though not with 3cm. The RNP and C18 formulations provided superior protection and no reduction in efficacy was seen with either the RNP or C18 formulations of ivermectin at either rainfall rate at any of the treatment concentrations applied.

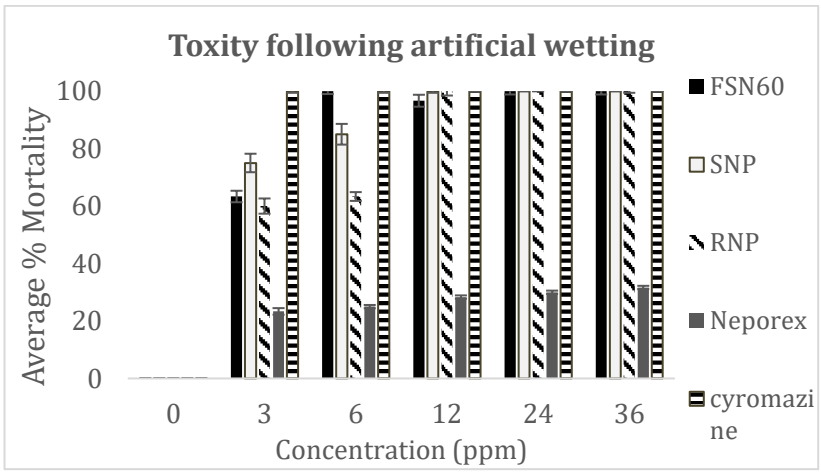
4.8 Effectiveness following weathering – cyromazine particles

4.8.1 Rainfastness

As cyromazine is water soluble and has been reported to be leached from the fleece by rainfall, a number of tests using different methods were conducted to test the effectiveness of nanoencapsulation with different particles and the persistence of larvicidal efficacy. In initial tests treated samples were immersed in water for 60 s, blotted in paper towelling and then left to air dry. In the first of the larval assays (Figure 38a) three candidate nanoparticle formulations (RNP, FSN60 and SNP) applied at a range of

concentrations were compared with technical cyromazine solubilised in water. In the second assays (Figure 38b), the same nanoparticle formulations were re-tested, but at a slightly lower range of concentrations. In the first assay, the concentration range tested was too high for good indication of the relative effectiveness of the different formulations with the three highest nanoparticle concentrations giving 100% mortality, or close to it with all four formulations tested. Technical cyromazine concentration gave 100% kill at all of the concentrations tested and likely reasons why the technical formulation gave such good effect in this assay system have been previously discussed. Neporex® is a cyromazine product formulated for house fly control in poultry manure, not for application to sheep and the poor effects noted with this product underline the importance that appropriate formulation can play in determining the effectiveness of different active ingredients.

In the second assay, at lower concentration (Figure 38b) there was clearer differentiation between the different nanoparticle treatments with the formulations with 'whiskers' giving better effect after wetting than the SNP formulation. Similar differences between the RNP and SNP formulations were seen in previously reported assays using ivermectin as the active ingredient.



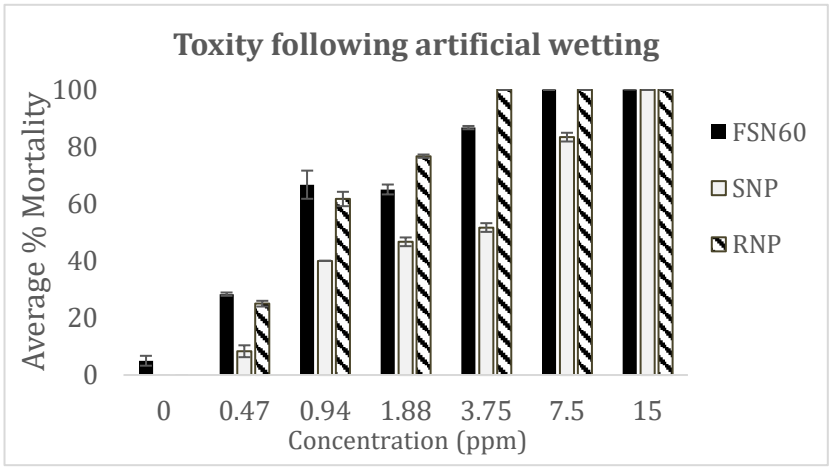


Figure 38. Larval toxicity with different formulations and concentrations of cyromazine following immersion in water to test the retention after wetting

The results for a more comprehensive range of formulations comparing efficacy before and after wetting (treated wool dipped in water, then shaken and blotted dry as above) are shown in Figure 39 and give results expected on the basis of previous studies with ivermectin.

At 25 ppm, whereas larval mortality dropped significantly after wetting from 100% prior to wetting by nearly 50% in the commercial formulation-treated wool and nearly 60% in the SNP-treated wool, for the FSN hydrophobic and RNP formulations, mortality remained at 100% and for FSN efficacy was reduced by

only 23% (Figure 39). At concentration of 3.125 ppm the results were not as clear without much difference between the five formulations and a significant drop in efficacy for all of the formulations. It should be noted that this result may have been partly an artefact of using a bioassay rather than direct measurement of cyromazine content to assess the differences in change in effect caused by wetting. The efficacy of the 3.125 ppm treatments was marginal before wetting and only a small change in concentration would have rendered this treatment ineffective. Most of the formulations having 80% efficacy or less prior to wetting, reduced to less than 20% after wetting. Clearer results may have been achieved if concentrations intermediate between 3.125 and 25 ppm had been included.

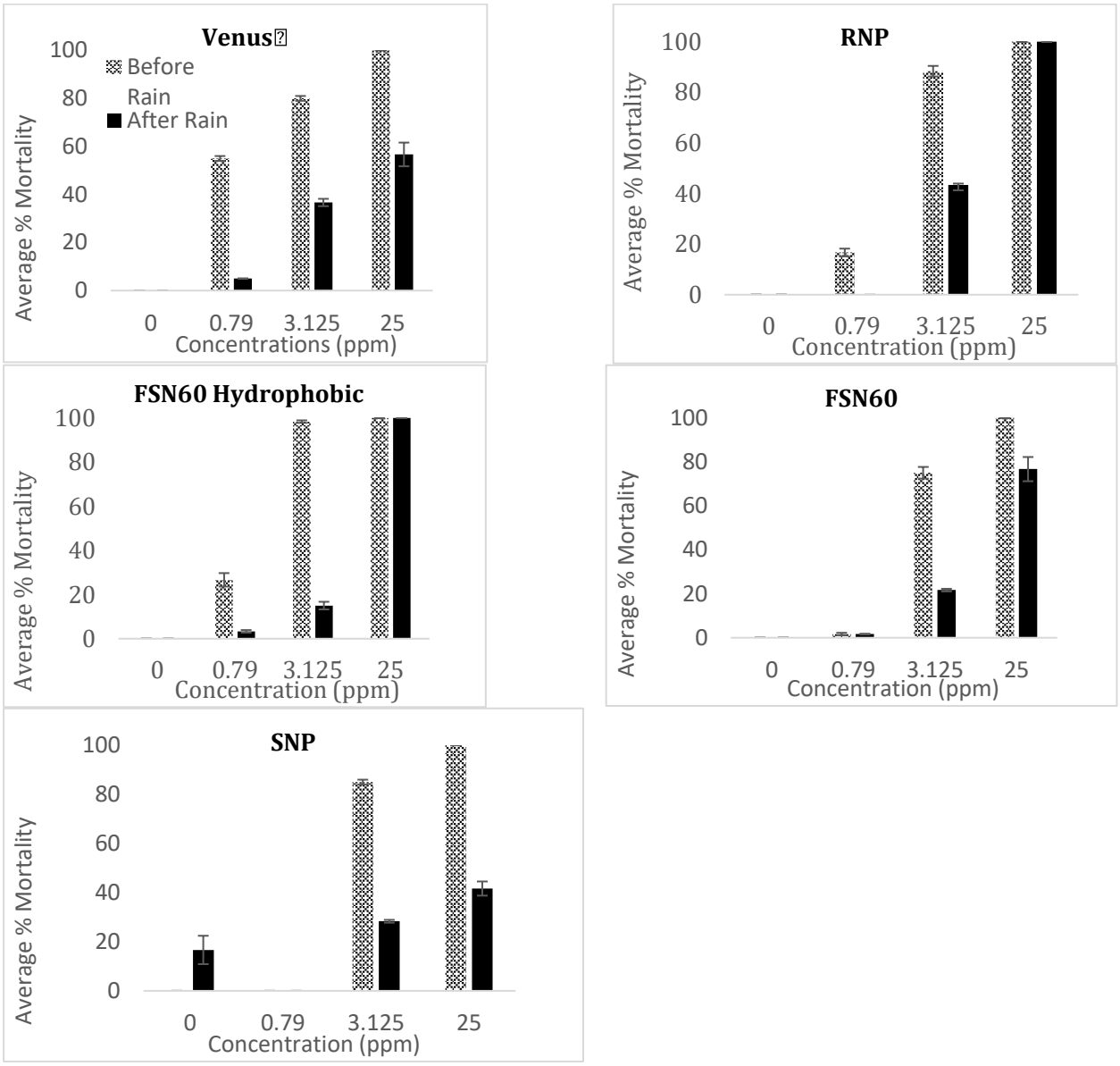


Figure 39. Mortality of *L. cuprina* larvae treated with different concentrations of cyromazine formulated in different designs of nanoparticles and with a commercial flystrike formulation (Venus®) before and after immersing the treated wool in water for 60sec

As a result, a second similar study was conducted with two of the formulations that performed best in the earlier studies (RNP and FSN60) and with a concentration of 7 and 14 ppm cyromazine included. 'Artificial rainfall' (6cm) was applied holding the wool samples on a gauze frame over a sink, spraying them with the calculated amounts from a hand-held wash bottle with a spray head on two occasions and allowing them to dry in a fume hood before testing. Approximately 6cm of artificial rainfall was applied on each occasion. A wool sample with no water applied, but otherwise treated similarly, was included as a control. The results are shown in Figure 40.

Wetting reduced efficacy of the commercial cyromazine formulation to between 27% and 68% whereas the FN60 treatment gave close to 100% effectiveness in both the wetted and unwetted treatments at all of the 3.125, 7, 14 and 25ppm cyromazine concentrations. A similar result was seen with the RNP at the 3 highest concentration, but effectiveness was lower than 50% at the 3.125 ppm concentration in both the wetted and unwetted treatments.

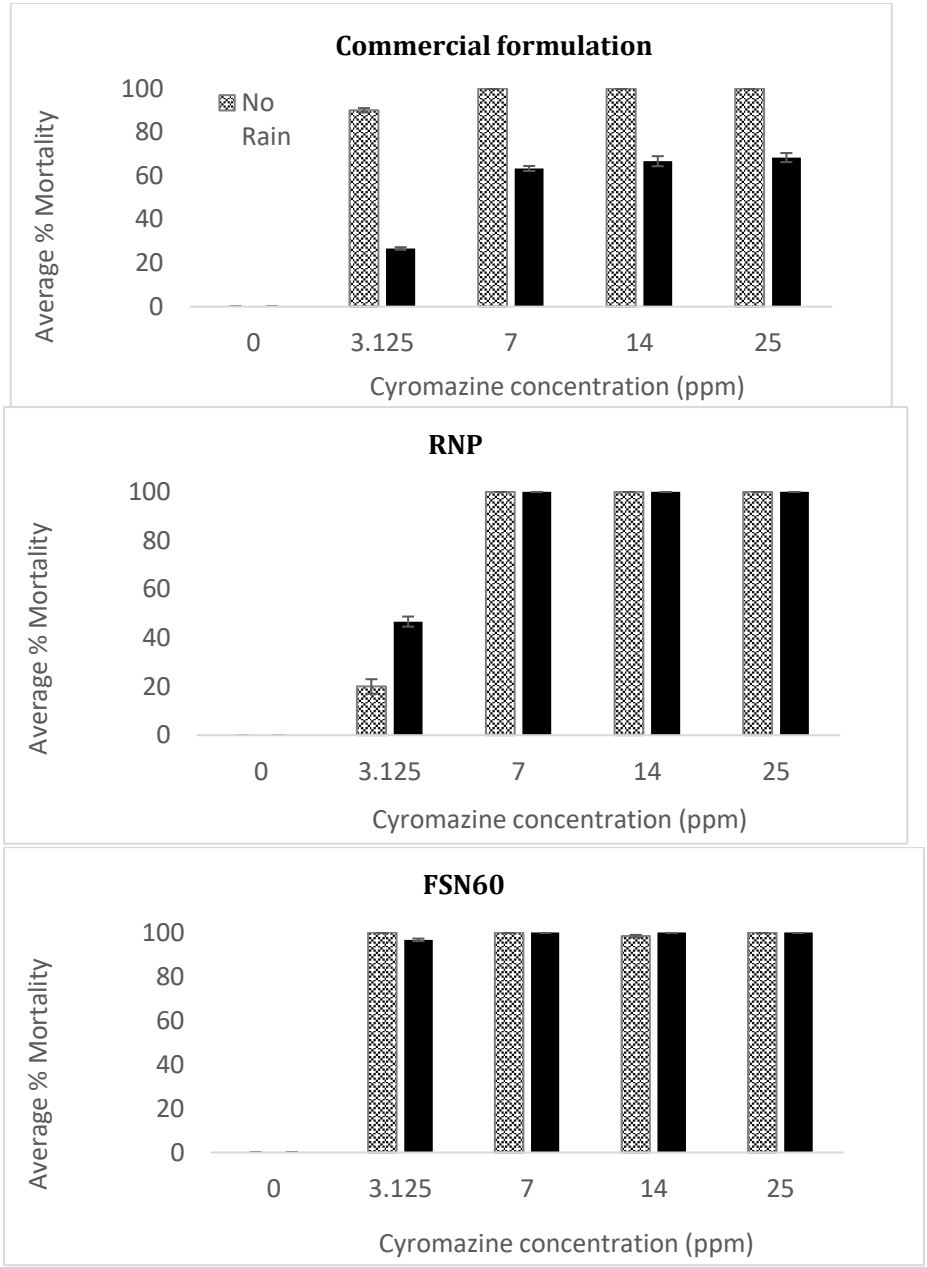


Figure 40. Larval toxicity in assays for rain fastness with wool treated with different formulations of cyromazine then exposed to simulated rainfall on two occasions

4.8.2 Photostability

Photostability was assessed by bioassay with *L.cuprina* on treated wool artificially weathered (exposed to high UV flux in a UV shielded cabinet) according to the previously described protocol. The UV exposure regime led to a significant reduction in the larvicidal efficacy of the commercial cyromazine formulation (Venus®) at all four cyromazine concentrations (Figure 41). Formulation in the FSN-hydrophobic and RNP particles, and to a lesser degree the SNP particles protected against photo degradation. There appeared

to be some reduction in efficiency with the FSN-60 formulation following UV exposure, although not to the same degree as with the commercial product.

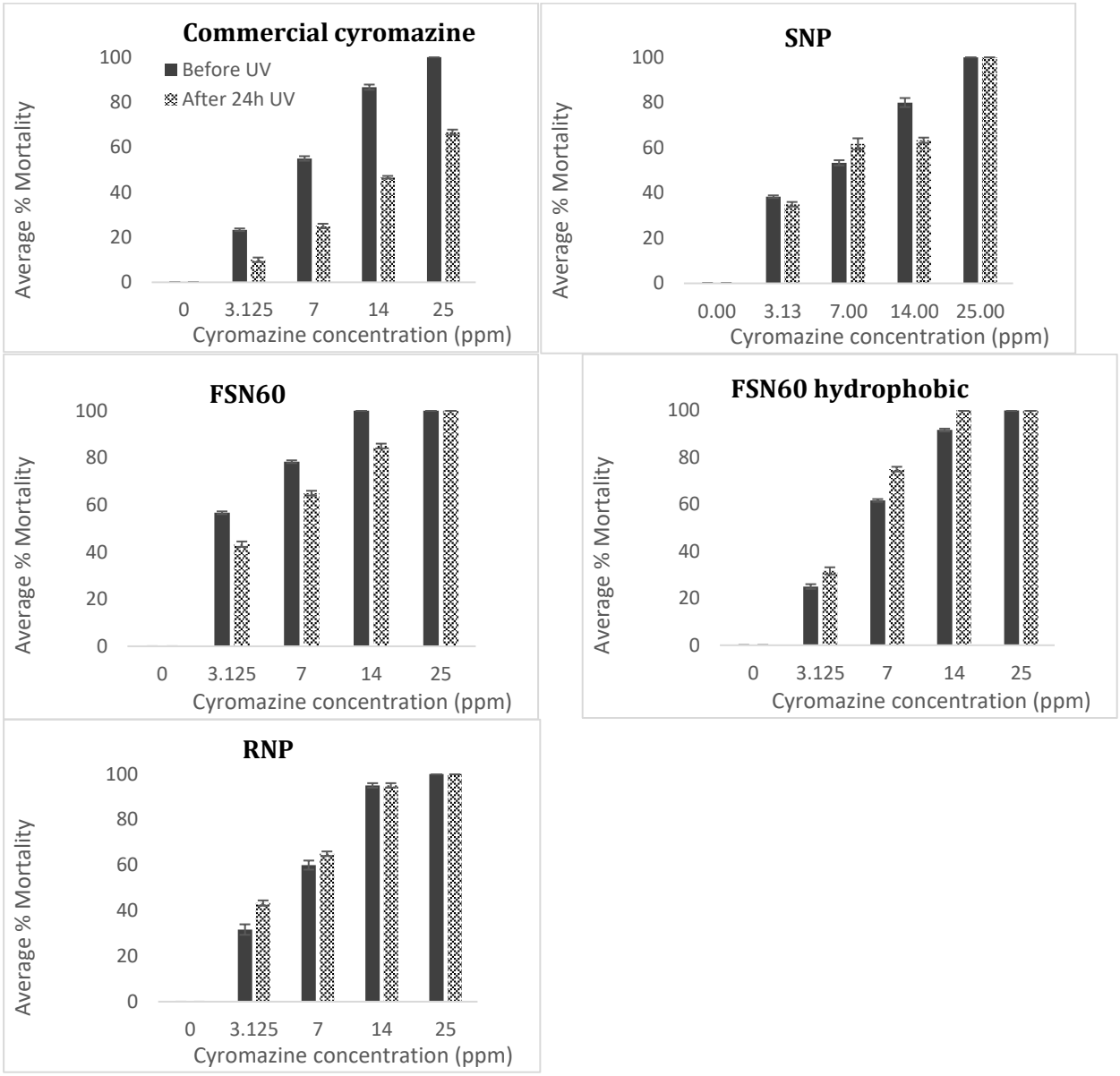


Figure 41. Effect of 24 h exposure to ultra-violet radiation on efficacy of nanoparticle and commercial formulations of cyromazine in larval assays

With the FSN60 hydrophobic and RNP particles there was a slight increase in efficiency at the 3 and 7 ppm concentrations, and with the FSN formulation at 14ppm. These increases were small, non-significant and would normally be attributed to experimental variability. However, a similar effect was also seen in a preliminary weathering study (Figure 42). In this assay there were quite large increases in larvicidal effect following UV irradiation at the lower cyromazine concentrations with the FN60, FN60-hydrophobic and RNP particles, though not with the SNP particles or with the Venus® formulation. Whether or not this effect is real or an experimental aberration is unclear at this stage. More notably, the FSN60, FN60 hydrophobic and the RNP particles all appeared to give protection against photo-degradation, whereas degradation was evident with both the commercial and SNP formulations, particularly at the lower concentrations.

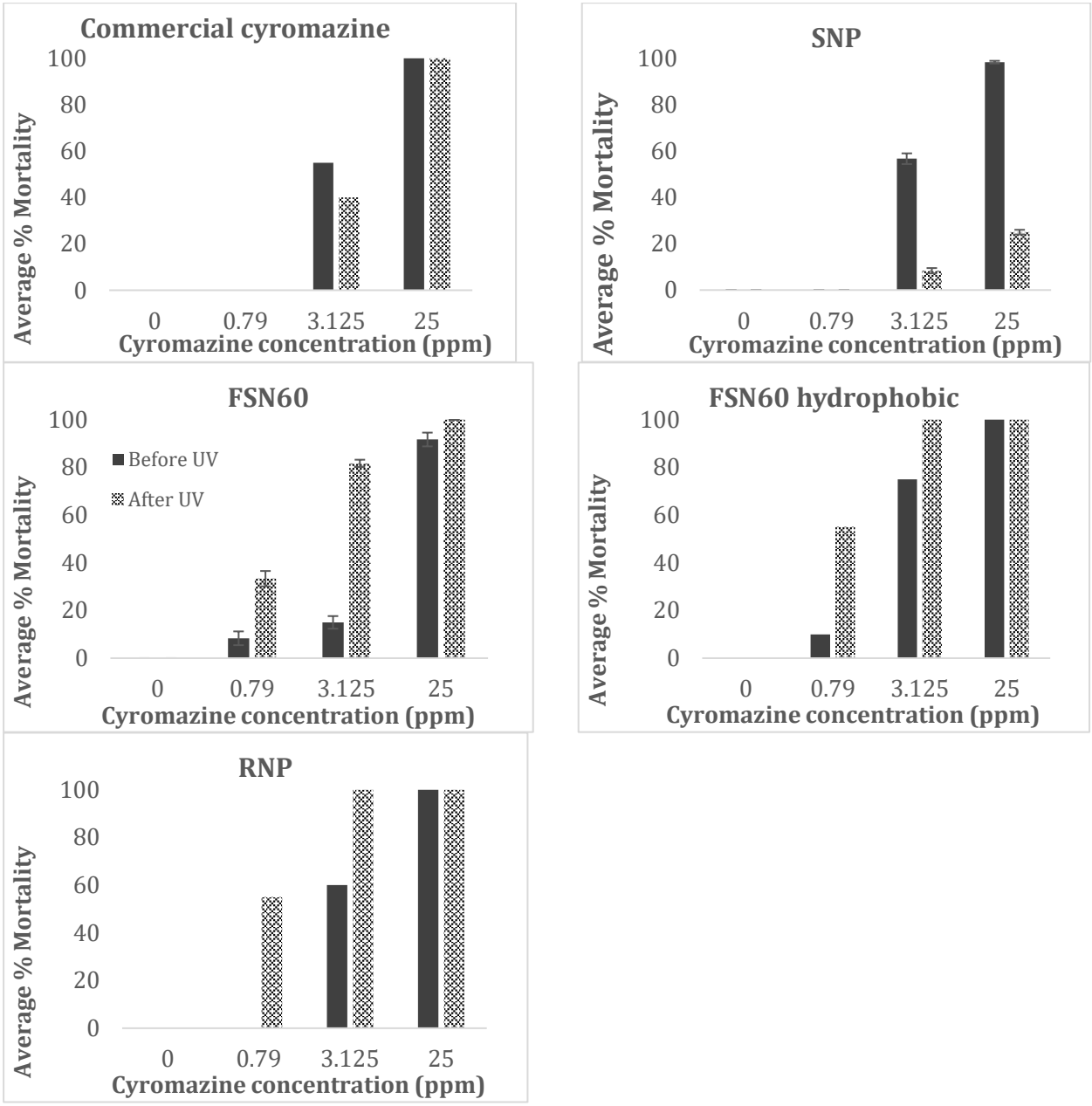


Figure 42. Mortality of *L. cuprina* larvae exposed to wool treated with different concentrations of cyromazine nanoparticles and a commercial formulation (Venus®) before and after 24 h exposure of the wool to artificial UV

4.8 Efficacy of Nanoparticles and after environmental weathering

4.8.1 Ivermectin particles

In the first of the extended environmental weathering trials with the SNP, RNP and RNP-C18 ivermectin particles there was only limited reduction in efficacy seen with all of the formulations after one day of exposure, with no significant differences between treatments (Figure 43). At 6w the protection provided by all had declined significantly, but all nanoparticle types were significantly better than the commercial formulation with the RNP and RNP-C formulations, providing approximately twice the level of protection provided by the commercial treatment. However, there was no significant difference between the three nanoparticle types (Figure 44). By 18w protection had been pretty much lost with all treatments. However, although the protection was low, the RNP-C18 was still providing significantly better protection than all of the other nanoparticle types and the commercial formulation with approximately 30% larval mortality at all concentrations ($P < 0.001$) (Figure 45). No rain was received on the first day of exposure and a cumulative amount of only 2 mm had been received up until 6w suggesting that the majority of

breakdown during these periods was due to photo-degradation or some other factor causing degradation. However, 130 mm was received in the last 12 weeks, which could have been important in contributing to the low effectiveness of all of the formulations at 18 weeks.

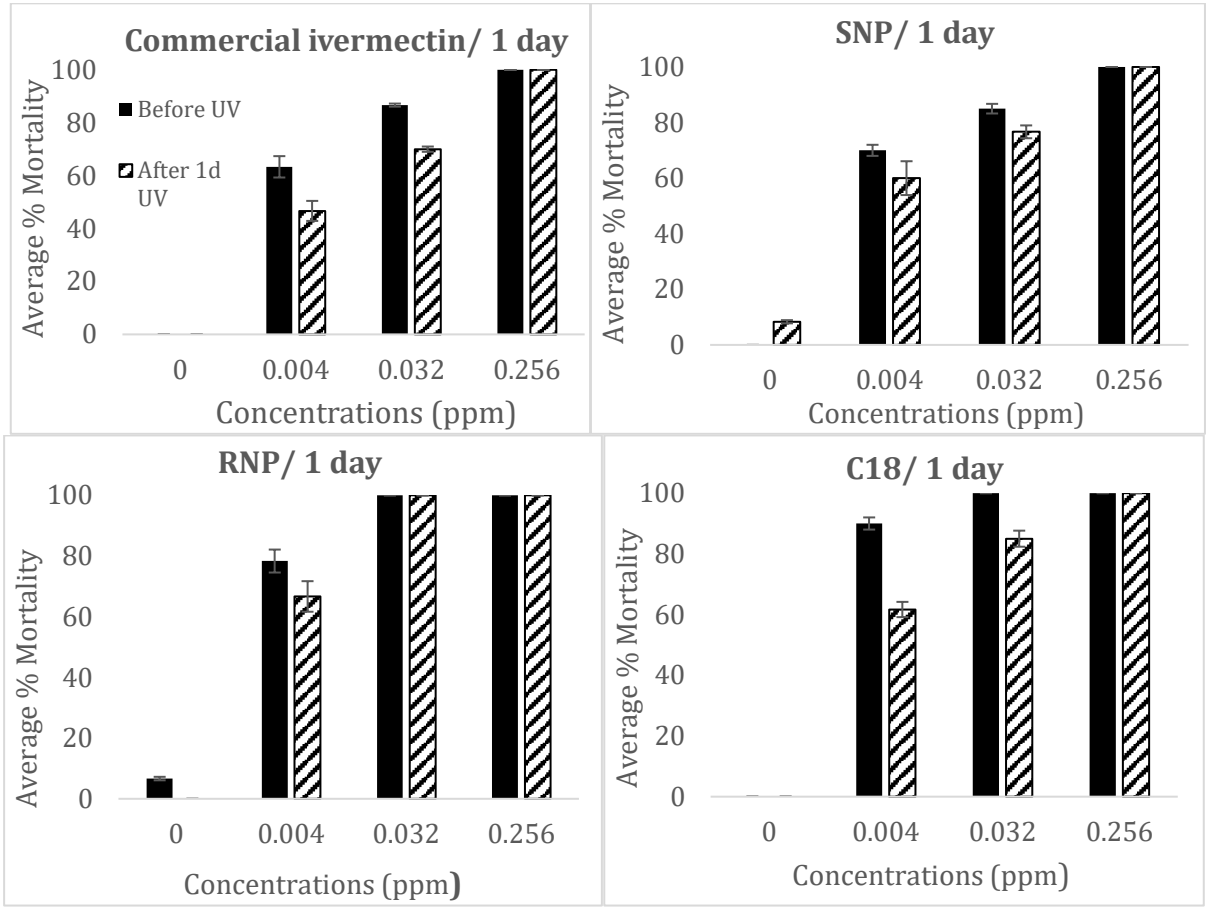


Figure 43. Larval toxicity with different formulations and concentrations of ivermectin following 24 hour exposure to the day light ultra-violet radiation

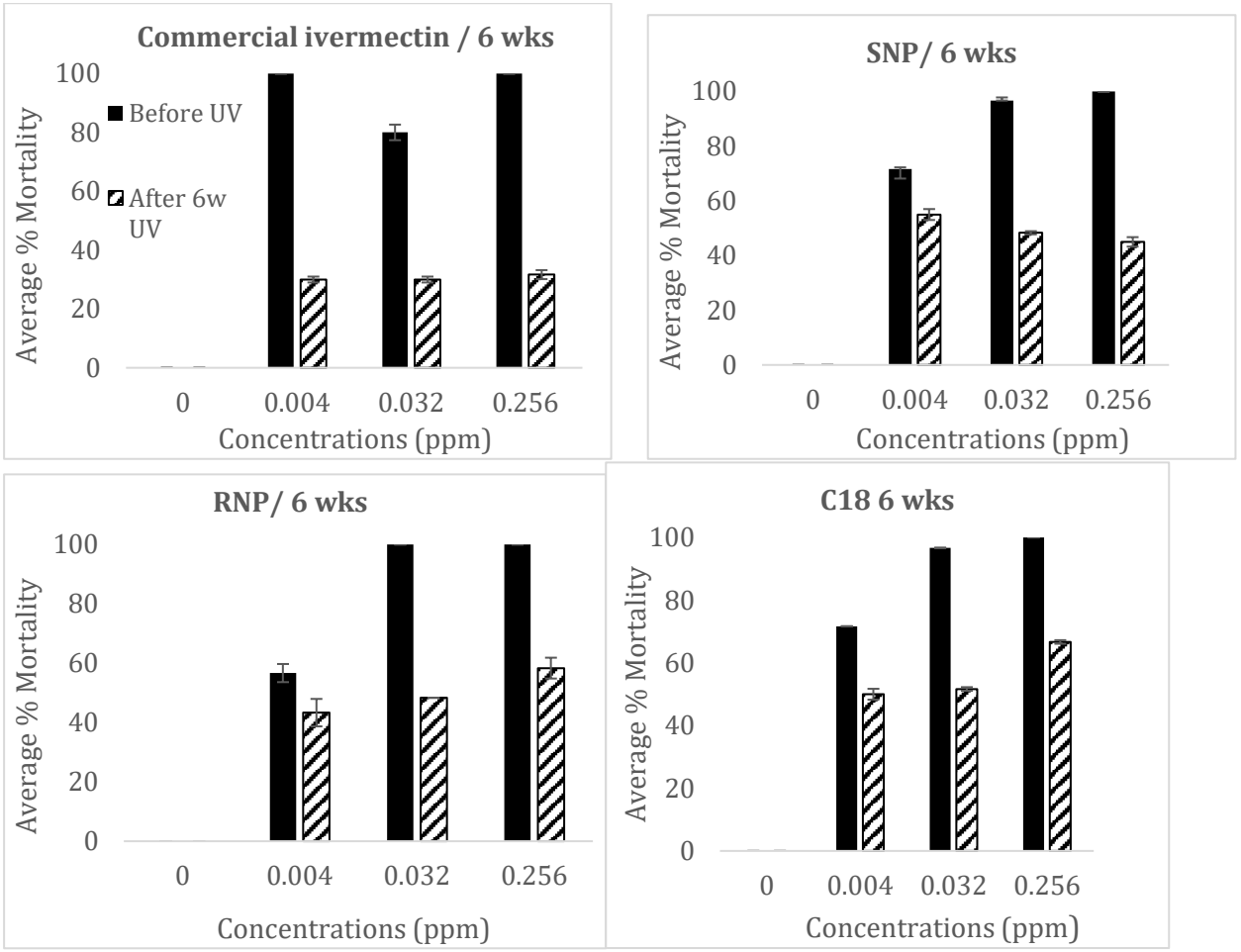


Figure 44. Larval toxicity with different formulations and concentrations of ivermectin following 6 weeks exposure to the day light ultra-violet radiation

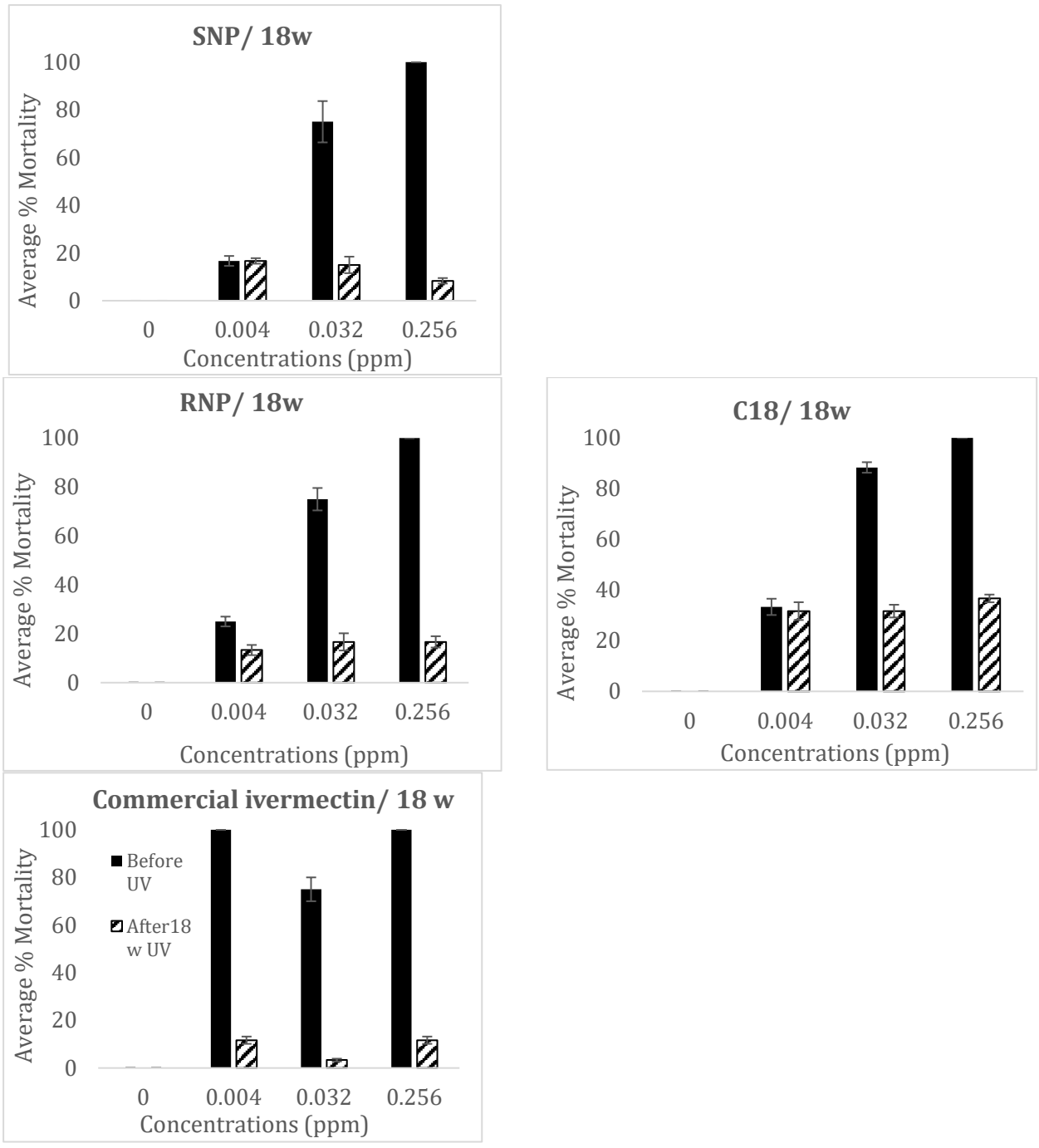


Figure 45. Larval toxicity with different formulations and concentrations of ivermectin following 18 weeks exposure to the day light ultra-violet radiation

4.8.2 Cyromazine particles

The influence of environmental weathering on efficacy of different nano-formulations of cyromazine was evaluated in a number of experiments over 21 weeks of exposure. In an initial experiment in which wool samples were exposed for 7 days and assessed at 24 hour and 1 week (Figure 46, 47), a clear reduction in efficacy was seen with the commercial formulation at all concentrations at 24h but there was little reduction apparent with the SNP, RNP or FSN60 hydrophobic nano-formulations (Figure 46).

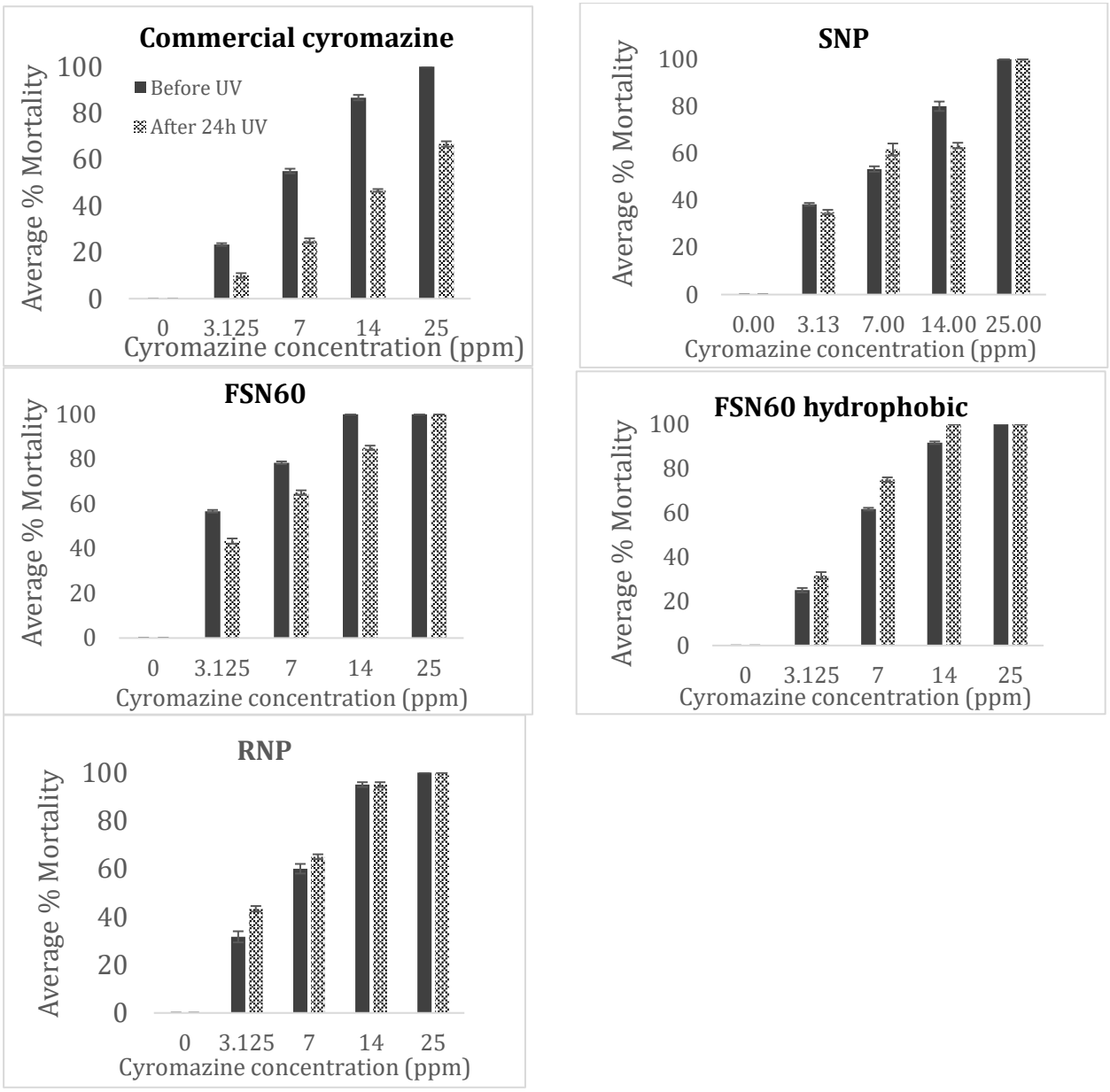


Figure 46. Mortality of *L. cuprina* larvae in assays with wool samples treated with nanoparticle formulations or a commercial cyromazine formulation and then exposed to environmental solar radiation for 24 h

The results for 1 week of exposure indicated that larvicidal effectiveness of the commercial formulation was reduced to approximately one third that of unweathered samples at the 7, 14 and 25 ppm concentrations and to one half in the 3.125 ppm concentration treatment (Figure 47). At this time there was some reduction in efficiency of the nanoparticle-treatment groups, but the reduction was less than for the commercial cyromazine formulation in most instances The 25ppm FSN60-hydrophobic formulation was still providing 100% protection at this time whereas the FSN60, RNP and SNP had reduced to 63, 48 and 68%.

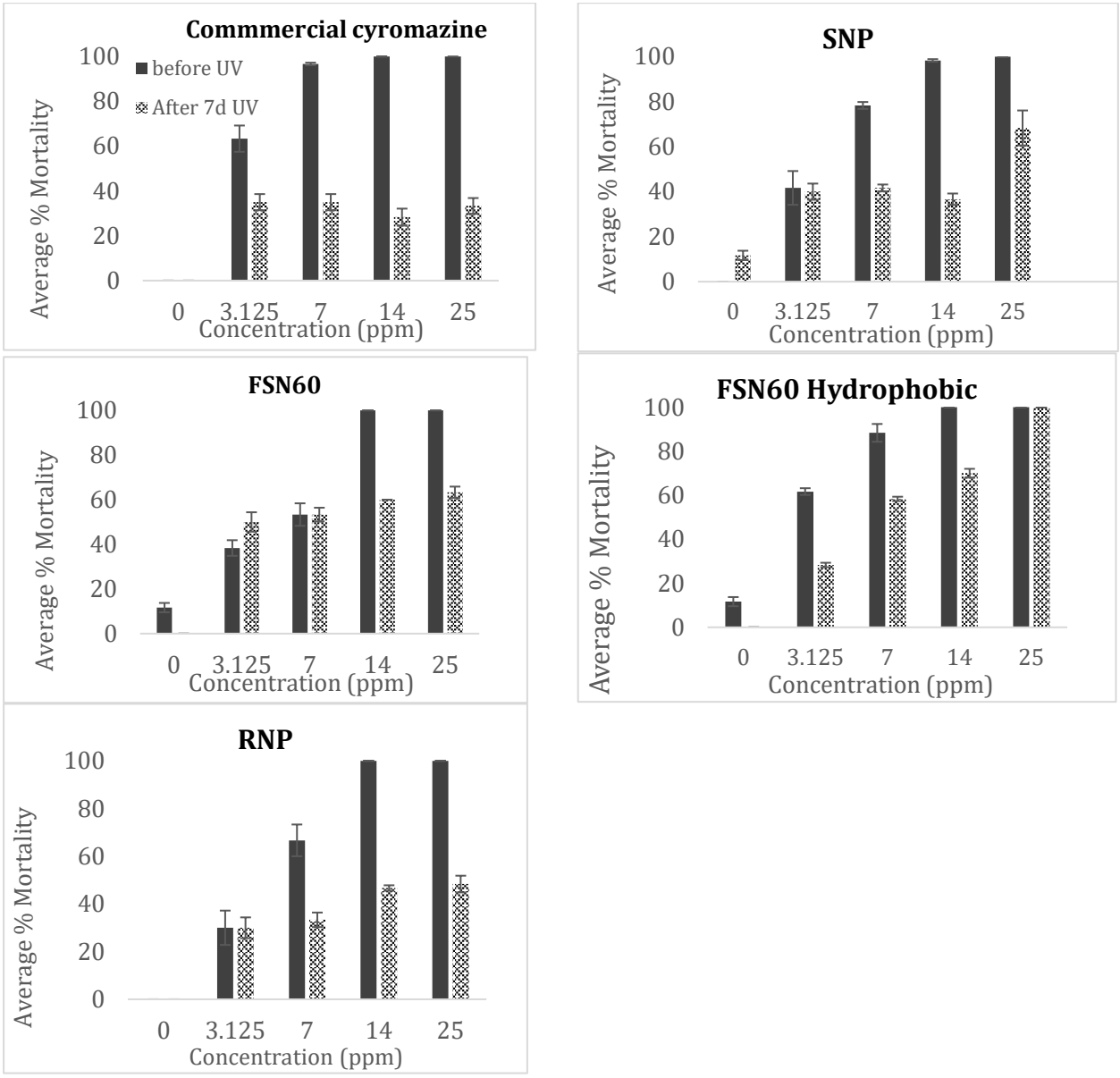


Figure 47. Mortality of *L. cuprina* larvae in assays with wool samples treated by different nanoparticle formulations or a commercial cyromazine formulation then exposed to environmental solar radiation for 7 days

In the second experiment (Figures 48-51), after 24h the effectiveness of all concentrations was reduced with the maximum protection at 25ppm (highest) concentration (63%). In contrast, with the nanoparticle treatments there was only a very minor reduction in efficacy in comparison to unweathered samples at all concentration. The one exception was with the FSN60 formulation where there was a significant reduction at the 3.125, 7 and 14 ppm concentrations although there was still 100% efficacy at the 25ppm. The reason for the lower efficacy than with the other nano-encapsulated formulations in this assay is unclear as this formulation gave relatively good effect in assays at later dates.

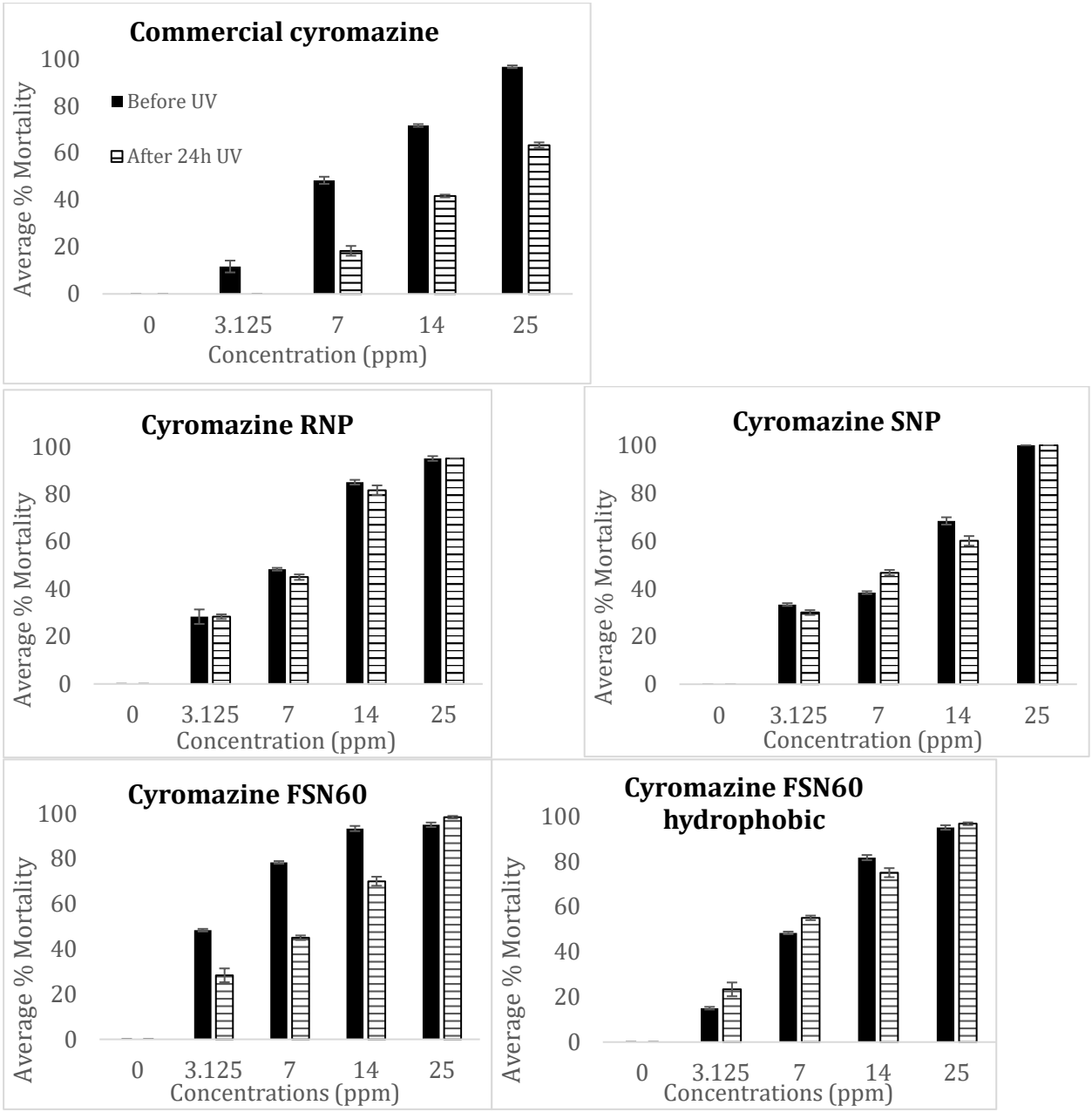


Figure 48. Larval toxicity with different formulations and concentrations of cyromazine following 24 hours exposure to the day light ultra-violet radiation

After 6 weeks, protection from the commercial formulation had been functionally lost whereas all of the nanoparticle formulations were still providing more than 50% protection at most concentrations and up to 78% protection in the case of the FSN60 hydrophobic formulation at 25ppm (Figure 49).

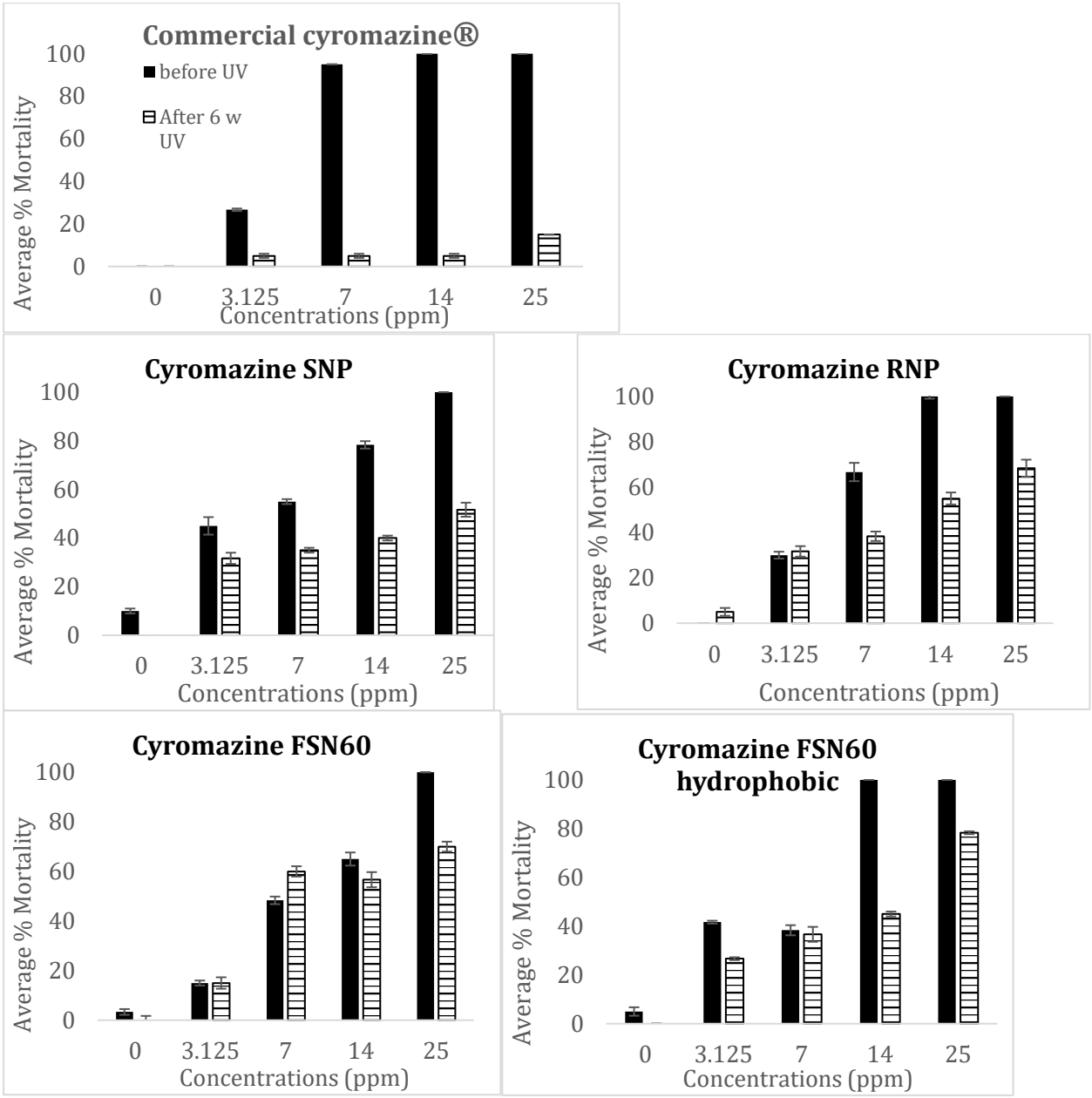


Figure 49. Larval toxicity with different formulations and concentrations of cyromazine following 6 weeks exposure to the day light ultra-violet radiation

At 16 weeks FSN60 and FSN60-hydrophobic had a mortality range of 45%-100% for the 3.125, 7, 14 and 25 ppm treatments whereas for the commercial formulation the protection at the 25ppm (highest concentration) was only 33% (Figure 50). In this case the mortality was higher than at 6 weeks in all treatments, which may have suggested that the larvae used for the test were 'weaker', or other factors were favouring mortality. However, the relativity of protection was maintained with higher levels of mortality in all of the nano-encapsulated formulations in comparison to the conventional formulation.

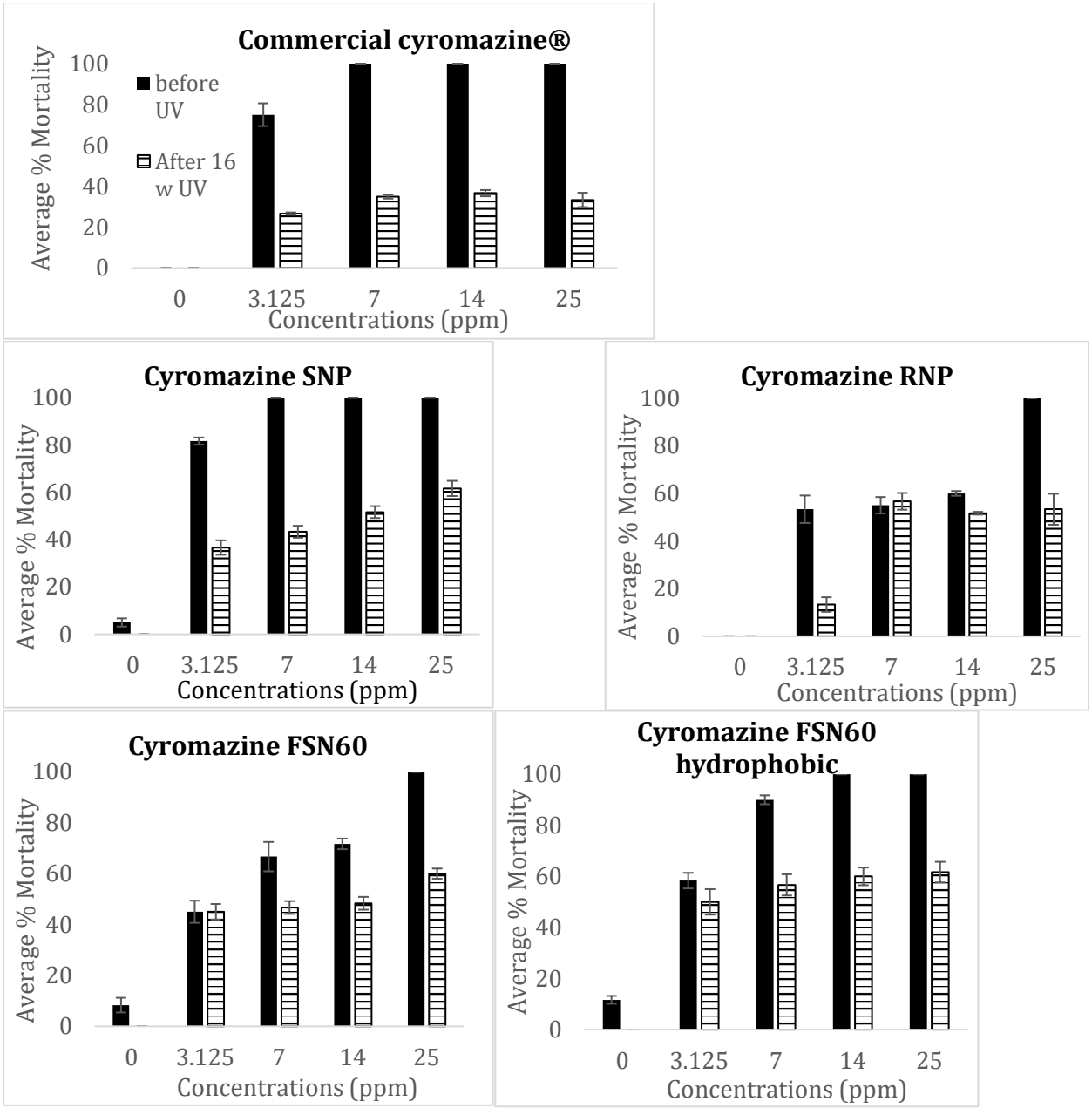


Figure 50. Larval toxicity with different formulations and concentrations of cyromazine following 16 weeks exposure to the day light ultra-violet radiation.

After 21 weeks of exposure, commercial formulation had lost effectiveness at all concentrations (maximum protection 8% at the 25ppm concentration, 0% at lower concentrations), while the best formulation (FSN-60 hydrophobic) was still providing 62% protection at the 25ppm concentration (Figure 51).

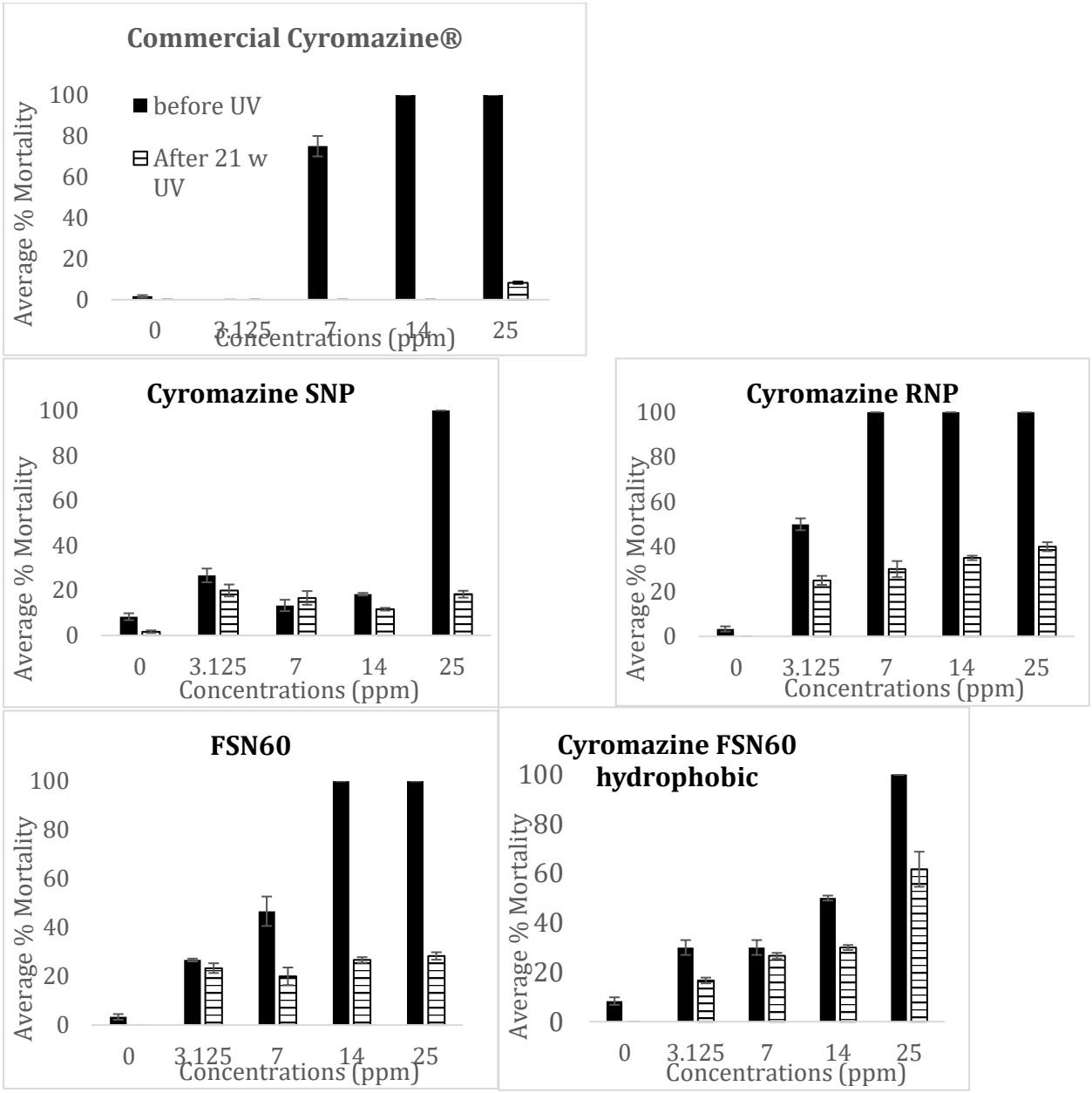


Figure 51. Larval toxicity with different formulations and concentrations of cyromazine following 21 weeks exposure to the day light ultra-violet radiation

In this experiment although the samples were exposed to relatively small amounts of rainfall in the early exposure periods in the periods between 6 to 16 weeks and 16 to 21 weeks, significant amounts of rainfall were received (51mm and 72mm respectively) (Table 2). This suggests that major weathering effect during the first 6 weeks was likely mainly due to photo-degradation whereas at the later times, the effects of rainfall may also have been important, particularly as cyromazine is reasonably water soluble.

Table 2. Cumulative rainfall up until each larval assay test in the cyromazine nanoparticle weathering study

Cyromazine	
Time after UV exposure	**Rainfall (mm)**
1day	0
1 week	2.4
6 weeks	7
16 weeks	58
21 weeks	130

4.9 Encapsulation of labile actives

The results showed a very significant advantage from nanoencapsulation (Figure 52). The highest concentration (0.2%) gave just over 50% mortality and at 0.01% mortality was less than 5%. In comparison, the encapsulated formulation gave 100% mortality at all concentrations tested from 0.2% down to 0.01%. In this instance this difference was evident without either artificial UV weathering or water rinsing. It is thought that a significant amount of the unencapsulated active was either degraded or volatilised during set up of the assay or during the 24 hour drying period and that formulating in the rough nanoparticles was protected against this loss.

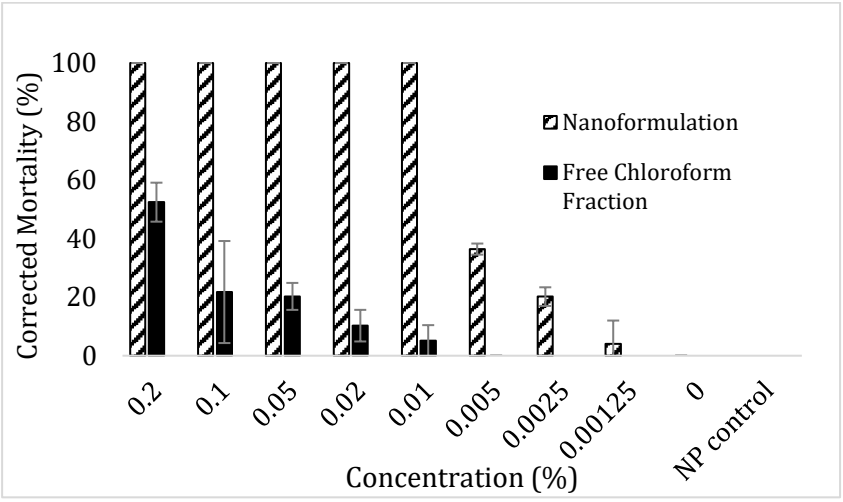


Figure 52. Mortality induced by a volatile plant compound presented as free chloroform fraction and encapsulated in rough nanoparticles in 1st instar *L. cuprina* larval assays following artificial UV exposure

4.10 Sheep studies

4.10.1 Weathering effects on sheep

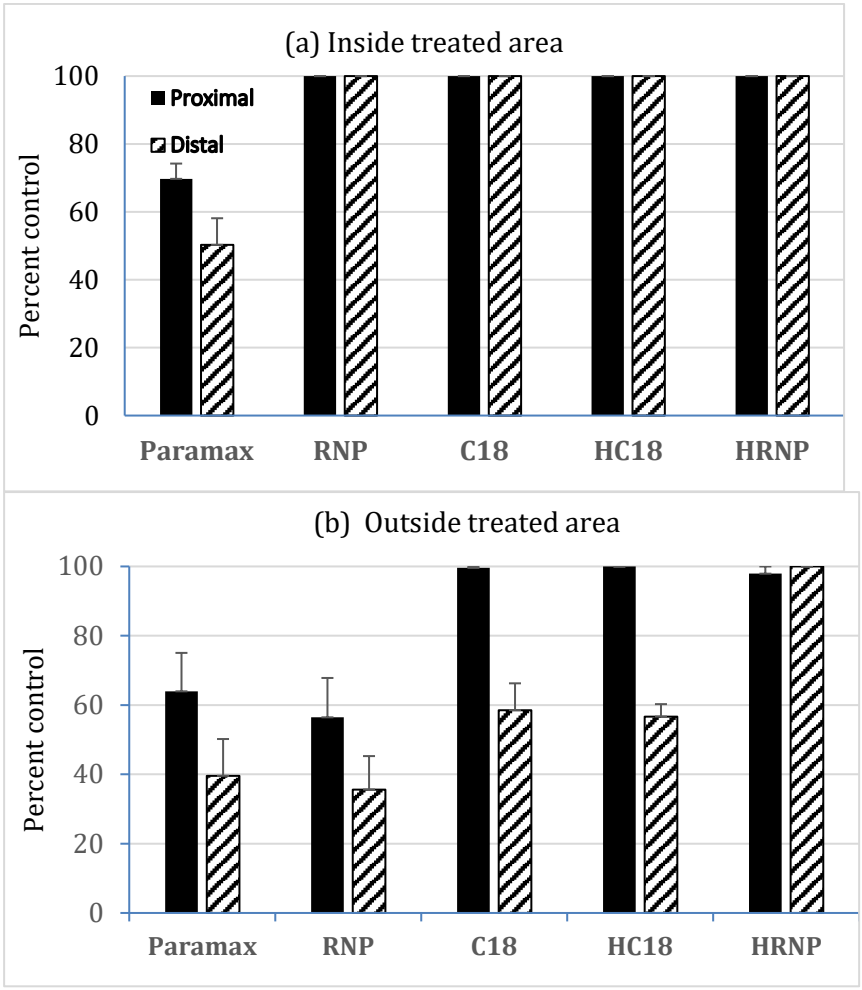


Figure 53. Percent mortality in *L. cuprina* larval assays using distal and proximal wool collected from areas on sheep treated with a commercial formulation of ivermectin mixed according to label instructions, rough nanoparticle formulation (RNP) and hydrophobic modified RNP particles (C18) at equivalent ivermectin concentration and RNP and C18 applied at half concentration

Results from the assays with wool collected from within the treated areas on sheep demonstrated a clear advantage for persistence of nanoparticle formulations over conventionally treated wool. Mortality was 100% with both distal and proximal wool in all assays on the sheep treated with the nanoparticle formulations, even when the chemical application rate with nanoparticles was half that used in the commercial treatment. In comparison, with the wool treated with the commercial formulation rate protection had dropped to 50% with the distal wool, probably as a result of photo-degradation of the unencapsulated chemical and had dropped to 70% with the proximal wool, less than with the distal wool probably because chemical deeper in the fleece was less exposed to sunlight.

When wool from outside of the treated area was tested there was a similar advantage for the nanoparticle formulations over the conventional formulation in most assays. Rugg (1995) showed that ivermectin spread laterally over the skin surface in a similar way as also shown for synthetic pyrethroids (Kettle et al. 1983, Jenkinson et al. 1986). The higher mortality in the proximal wool may have resulted from lateral spread of ivermectin released from the nanoparticles. Previous studies have also shown that there is often a very uneven pattern of chemical that spreads away from the application point (Kettle et al. 1983, Johnson et al. 1995, 1996) and Rugg (1995) also showed that the amount of spread varied markedly between sheep. In addition, there are frequently 'runs' of chemical in the fleece at application which could also contribute to variations in concentration of chemical in the fleece. Unevenness in spread or

application could account for the very good effect from the half dose RNP treatment compared with the full dose RNP when wool from outside of the application area was tested.

5. Discussion

With ever growing requirement for cost of production efficiencies and low labour inputs, flystrike protection requires control methods that can give extended periods of protection. In addition, many wool producers are seeking to reduce reliance on mulesing and the ability to do this in many flocks relies on the availability of chemical treatments that can give prolonged protection against flystrike. A recent survey of sheep producers indicated that 76% used preventative chemical treatments to control flystrike (Colvin et al. 2021. Two chemical actives that can give long periods of protection, dicyclanil and cyromazine, account for a very high proportion of these preventative treatments (79% for growers that treat at the same time each year, 72% for those that treat when the risk of strike is high, 86% of those that treat once flystrike is detected and 69% of growers who treat to protect sheep during periods when they are unable to regularly check their sheep (Colvin et al. 2020. Thus, these two chemicals are particularly important for flystrike control and reliance on chemicals has increased over the last five years as growers seek to reduce reliance on mulesing as a means of flystrike control (Colvin 2020). Unfortunately, resistance to these chemicals has emerged in sheep blowfly populations and already appears to be relatively widespread (Levot et al. 2014, Waghorn et al. 2013, Sales et al. 2020). This resistance is manifest in the field by reduced periods of protection and protection periods were reduced by between 69% and 78% in larval implant trials for dicyclanil and 33% to 50% for cyromazine when compared to registered label claims (Sales et al. 2020).

Nanotechnology offers a means of extending periods of protection from chemical methods of flystrike and of potentially making environmentally 'softer' or 'natural' chemicals, which generally have short periods of protection, a practical option for controlling flystrike. The UQ nanoparticles with a large hollow cavity and porous silica shell with numerous spikes on the surface studied in this project represent a new patented technology (Australian Patent Appl No. 2015901379). The silica shell protects the internal active payload against degradation, while pores in the shell allow easy loading of chemical actives into the hollow cavity and sustained release of the active compound. Silica spikes (or whiskers) covering the surface of the 'rough' particles evenly, were shown to aid retention of the capsules on wool fibres and the exocuticles of blowfly larvae and sheep lice and may also have aided the accumulation of particles in the guts of these parasites.

Silica has been well recognized as inert and abundant in the environment with good bio-compatibility and is approved by Food and Drug Administration (FDA) for oral delivery of human pharmaceuticals and bio-active compounds. Silica comes in two main forms, crystalline and amorphous. The crystalline form has a negligible rate of biodegradation and can have cytotoxic effects (Rushton 2007). There is also a dose effect and silicosis, generally occurs following the inhalation of crystalline forms over extended periods of time (Byrne and Baugh 2008). The silica used in our particles is amorphous silica. Amorphous silica is readily biodegraded and breaks down to silicic acid ($Si(OH)_4$) which can diffuse through to the blood stream and lymph and is excreted in the urine (Lai et al. 1998). Mesoporous silica with high surface area and large mesopores, such as in our particles, is the most amenable to breakdown and the breakdown rate is controllable using various synthesis strategies (Savi and Putz 2011).

It is also important to note that most concerns about nanoparticles relate to much smaller particles than described in this report. Very small particles can migrate into organisms and body tissues and even across cell membranes which leads to health concerns (APVMA 2003), but there are very many other factors, particularly relating to surface characteristics of the particles that need to be taken into account to accurately determine the toxic potential of nanomaterials (Savi and Putz 2011). The term nanoparticles is most often applied to particles in the 1-100 nm range in size whereas our particles are 200-300 nm,

which minimises any likelihood of the particles moving across the skin /body tissue barrier. The definition of nanoparticles as less than 100 nm has also been adopted by the Australian Pesticides and Veterinary Medicines authority and under current recommendations (APVMA 2015) our particles would not be considered as nanoformulations in the registration process. However, they would still be subject to the normal safety assessments required for registration of pesticides and veterinary medicines (APVMA 2015). In addition, formulation of the particles for application to sheep, for example in a backline or pour-on formulation or in a liquid vehicle as a spray or immersion dip, would be expected to minimise the inhalation risk of particles and instructions to minimise exposure risk by other means should be covered under current safety guidelines for pesticide applications.

Moreover, the UQ patented technology provides a relatively simple approach to the fabrication of nanoparticles, employing cheap industrial chemicals, which is ideal for adaptation to large scale commercial oriented production. The two chemicals investigated in this study were quite different in their physico-chemical profiles with ivermectin a highly lipophilic neurotoxin, acting on glutamate-gated chloride channels of insect nerve and muscle cells with very low water solubility and cyromazine, a water-soluble insect growth regulator chemical acting primarily by disrupting the moulting process in early-stage fly larvae. Incorporation of a volatile plant extract in a rough-surface nanoparticle formulation was also shown to significantly improve its effect. This suggests that the formulation types described here should be compatible with a wide range of chemistries and may also increase the longevity of volatile pesticide actives, botanical extracts and repellents.

One of the advantages of the silica nanoparticles is that they provide significant flexibility of design and can be 'tuned' for a number of characteristics. For example, the FSN and FSN hydrophobic particles prepared as part of this project had a similar pore size to the previous RNP particles, but no hollow central core, a much denser whisker configuration, a greater surface area, and a higher pore volume which provided approximately twice the chemical loading capacity compared to the RNP particles. These particles provided better effect than the RNP particles in most tests, even when loaded at 23% chemical, well below their maximum capacity to enable direct comparison with the earlier particle types.

Size of the particles is potentially an important factor as it influences parameters such as chemical payload, distribution on, or movement across, surfaces following application and the likelihood of absorption through the cuticle of fly larvae. In the study reported here, when the different sized particles were tested in the artificial weathering studies, there appeared to be relatively little difference in performance between the different sized particles. Although the 180 nm particles appeared to provide better effect overall than the other sized particles, the difference was small. It should be noted that in the efficacy studies with different particle sizes the different formulations were applied at similar concentration so that the effect of particle size was confounded with the density of particles. Higher chemical loadings are possible with larger particles and it is expected that the release dynamics would be different, potentially providing a longer period of protection, but this requires testing.

Efficacy of the silica nanoparticles following environmental exposure

All of our studies have indicated significantly prolonged persistence of the rough topography nanoparticle formulations (RNP, FSN60, RNP-C18 and FSN60-hydrophobic) in comparison with the commercial formulations and compared to smooth silica nanoparticles. There appeared to be only marginal differences in effectiveness between the different designs of rough topography formulations with small and inconsistent differences between the RNP and the RNP-C18 in assays where there was a direct comparison. Overall, the FSN formulations appeared to give somewhat better protection than the RNP formulations, even when loaded with chemical at well below their maximum insecticide capacity to enable direct comparison with the other particle types. In addition, there was indication of a slight advantage for the FSN60-hydrophobic formulation in comparison with FSN60 non-hydrophobic formulation in some assays and it is expected that higher chemical loading could be used to further improve longevity of effect of these particles.

One of the main effects of micro encapsulation and nano-encapsulation is the prevention of photo-degradation (Kah and Hofman 2014, AVPMA 2015, Mitter et al. 2017, Zhang et al. 2020). Avermectins are known to be subject to photo degradation (Li et al. 2007, Rugg 1995) and when ivermectin was applied to the fleece and tested for distribution along the fibre, the chemical was in highest concentration midway along the fibre (Rugg 1995). Ivermectin has low water solubility and has been reported to not translocate proximally along wool fibres in the fleece to any degree (Rugg 1995), although this is likely to be influenced by formulation. Lower concentration in the surface third of the wool fibres was thought to be due to photo-degradation whereas the lower concentration in the proximal wool was thought to be due to growth of the wool away from the skin, with little translocation of the chemical into wax at the proximal end of the fibre.

A previous study showed that encapsulating avermectins in smooth-surface silica nanoparticles can significantly reduce photo-degradation (Li et al. 2007). The studies reported here showed only small advantage from encapsulation in the smooth particles in some experiments but indicated that the particles with rough surface topography (RNP, RNP-C18, FSN-60 and FSN-60 hydrophobic) can significantly improve the level of protection provided against breakdown by sunlight above that offered by both the smooth particles and commercial formulations. Cyromazine is also subject to photo-degradation (Goutailler et al. 2001) and the UV degradation studies showed a similar advantage for the rough surface particles in comparison with conventional and smooth nanoparticle formulations.

A clear advantage from reduction in photo-degradation was also seen in our previous studies with similar silica nanoparticles containing spinosad applied to cattle skin in an *in vitro* assay (Zhang et al., 2020). Spinosad is also registered for control of sheep blowfly and has a favourable environmental and low residue profile, which makes it particularly attractive for use where wool is targeting low residue markets. However, its rapid degradation in the environment is also associated with relatively short flystrike protection times (Sandeman et al. 2014). Incorporating spinosad in rough-surface silica nanoparticles could also greatly increase its functionality as a flystrike preventative.

Leaching from the fleece during heavy rainfall has previously been suggested to reduce periods of protection from cyromazine, which is considered to be particularly susceptible to leaching due to its relatively high-water solubility (Nottingham et al. 2001). In this study, encapsulation in rough-surface nanoparticles was shown to reduce cyromazine leaching and longer persistence was measured with the rough surface nano-formulations in bioassays following wetting by both immersion in water and the application of artificial rainfall.

The improved persistence after artificial wetting or immersion was also seen with ivermectin. The 'suint' fraction of the wool yolk is known to contain potassium salts and other compounds that can give it a detergent-like action. During immersion, dipping of sheep to control lice, the washing of suint compounds into the dipping fluid is considered to aid thorough wetting of the sheep and improve the effectiveness of lice control, leading to recommendations with some formulations that the first pen of sheep should be re-dipped to benefit from this effect. This effect could also contribute to leaching of more lipophilic compounds and help explain the prolonged periods of effect also observed with the ivermectin RNP in the wetting studies.

It has been suggested that rainfall can also play a part in extending periods of protection from cyromazine by moving chemical down into the proximal wool and onto the skin where strikes develop (Levot et al. 2014). It is possible that the spiky-surface particles could help prolong this effect with their pollen type configuration (Song et al. 2016), helping to 'anchor' the capsules in the fleece, protect against photo-degradation and maintain a 'depot' of chemical in the fleece. The rough surface nanoparticles were shown to provide slow release of cyromazine for up to 6 days when immersed in water. When applied to sheep fleece they could both reduce photo-degradation of chemical as well as release cyromazine into the wool over subsequent wettings, potentially leading to extended protection by this means.

Effect of application technique

Periods of protection in the field are also strongly influenced by application method and protection periods are generally shorter when chemical is applied off shears or in backline formulations than when sheep are treated in long wool. For example, the registered protection period from spray-on formulations of cyromazine is 11 weeks whereas when sheep are treated by hand jetting protection periods of up to 14 weeks can be achieved. In addition, periods of protection from hand jetting where the chemical is applied deep into the fleece are generally longer than with jetting races where a greater proportion of chemical is applied to the distal portions of the fleece (James et al. 1980). Campbell et al. (1998) showed that when cyromazine was applied to sheep with 6-8 months wool by hand jetting, which delivers chemical into the fleece and along the length of each fibre, the half-life was 79-96 days whereas with jetting races which apply a much greater proportion of chemical to the distal wool, half-life was much shorter at 39-66 days depending on the effectiveness of the race. The reason for this is likely mainly due to greater photo-degradation as a result of the higher proportion of the chemical near the tip of the fleece, although greater susceptibility to leaching could also reduce protection periods when sheep are exposed to heavy rainfall. The use of the nanoparticle types described here could be of particular benefit in improving the longevity of effect from off-shears and spray on formulations, favoured by sheep producers because of their labour-saving attributes and could also help in increasing the longevity of protection from jetting race treatments.

Uptake by insects

Studies with both blowfly larvae and lice indicated accumulation of the fluorescent particles in the gut following exposure to SNP or RNP nanoparticle-treated wool fibres indicating that both parasites are ingesting significant amounts of the labelled particles. Whether the particles are transient, possibly attaching to gut lining or peritrophic membrane, or whether both insects are actively accumulating the particles during feeding is currently unclear. Similarly, whether the accumulation of insecticide is more efficient with the RNP nanoparticle formulations than conventional or smooth particulate formulations is not clear at this stage.

The feeding habits of both insects would seem to favour active accumulation of particles from surfaces. When sheep blowflies oviposit, most eggs are deposited in cavities in the distal portion of the fleece (Browne 1979). If the fleece remains moist, eggs will hatch in 12 to 24 hours and the newly hatched larvae move down through the wool fibres to the skin where a strike is initiated. In addition, the young larvae may move laterally through the wool fibres to locate feeding foci associated with fleece rot or lumpy wool lesions, urine scalding or an existing strike. It seems likely that the young larvae feed during this migration, for example on bacteria or skin secretions. During feeding, blowfly larvae 'scrape' at the food surface, the 1st instar using (Sandeman et al. 1987) and later stage larvae using mouth hooks adapted for this purpose. This behaviour was thought to be the reason for the better effects seen when nano-formulations were applied to the wool surface than when applied in the serum in the assays system in our studies. Sheep lice feed from the skin surface and the surfaces of the wool fibres using sclerotised mandibles to scarify the skin and wool fibres, ingesting lipid, detached skin debris, stratum corneal cells and bacteria (Sinclair et al. 1989). It seems that application strategies would need to be designed to deliver the particles to places where the insects feed to have best effect. Whether there is a difference between the different types or designs or possibly sizes of particles in the degree to which they are ingested or accumulate awaits further studies. However, greater adhesion of the rough-surface particles to treated surfaces would seem to facilitate uptake by both feeding blowfly larvae and lice.

The different studies of cuticular adherence suggest that there was definitely some 'sticking' of the particles to the exocuticle of both fly larvae and lice and furthermore that this was greater in the particles with rough surface topology than the smooth particles and appeared to be higher with the (hydrophobic particles. Whether this translates to topical uptake of the chemical into the insect or not is uncertain. The movement of larvae in the fleece is noted above and sheep lice move up and down the wool fibres to thermoregulate (Murray 1968), as well as laterally through the fleece. Thus, there is considerable cuticular contact with wool fibres and the skin during movement through the fleece that facilitates the acquisition of insecticide topically by both of these parasites.

The immersion studies with all stages of the blowfly larvae suggested that conventional formulations were significantly more toxic at equivalent concentration than the nanoparticle formulations when applied topically. Similarly, the adult immersion studies showed that although there was a level of toxicity conferred by the rough particles, toxicity was lower than with the conventional chemical formulation. It is also possible that there may have been some oral acquisition of particles resulting from fly 'grooming' activities after treatment. It should also be noted however that in these assays the insects were only exposed for short periods of time to minimise the likelihood of ingestion and greater adhesion is likely when exposure is for extended periods of time. In addition, it is likely that the pick-up would be greater with the rough surface or rough hydrophobic particles, given the lipid nature of the exocuticle of most insects (Chapman 2013). Although cuticular acquisition potentially contributes to the overall toxic effect, the much higher levels of fluorescence from the labelled particles seen in the guts of both blowflies and lice suggests that an oral route of exposure is much more important, and it seems unlikely that insects would accumulate a toxic dose of chemical by topical absorption alone. This could be an important consideration when designing application strategies.

Fleece dynamics

The weathering system used in these studies exposed wool samples laid flat on the trays so that the full length of the wool fibre was exposed to the effects of rainfall and photo-degradation. In practice on sheep, fibres are exposed closely packed together in the fleece in a proximal-distal orientation. In this case most sunlight and rainfall would impact at the tip of the fleeces and a lower overall amount of exposure would be expected, than in the *in vitro* studies reported here. The dynamics of pesticides in the fleece can be a key determinant of the efficiency of parasite control and it is known that when insecticides are applied to the wool or skin surface of sheep that there can be considerable movement along the fibre with organochlorine and organophosphate chemicals (Fiedler and Du Toit 1951). This is a key factor in the effectiveness and duration of parasite protection from these chemicals. However, Rugg (1995) noted that in the case of ivermectin there is limited movement of chemical into new wool growth in the fleece, and it seems likely that this would also be the case with the nanoparticles. It should also be noted that it has also been suggested that lipophilic chemicals applied at skin level could be absorbed into sebaceous glands and that later secretion with sebum onto the wool fibre surface and skin may contribute to the longevity of protection (Harrison and Rundle 1983). Whether a similar mechanism could occur with appropriately designed nanoparticle formulations (most likely the hydrophobic configurations) when they are applied to the skin surface in backline formulations or by hand jetting is worthy of investigation.

In the case of synthetic pyrethroids, which are more lipophilic, it has been shown that there is considerable lateral spread of chemical across the skin (Jenkinson et al. 1986, Hennessy et al. 2000, Johnson et al. 1995, 1996). It has been suggested that this is due to movement through channels in the stratum corneum (Jenkinson et al. 1986) and Rugg (1995) showed a similar mechanism of lateral spread with ivermectin. Lateral movement of chemicals can be an important consideration in the effectiveness of formulations against sheep lice when applied as a backline or long wool treatment and the potential for movement of the silica particles, and the chemicals formulated in them once released from the particles in the fleece and across the skin could be an important consideration in the efficacy of the formulations once applied to sheep. In contrast, it is interesting to hypothesise that strong 'anchoring' in the fleece could be advantageous for water soluble chemicals such as cyromazine that can be leached out of the fleece, or down to skin level under the effects of rainfall. 'Anchoring' the encapsulated chemical, protected from the effects of photo-degradation, in the fleece using the slow-release spiky surface nanoparticles could potentially extend periods of protection by allowing release of chemical into the fleece over multiple wetting events.

The dynamics of chemicals in the fleece and on the skin surface are important factors affecting the efficiency of sheep blowfly larvicides and how this might be affected by nano-encapsulation of chemicals with different mobilities and solubilities in the fleece will be an important consideration in the development of nano-formulations for sheep blowfly strike control. The results of the preliminary studies to investigate weathering of the formulations on sheep matched well with the results achieved in the *in*

vitro systems and confirmed a significant advantage in longevity of effectiveness from the rough surface formulations. In addition, the results to date suggest that there is significant spread of ivermectin both along fibres and across the skin surface from the rough surface particles. Clearly a range of other factors such as the effect of carrier formulation, application method and availability and uptake of the particles or released chemical by larvae on sheep will also impact on the ultimate effectiveness of flystrike control from the nanoparticle formulations and preliminary pen studies using larval implants or other controlled flystrike challenge are now required.

6. Conclusions and Recommendations

This project has clearly demonstrated that encapsulating ivermectin and cyromazine in silica nanoparticles can provide extended protection in comparison to 'conventional' commercial formulations when compared in an in-vitro system. The advantage is greatest with more labile or volatile chemicals that generally have impractically short protection periods but are more environmentally 'attractive' because they break down quickly in the environment. Further these results suggest a significant advantage for the 'rough' surface formulations in comparison with smooth surface nanoparticles. Although there were generally rather small and inconsistent differences between the different rough surface particles, the FSN60 and FSN60 hydrophobic formulations generally gave the best effect. Furthermore, the FSN particles tested in this project were loaded at 23% chemical content to enable comparison with the other particle types for which this was the maximum loading capacity, However, the FSN particles have maximum loading capacity of approximately 50% which offers the flexibility of a much higher insecticide loading capacity than with the other particle types and which would be expected to further extend the protection period.

The effects of sheep factors, fleece dynamics and differences in the level of environmental exposure when flystrike prevention formulations are applied to sheep make it difficult to relate the advantages in protection efficiency demonstrated in laboratory assays to effectiveness and duration of protection under field conditions. Also, there is likely to be interaction between the method of application and the relative advantage realised from different nanoparticle formulations and the relative advantage in field protection could be much larger, or alternatively less, than suggested by the results reported here. Clearly the next stage in this work is pen studies with these formulations applied to sheep. It is suggested that FSN60 particles, with a higher chemical loading, should be tested in these studies.

7. References

Anderson, N., McKenzie, J.A., Laby, R.H., Strong, M.B., Jarrett, R.G., 1989. Intraruminal controlled release of cyromazine for the prevention of *Lucilia cuprina* myiasis in sheep. Research in Veterinary Science 46, 131-138.

A.P.V.M.A. (Australian Pesticides and Veterinary Medicines Authority) 2015. Nanotechnologies for pesticides and veterinary medicines regulatory consideration, AVPMA, ed. (Kingston ACT Australia, Australian Veterinary Medicines Authority), p. 237.

Browne, L.B., 1979. The behaviour and nutritional requirements of adults of *Lucilia cuprina* - possibilities for modification. In: National Symposium on the Sheep Blowfly and Flystrike in Sheep, Sydney, pp. 45-57.

Byrne, J.D. and Baugh J.A. 2008. The significance of nanoparticles in particle induced pulmonary fibrosis. McGill Journal of Medicine 11, 43-50.

Campbell, N.J., Hanrahan, P.D., Russell, I.M., Roberts, G.S., Horton, B.J., 1998. Modelling pesticide residues on greasy wool: experimental studies. Australian Journal of Experimental Agriculture 38, 441-449.

Chapman, R.F., 2013. The Insects Structure and Function, 5th Edition. Cambridge University Press, Cambridge UK, 959p.

Colvin, A.F., Walkden-Brown, S.W., Reeve, I., 2020. Benchmarking Australian Sheep Parasite Control: Project final report. Australian Wool Innovation Ltd., University of New England, Armidale. https://www.wool.com/globalassets/wool/sheep/research -publications/welfare/surveys/2018-benchmarking-australian-sheep-parasite-co ntrol-survey.pdf, 17 March 2021b.

Colvin, A.F., Reeve, I., Peachey, B., Walkden-Brown, S.W., 2021. Benchmarking Australian sheep parasite control practices - a national online survey. Anim. Prod. Sci. 61 (3), 237–245. https://doi.org/10.1071/AN20171.

Fiedler, O.G.H., Du Toit, R., 1951. A new biological method for evaluating the efficiency of insecticides for the protection of sheep against blowfly strike. Nature 168, 608-609.

Goutailler, G., Valette, J.C., Guillard, C., Paisse, O., Faure, R., 2001. Photocatalysed degradation of cyromazine in aqueous titanium dioxide suspensions: comparison with photolysis. Journal of Photochemistry and Photobiology a-Chemistry 141, 79-84.

Harrison P.M., Rundle, J.C. 1983. The mechanism and importance of insecticide translocation on wool. In: Second National Symposium on the Sheep Blowfly and Flystrike in Sheep, Sydney, pp. 180-185.

Hennessy, D.R., Darwish, A., Maxwell, C.A., 2000. Increased control of the sheep biting louse *Bovicola* (*Damalinia*) *ovis* with deltamethrin formulated in a fractionated wool grease carrier. Veterinary Parasitology 89, 117-127.

Hughes, P.B., Levot, G.W., 1987. Simulation of fly-waves to assess the ability of diflubenzuron to protect sheep against flystrike by lucilia-cuprina. Veterinary Parasitology 24, 275-284.

Iezzi, S.; Purslow, P.; Sara, C., Lannusse, C., Lifschitz, A. 2017. Relationship between ivermectin concentrations at the injection site, muscle and fat of steers treated with traditional and long-acting preparations. Food and Chemical Toxicology 105, 319-321.

Jackson, J.E., Kopecki, Z., Cowin, A.J., 2013. Nanotechnological Advances in Cutaneous Medicine. Journal of Nanomaterials.

James, P.J., Erkerlenz, P., Meade, R.J., 1990. Evaluation of ear tags impregnated with cypermethrin for the control of sheep body lice (*Damalinia-ovis*). Australian Veterinary Journal 67, 128-131.

James, P.J., Meade, R.J., Powell, D., 1989. Effect of insecticidal ear tags on populations of lice (*Damalinia-ovis)* infesting sheep. Australian Veterinary Journal 66, 134-137.

James, P.J., Mitchell, H.K., Cockrum, K.S., Ancell, P.M.C., 1994. Controlled-release insecticide devices for protection of sheep against head strike caused by Lucilia-cuprina. Veterinary Parasitology 52, 113-128.

James, P.J., Russell, D.W., 1980. Comparative efficiency of hand jetting, race jetting and shower dipping for application of insecticide to the fleece of sheep. Agricultural Record 7, 59-64.

Jenkinson, D.M., Hutchison, G., Jackson, D., McQueen, L., 1986. Route of passage of cypermethrin across the surface of sheep skin. Research in Veterinary Science 41, 237-241.

Johnson, P.W., Darwish, A., Dixon, R., Steel, J.W., 1995. Kinetic disposition of xylene-based or aqueous formulations of deltamethrin applied to the dorsal midline of sheep and their effect on lice. International Journal for Parasitology 25, 471-482.

Johnson, P.W., Darwish, A., Dixon, R., Steel, J.W., 1996. Kinetic disposition of an aqueous formulation of alphacypermethrin applied to the dorsal mid-line of sheep with long wool and its effect on lice (vol 26, pg 1369, 1996). International Journal for Parasitology 27, 455-455.

Kah, M, Hofmann T (2014). Nanopesticide research: Current trends and future priorities. *Environment International* 63, 224–235.

Kettle, P.R., Watson, A.J., White, D.A., 1983. Evaluation of a deltamethrin formulation as a back-line treatment of sheep for the control of the sheep body louse (*Damalinia-ovis*) New Zealand Journal of Experimental Agriculture 11, 321-324.

Lai C.Y., Trewyn B.G., Jeftinija D.M., Jeftinija K., Xu S., Jeftinija S., Lin V.S.Y. A mesoporous silica nanosphere-based carrier system with chemically removable CdS nanoparticle caps for stimuli-responsive controlled release of neurotransmitters and drug molecules. Journal of the American Chemical Society 125, 4451-4459.Levot, G.W., Langfield, B.J., Aiken, D.J., 2014. Survival advantage of cyromazine-resistant sheep blowfly larvae on dicyclanil- and cyromazine-treated Merinos. Australian Veterinary Journal 92, 421-426.

Li, Z.Z. Chen, J.F., Liu, F., Liu, A.Q. Wang, Q., Sun, H.Y. Wen, L.X. 2007. Study of UV shielding properties of novel porous hollow silica nanoparticle carriers for avermectin. Pest Management Science 63, 241-246.

Mitter, N., Worrall, E.A., Robinson, K.E., Li, P., Jain, R.G., Taochy, C., Fletcher, S.J., Carroll, B.J., Lu, G.Q., Xu, Z.P., 2017. Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. Nature Plants 3.

Murray, M.D., 1968. Ecology of lice on sheep. VI. The influence of shearing and solar radiation on populations and transmission of Damalinia ovis. Australian Journal of Zoology 16, 725-738.

Nottingham, R.M., Hosking, B.C., Schmid, H.R., Strehlau, G., Junquera, P., 2001. Prevention of blowfly strike on coarse and fine woolled sheep with the insect growth regulator dicyclanil. Australian Veterinary Journal 79, 51-57.

Rugg, D.R., 1995. Toxicity and tolerance to ivermectin in some insects associated with livestock and the interaction of ivermectin with the skin and fleece of sheep. Ph.D. Thesis, University of Sydney, 195pp.

Rugg, D., Thompson, D., Gogolewski, R.P., Allerton, G.R., Barrick, R.A., Eagleson, J.S., 1998. Efficacy of ivermectin in a controlled-release capsule for the control of breech strike in sheep. Australian Veterinary Journal 76, 350-354.

Rushton, L. 2007. Chronic obstructive pulmonary disease and occupational exposure to silica. Review of Environmental Health 22, 255–272.

Sales, N., Suann, M., Koeford, K., 2020. Dicyclanil resistance in the Australian sheep blowfly, Lucilia cuprina, substantially reduces flystrike protection by dicyclanil and cyromazine based products. International Journal for Parasitology-Drugs and Drug Resistance 14, 118-125.

Sandeman, R.M., Collins, B.J., Carnegie, P.R., 1987. A scanning electron-microscope study of *L. cuprina* larvae and the development of blowfly strike in sheep. International Journal for Parasitology 17, 759-765.

Sandeman, R.M., Levot, G.W., Heath, A.C.G., James, P.J., Greeff, J.C., Scott, M.J., Batterham, P., Bowles, V.M., 2014. Control of the sheep blowfly in Australia and New Zealand - are we there yet? International Journal for Parasitology 44, 879-891.

Savi,C., Putz, A. (2011) Recent Advances in Bioresponsive Nanomaterials. In: Putz, M, Ed. "Carbon Bonding and Structures, Advances in Physics and Chemistry" 5 (Springer) pp 379-436.

Sinclair, A.N., Butler, R.W., Picton, J., 1989. Feeding of the chewing louse Damalinia-ovis (schrank) (Phthiraptera, Trichodectidae) on sheep. Veterinary Parasitology 30, 233-251.

Song, H., Nor, Y.A., Yu, M., Yang, Y., Zhang, J., Zhang, H., Xu, C., Mitter, N., Yu, C., 2016. Silica Nanopollens Enhance Adhesion for Long-Term Bacterial Inhibition. Journal of the American Chemical Society 138, 6455-6462.

Swiger, S.L., Payne, R.D., 2017. Selected Insecticide Delivery Devices for Management of Horn Flies (Haematobia irritans) (Diptera: Muscidae) on Beef Cattle. Journal of Medical Entomology 54, 173-177.

Waghorn, T.S., McKay, C.H., Heath, A.C.G., 2013. The *in vitro* response of field strains of sheep blowflies Lucilia sericata and L-cuprina (Calliphoridae) in New Zealand to dicyclanil and triflumuron. New Zealand Veterinary Journal 61, 274-280.

Witchey-Lakshmanan, L.C., 1999. Long-acting control of ectoparasites: a review of collar technologies for companion animals. Advanced Drug Delivery Reviews 38, 113-122.

Zhang, J., Brown, G., Fu G, James, P., Mukandiwa, L., Huang, X., Yu, C., 2020. Nanobiopesticides: Silica nanoparticles with spiky surfaces enable dual adhesion and enhanced performance. EcoMat 2: e12028.

Yu, M.H., Karmakar, S., Yang, J., Zhang, H.W,;Yang, Y.N., Thorn, P., Yu, C.Z. 2014 Facile synthesis of ultra-small hybrid silica spheres for enhanced penetration in 3D glioma spheroids. Chemical Communications 50: 1527-1529. DOI 10.1039/c3cc48416e

8. Appendices

a. **Appendix 1 – 2020 Flystrike Prevention RD&E Program – Project Summary Report**

AWI PROJECT NO: ON-00549

NANOTECHNOLOGY FOR FLYSTRIKE AND LICE CONTROL

AUTHORS

Peter James[1], Mona Moradi-Vargarah[1], Hao Song[2], Jun Zhang[2], Geoff Brown[3], Neena Mitter[1] and Chengzhong Yu[2].

[1]Queensland Alliance for Agriculture and Food Innovation, University of Queensland, Dutton Park, QLD 4101
[2]Australian Institute for Bioengineering and Nanotechnology, University of Queensland, St Lucia, QLD 4072
[3]Department of Agriculture and Fisheries, Dutton Park, QLD 4101

SUMMARY

New chemical formulations for flystrike control are required to support the phaseout of mulesing and because of the development of resistance to the most widely used flystrike control compounds. Control of sheep lice has suffered similar resistance problems and remains an issue in the sheep industries. Nanotechnology offers a means of providing extended and 'softer' protection of sheep against flystrike and lice. This project is designing and testing unique silica nanocapsule formulations with spikes on the particle surface and purpose-designed release characteristics to give prolonged periods of protection against flystrike and lice, with minimal residues and off-target effects. This will provide new, labour efficient, options for managing flystrike in unmulesed sheep and countering resistance in sheep blowflies and lice.

Background

With ongoing requirements to increase production efficiency and constraints on the availability of labour livestock producers increasingly favour parasite treatments that can provide extended periods of protection. For this reason there has been much interest in controlled release technology such as long-acting injectable formulations for internal and blood feeding ectoparasite control, slow release polymer matrix devices such as ear tags and collars for prolonged buffalo fly control in cattle and flea control in dogs and cats, rumen capsules for helminth and tick control and more recently, microencapsulated and nanoparticle formulations.

Whereas traditional formulations of pesticide depend for prolonged action on a single initial high level treatment so that control is maintained until concentrations decay below effective levels, controlled release systems aim to release pesticides in steady amounts at active levels or to release only at times of infestation risk. This approach has a number of advantages in addition to prolonged control. Doses need not be as large so there is less risk of tissue residues. There is generally a lower risk to the operator and of environmental contamination and there is a reduced chance of subclinical toxicity or accidental poisoning of animals. In addition, there are a number of 'softer' chemistries, including plant extracts that have been shown to have activity against *Lucilia* spp. These compounds are often favoured in pest control, particularly by organic producers, because of their rapid degradation in the environment and lower potential for tissue residues but are of limited practical usefulness because of their limited persistence. Suitable controlled release systems may enable the use of insecticides which have not previously been suitable for use because of poor persistence in the fleece. Micro or nanoencapsulation

technology can protect these compounds against environmental degradation and release them strategically at times of flystrike risk, or over an extended period of time to provide practically significant periods of protection against flystrike.

A wider choice of insecticides would be valuable in providing additional options in planning insecticide usage programs to minimise resistance development. In addition, controlled release systems that maintain insecticide at high concentration and then give a rapid residue decay avoiding resistance-selecting 'decay tails' (Anderson et al 1989), particulate controlled release system that could sit inert in the fleece and only release in the presence of moisture, systems that maintain high levels of insecticide through the fly season and then decay during the winter when no flies are present, or systems containing insecticides that degrade rapidly once released could also reduce the risk of resistance development.

Major innovations in the area of nanotechnology have led to the development of a variety of nanoparticle-based pesticide formulations, including polymeric/cellulose nanocrystals and lipid nanoparticles. By encapsulating active ingredients into nanocapsules, breakdown due to environmental pesticides can be reduced and chemical can be delivered at steady active levels over a prolonged period or designed to release only at times and sites where they are needed. Nanoencapsulated formulations also have the important attribute that they can generally be applied using existing application equipment.

UQ Silica nanoparticles

The UQ silica nanoparticles are a patented technology to fabricate novel hollow silica (SiO_2) nanocapsules that can be loaded with active molecules to enable superior protection against insect pests (Australian Patent Appl No. 2015901379). The nanocapsules have a large hollow cavity and porous silica shell which protects the internal active payload against degradation, while pores in the shell allow easy active loading into the hollow cavity and sustained release of the active compound. A number of designs of particle have been tested. The basic design is the smooth nanoparticle (SNP) as described above. However, a number of more recent designs of rough-surface nanoparticles (RNP) have a more pollen grain like topology (Figure 1a) with silica spikes (or 'whiskers') covering the nanocapsule outer surface. Similar to pollen grains, these spikes aid retention of the capsules on surfaces. The characteristics of these particles are 'tunable' and the particles can be designed with different characteristics such as with different chemical payloads, different size, different wall thicknesses and pore sizes, and different silica 'whisker' characteristics to optimise their functionality for different uses. This project is developing and testing silica nanocapsule formulations that can potentially provide prolonged, safe and residue free protection against sheep flystrike and lice and provide new, labour efficient, options for managing these pests. The UQ nanocapsules also possess advantages compared to other types of nanoparticles for translation to a viable commercial product. Polymer or lipid nanocapsules are often expensive or unstable under field conditions, whereas silica has been well recognized as inert and abundant in the environment with good bio-compatibility and is approved by Food and Drug Administration (FDA) for oral delivery. Moreover, the UQ patented technology provides a relatively simple approach to the fabrication of nanocapsules, employing cheap industrial chemicals, which is ideal for large scale commercial oriented production.

Three types of silica nanoparticles were initially studied in this project, smooth surface silica nanoparticles (SNP's), silica nanoparticles with silica spikes on the surface (RNP's) and RNPs with a surface modification to provide hydrophobic surface characteristic (RNP-C18) (Figure 1). The initial particles were 200-300 nm in diameter, but a number of other diameter particles with diameter from 180 – 800 nm have been fabricated and tested.

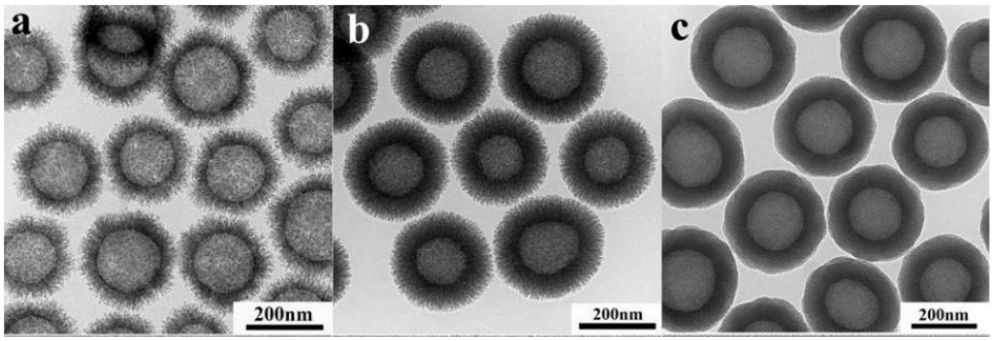


Figure 1. Transmission electron micrograph images of (a) rough nanoparticles; (b) rough particles after C18 surface modification and (c) smooth nanoparticles.

More recently, two new types of particles (FNS-60 and FNS-60-H) with-hydrophobic surface characteristics have been developed and are being tested. The FSN-60 particles have a higher pore volume than the previous formulations allowing higher chemical loading which, depending on release dynamics, is expected to provide further improvements in longevity of effect.

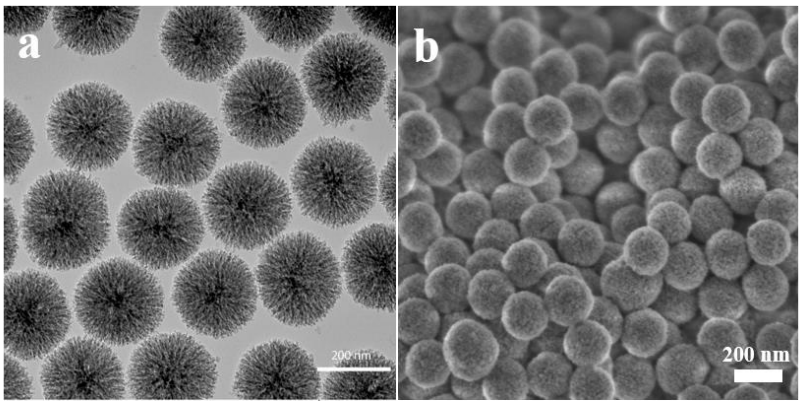


Figure 2. a) Transmission electron micrograph and, b) scanning electron micrograph images of the FSN-60 silica nanoparticles.

As noted above, it is expected that the silica nanoparticles will be able to provide greater persistence of protection by protecting encapsulated chemicals from environmental breakdown and in the case of the rough nanoparticle types, superior adherence to wool and to the cuticle of insects. Adherence to wool fibres is shown below. The electron micrographs (Figure 3) show the nanoparticles adhering to the wool fibres after water rinsing. This effect appears to be most marked with the C18 nanoparticles (Figure 3c) with the remaining particles more evident than with the smooth and rough particles.

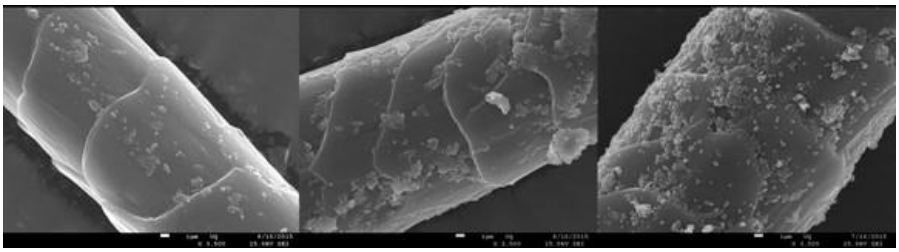


Figure 3. Electron microscope images of nanoparticles adhered to wool after water rinsing; (a) smooth nanoparticles (b) rough nanoparticles (c) RNP- C 18 nanoparticles.

We have also investigated the distribution and cuticular adherence of the different silica nanoparticles following treatment of *L. cuprina* larvae using fluorescence microscopy. Blowfly larvae were exposed to the fluorescein-labelled nanoparticles using a standard larval wool assay whereas sheep body lice were exposed by either being placed in wool that had been dipped in the nanoparticle solutions or by exposing them to a lice diet that had been

treated with the nanoparticles. Figure 4 shows a high density of fluorescein-labelled particles (RNP) in the guts of both first stage blowfly maggots and lice. This indicates that both the insects are ingesting significant amounts of the labelled particles. The feeding habits of both insects would seem to favour active accumulation of particles but whether the particles are attaching to gut lining or peritrophic membrane, or just accumulating as the insects feed is currently unclear.

Cuticular adhesion was also noted in the assays with both blowfly larvae and lice, but the fluorescence was much lower, than in the gut. This is expected as ingestion of particles occurs actively as the insects feed whereas the particles on the cuticle would be acquired passively and presumably more slowly as the larvae or lice contact particles as they move through the wool or on the skin surface. Cuticular electron micrographs for both blowflies and lice suggest that the C18 and rough nanoparticles both adhere more strongly to cuticle than the smooth particles and that the C18 particles adhere more strongly than the rough particles. These results suggest that best effect against both blowfly larvae and lice is likely to be achieved when the nanoparticles are administered with the objective of oral toxicity. However, the rough or C18 particles could also be expected to add to the toxic dose delivered, particularly with purpose designed chemical payload and release characteristics.

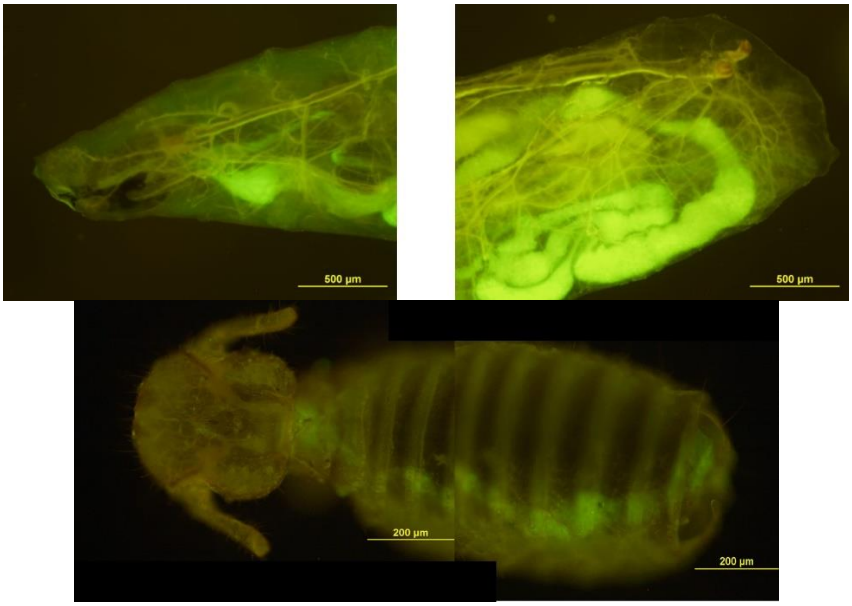


Figure 4. Fluorescein-labelled rough nanoparticles ingested during feeding in assays with first stage sheep blowfly larva (fluorescence in the anterior and posterior sections of the gut shown) and an adult sheep louse.

Testing against sheep blowflies

To test the relative efficacy of different formulations in the presence of environmental influence such as photo-degradation and leaching from the fleece by rainfall a series of laboratory tests with *L. cuprina* larvae have been conducted. Formulations for the tests were dispersed in the carrier compound (water for lipophilic pesticides, hexane for water soluble pesticides) by ultrasonication for 1 hour and applied to wool staples collected from a Merino fleece known to have had no previous chemical treatment. First stage blowfly maggots were then exposed to the treated wool using standard larval assays. To test the effects of photodegradation with the different nanoparticle formulations the treated wool samples were first exposed to ultra-violet radiation by two methods, an artificial UV exposure regime in the laboratory, or extended exposure to natural sunlight on the roof of the EcoSciences precinct in Brisbane (Figure 5).

The incorporation of water soluble chemicals may offer potential for development of a formulation that is strategically released under moist conditions, but which remains inert in the fleece when there is no moisture and therefore no flystrike risk, or which is only released in the insect gut following ingestion. That is, a formulation with a longer presence in the fleece and designed to release only at times and in sites where control is needed.

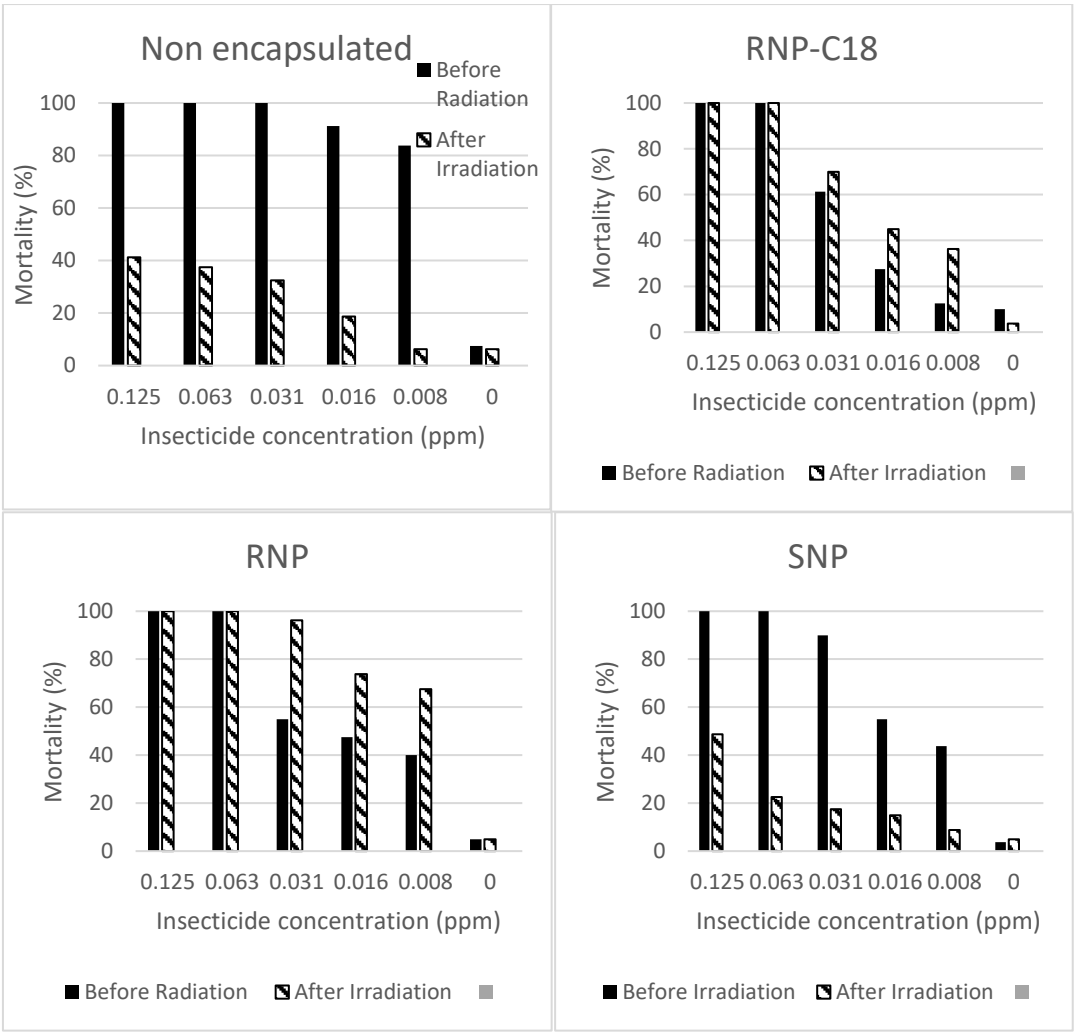


Figure 5. Effect of UV exposure on efficacy of nanoparticle formulations of lipophilic insecticide and a commercial formulation in larval assays.

Figure 5 shows the mortality of larvae exposed to wool treated with nanoparticles containing a lipophilic chemical following exposure of the wool to high-level UV radiation. As with most of the assays conducted, the rough nanoparticle formulations suffered much less degradation, and remained effective against the exposed larvae whereas the effectiveness of the unencapsulated chemical and the smooth nanoparticle formulations larvae was considerably reduced after irradiation.

Figure 6 suggests that the rough-surface particles also assist in reducing leaching of water-soluble chemical from the wool. After the wool samples had been exposed to approximately 6 cm of simulated rainfall on two occasions there was a significant decrease in efficacy of the unencapsulated chemical whereas the decrease was relatively small with the FSN-60 and RNP chemicals.

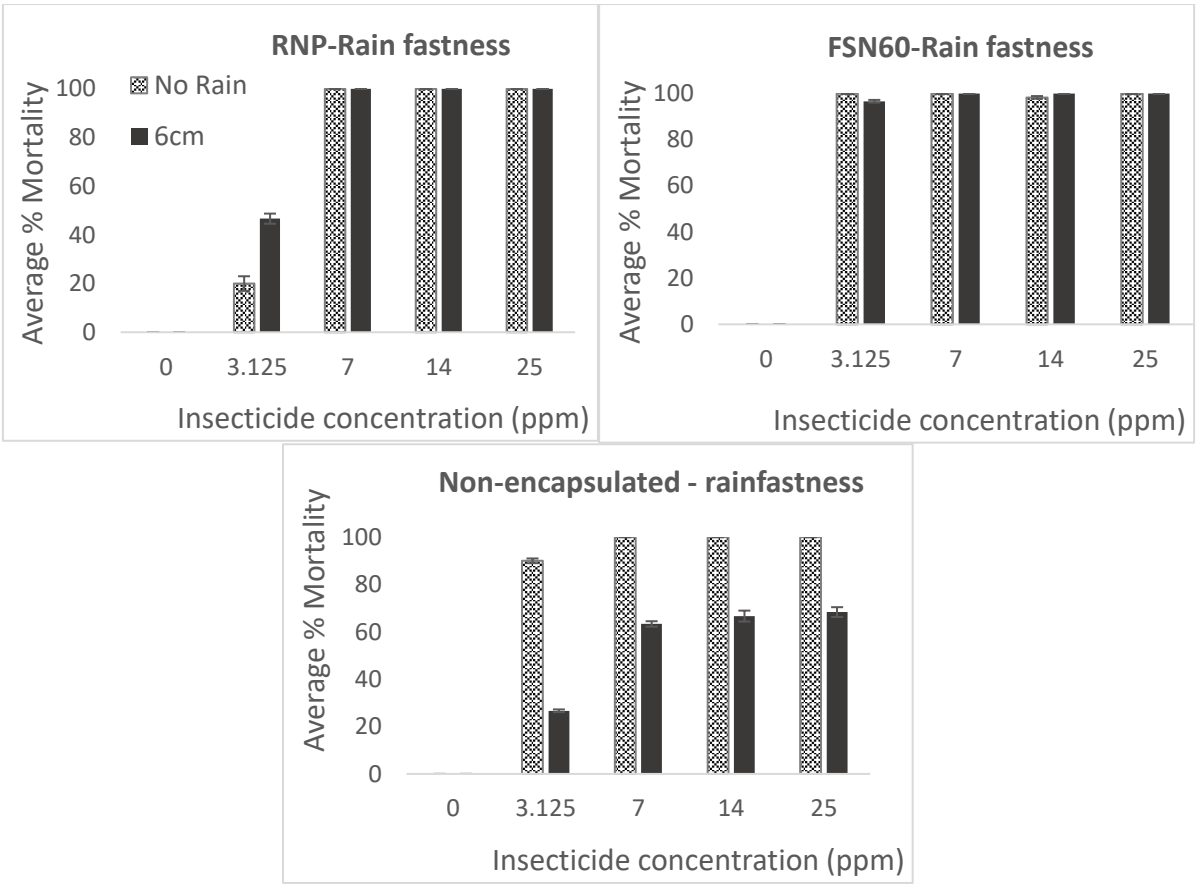


Figure 6. Larval toxicity in assays for rain fastness with wool treated with different formulations of water-soluble pesticide then exposed to simulated rainfall on two occasions.

Low residue chemicals and plant extracts

A large range of plant extracts and other chemical compounds have been shown to have insecticidal and repellent effects against *L. cuprina*. Although these compounds can often give short term protection, their effectiveness is usually rapidly lost due to volatilisation and environmental degradation. However, our results to date suggest that degradation can be significantly reduced by incorporation in rough silica nanoparticles and that appropriate formulation may be able to make their decay profile more favourable for practical use (Figure 7).

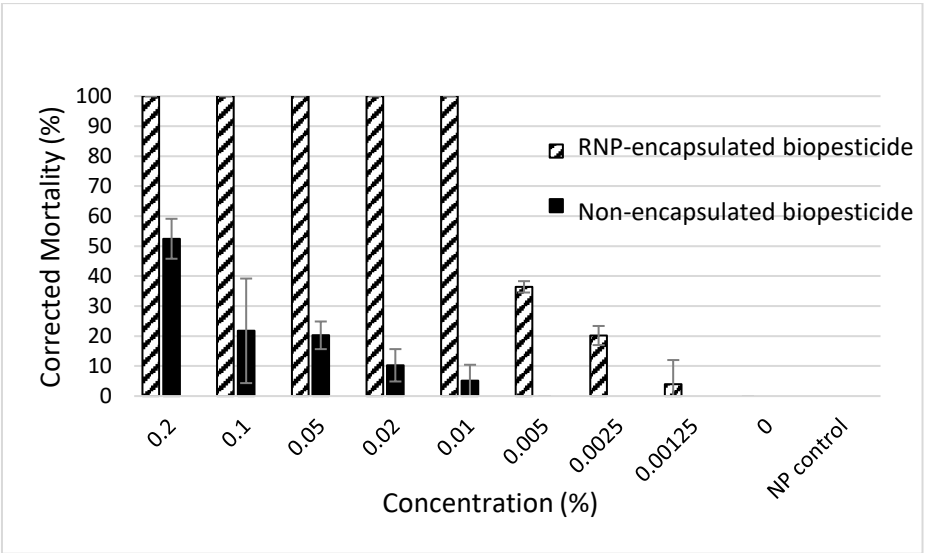


Figure 7. Mortality induced by a photo-labile volatile plant compound presented as free plant extract and encapsulated in rough nanoparticles in first instar *L. cuprina* larval assays.

CONCLUSION

Huge advances in controlled release technology for a wide range of applications, and in particular nanotechnology, offer significant opportunities for the development of new or enhanced sheep blowfly and lice control strategies. Although there have been some studies in this area in the past (Anderson et al. 1989, James et al. 1990, 1994, Rugg et al. 1998) for a range of reasons these have largely not been pursued.

The silica nanoparticles described here are environmentally degradable, have low health risk and importantly can be applied by conventional application equipment. As shown here they provided better protection in the presence of environmental challenge in laboratory tests. Studies are now required to test the behaviour of the particles in the sheep wool-skin environment to see if extended protection can be obtained from these formulations under more practical conditions.

What has long been considered the cardinal rule of toxicity, 'dose makes the poison' has been attributed to Paracelsus, a 15th century Swiss physician. This has more recently been elaborated to 'Dose makes the poison – but formulation is the key'. Nowhere would this seem to be more appropriate than with the possibilities presented by nanotechnology.

REFERENCES

Anderson, N., McKenzie, J. A., Laby, R. H., Strong, M. B., Jarrett, R. G. 1989. Intraruminal controlled release of cyromazine for the prevention of lucilia-cuprina myiasis in sheep. *Research in Veterinary Science* 46: 131-138.

James, P. J., Erkerlenz, P., Meade, R. J. 1990. Evaluation of ear tags impregnated with cypermethrin for the control of sheep body lice (damalinia-ovis). *Australian Veterinary Journal* 67: 128-131.

James, P. J., Mitchell, H. K., Cockrum, K. S., Ancell, P. M. C. 1994. Controlled-release insecticide devices for protection of sheep against head strike caused by lucilia-cuprina. *Veterinary Parasitology* 52: 113-128.

Rugg, D., Thompson, D., Gogolewski, R. P., Allerton, G. R., Barrick, R. A., Eagleson, J. S. 1998. Efficacy of ivermectin in a controlled-release capsule for the control of breech strike in sheep. *Australian Veterinary Journal* 76: 350-354.

Published by Australian Wool Innovation Limited, Level 6, 68 Harrington Street, THE ROCKS, NSW, 2000