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# The influence of size on the toxicity of an encapsulated pesticide: a comparison of micron- and nano-sized capsules

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ABSTRACT

Encapsulation technology involves entrapping a chemical active ingredient (a.i.) inside a hollow polymeric shell and has been applied to commercial pesticide manufacturing for years to produce capsule suspension (CS) formulations with average particle sizes in the micron-scale. The few literature sources that investigate the environmental fate and toxicity to non-target organisms of encapsulated commercially available pesticide products with regard to capsule size report on average sizes between 20 and 50 μm. Here, we have identified a CS formulation with an average capsule size of approximately 2 μm with some capsules extending into the nanometer scale (~200 nm). Determining how carrier size influences toxicity is important to understanding if current pesticide risk assessments are sufficient to protect against products that incorporate encapsulation technology. Here, a commercial pyrethroid CS pesticide with lambda-cyhalothrin (λ-Cy) as the a.i. was separated into two suspensions, a fraction consisting of nano-sized capsules (~250 nm) and a fraction of micron-sized capsules (~2200 nm) in order to investigate the influence of capsule size on toxicity to embryonic zebrafish, *Danio rerio*. Toxicity was evaluated 24 h after exposure to equivalent amounts of a.i. by the presence and severity of pyrethroid-specific tremors, 14 sublethal developmental impacts and mortality. Fish exposed to greater than 20 μg a.i. $L^{-1}$ technical λ-Cy or formulated product experienced curvature of the body axis, pericardial edema, craniofacial malformations, and mortality. Exposure to the unfractionated formulation, micro fraction, nano fraction and technical a.i. resulted in no significant differences in the occurrence of sublethal impacts or mortality; however, the technical a.i. exposure resulted in significantly less fish experiencing tremors and shorter tremors compared to any of the formulated product exposures. This suggests that the capsule size does not influence the toxic response of the entrapped λ-Cy, but the presence or absence of the capsules does. Testing across other encapsulated products is needed to determine if size does not have influence on toxicity regardless of encapsulation technology.



## 1. Introduction

Nanotechnology's involvement in agriculture is not limited to nanoparticulate active ingredients (a.i.), but includes a wide array of formulation chemistries and nanocarriers intended to better protect and disperse already on the market chemical a.i. Nanotechnology-based pesticides include formulations that incorporate nanoscale shells, capsules, coatings, particulate materials such as nano clays, inorganic additives and others (Kah et al., 2013). Pesticides engineered to utilize such complex formulation chemistries have the potential for unforeseen consequences to the environment and public health (Stone et al., 2010; Grillo et al., 2015; Mehrazar et al., 2015).

In pesticide risk assessment, toxicity and exposure are often well understood for the chemical a.i. alone, with little environmental data required to assess the risk of the complete formulation (Surgan and Cox, 2006; Kookana et al., 2014; Mullin et al., 2015). Meanwhile, many pesticide formulations are being developed with novel chemistry and nanotechnology to change the way the a.i. interacts with the environment and biota, limiting the applicability of a.i. specific partitioning coefficients (like $K_{ow}$) and degradation rates for estimating environmental persistence, mobility, bioconcentration potential and other risks after formulated.

Encapsulation technology involves entrapping a chemical a.i. inside a hollow polymeric shell and has been applied to commercial pesticide manufacturing for years to produce capsule suspension (CS) formulations with average particle sizes in the micron-scale. The few literature sources that investigate the environmental fate and toxicity to non-target organisms of encapsulated commercially available pesticide products with regard to capsule size report on average sizes between 20 and 50 μm (Jarvinen and Tanner, 1982; Sibley and Kaushik, 1991; Stejskal et al., 2009). Here, we have identified a CS formulation with an average capsule size of approximately 2 μm with some capsules

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extending into the nanometer scale (~200 nm). Pesticide toxicity, partially due to capsule rigidity and release, can be dependent on particle size (Tsuji, 2001; Roy et al., 2014; Mehrazar et al., 2015), yet little data exists on the toxicity or fate of nano-scale capsules from commercial pesticides. Entrapping the chemical a.i. in a nano-sized polymer capsule has the potential to change the biological distribution and persistence of the chemical, even relative to micron-sized particles of the same composition.

Size is known to influence biological mobility in terms of adsorption, distribution, metabolism and excretion (ADME) (Zolnik and Sadrieh, 2009). For example, the subcellular fate of particles is size dependent with particles greater than 500 nm being engulfed by phagocytes, while smaller particles are taken up by pinocytosis (Zhao, Zhao et al., 2011; Oh and Park, 2014). Polymeric microspheres with diameters between 2 and 3 μm have been shown to exhibit maximal phagocytosis compared to larger and smaller particles of the same composition (Champion et al., 2008). It is likely that entrapping a chemical in a nano-sized organic carrier can result in altered uptake, biodistribution and toxicity compared to submicron-sized organic carriers and the non-encapsulated chemicals.

The growing body of literature on nanoencapsulations for the targeted delivery of therapeutics supports the hypothesis that the toxic response of a chemical can be influenced by the size of its polymeric carrier (Kowalczuk et al., 2014). The Food and Drug Administration (FDA) requires extensive research and development to bring a drug reformulated with a nanocarrier to clinical trials including reevaluation of ADME and toxicity (Zolnik and Sadrieh, 2009). An equivalent safety assessment is not required for nano-sized carriers in pesticide formulations (USEPA, 2015). This is problematic considering the widespread use of these formulations and their inevitable increased presence in surface water and sediment (Stone et al., 2014; Tu et al., 2014; Stehle and Schulz, 2015) and as residues on crops intended for consumption (Ripley et al., 2001).

Size dependent toxicity for inorganic nanoparticles is well documented in the literature (Jiang et al., 2008; Jin et al., 2009; Oh and Park, 2014), but there has yet to be efforts to understand the relative toxicological differences of micron- and nano-sized polymeric capsules of commercial pesticide formulations. Extraction and concentration of nanocapsules from existing pesticide products allows for experimentation into both the risks and benefits of nanoencapsulation technology in relation to currently employed microencapsulation technology. Here, a pyrethroid CS insecticide was separated into two fractions, differing only in size, to investigate the influence of carrier diameter on the toxicity of λ-cyhalothrin (λ-Cy) to embryonic zebrafish. The aim of this paper is to provide some of the first data on the relative toxicity of micro- and nano-sized polymeric capsules that are commercially used as carriers for agricultural pesticides.

Chemical λ-Cy was first marketed in 1985 and in addition to its current use as an agricultural pesticide, it also has registered uses for controlling public health pests (Farmer et al., 1995; WHO, 2013). According to the Environmental Protection Agency (EPA), there are an additional 3500 pyrethrin and pyrethroid products registered in the United States, many of which are also encapsulated formulations and are used globally. Therefore, contamination by encapsulated pyrethroids, including nano-size capsules, in surface water is plausible. Currently, pyrethroids can be detected in natural waters throughout the world after agricultural, urban and residential applications (Weston et al., 2009; Domagalski et al., 2010; Weston and Lydy, 2012; Jabeen et al., 2015; Stehle and Schulz, 2015).

Class II pyrethroids, including λ-Cy are known to have detrimental neurotoxic effects on aquatic organisms (Toumi et al., 2013; Tu et al., 2014), including fish (Bradbury and Coats, 1989; Haya, 1989). As such, we are performing our toxicity assessments with embryonic zebrafish (*Danio rerio*). Zebrafish are commonly utilized for nanotoxicology studies (Harper et al., 2011; Lin et al., 2013; Rizzo et al., 2013) and as a developmental model for nervous system physiology and neurotoxicity studies (Ton et al., 2006; Chopra et al., 2010).

## 2. Materials and methods

### 2.1. Materials

An EPA registered capsule suspension insecticide with 22.8% λ-cyhalothrin was used (EPA Reg. Number 100-1295, Greensboro, NC, USA). Analytical standard grade λ-cyhalothrin [3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethyl-cyano(3-phenoxyphenyl)methyl cyclopropanecarboxylate], 97.8% purity (CAS number 91,465-08-6) was purchased from Sigma-Aldrich (St. Louis, MO, USA). For enzymatic removal of the chorionic membrane of the zebrafish, protease enzyme from *Streptomyces griseus* (cat #81,750) was purchased from Sigma-Aldrich. 3-aminobenzoate ethyl ester methanesulfonate salt (tricaine, cat # A-5040) and dimethyl sulfoxide (DMSO) (CAS number 67-68-5) were also purchased from Sigma-Aldrich.

### 2.2. Isolating and concentrating capsules by size

In order to isolate and concentrate the nano-sized capsules in the commercial CS formulation, the formulation (2.08 lbs. a.i./gallon per product label) was diluted to 1000 mg a.i. $L^{-1}$ with Milli-Q water (Milli-Q Gradient A10 water purification system equipped with a Q-Gard® 2 and a Quantum™ IX Ultrapure Organex cartridge, Millipore Corp., Billerica, MA, USA). Three 10 mL aliquots of the diluted stock were placed in 15 ml tubes and centrifuged for 7 min at 1454 g with a benchtop Eppendorf 5430 centrifuge. For two of the aliquots, the supernatants were collected to represent the nano fraction (NF). The remaining pellets in the two aliquots were resuspended in Milli-Q water, combined and labeled the micro fraction (MF). The pellet and the supernatant of the remaining aliquot were mixed back together to provide an unfractioned formulation (UF) control that contained both the nano and micron-sized capsules which had been subjected to the same centrifugation process as the other fractions. The UF, MF and NF were diluted with Milli-Q water to similar opaqueness and stored in the dark at 4 °C in glass vials.

### 2.3. Fraction characterization

The hydrodynamic diameter, polydispersity index (PDI) and zeta potential of the three suspensions (UF, NF and MF) were measured in triplicate using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) at 25 °C after dilution to 50 mg a.i. $L^{-1}$. Statistical differences between fractions were determined with a one-way ANOVA. To quantify the amount of a.i. in the three suspensions, λ-Cy was extracted from the capsules by mixing with toluene and continually agitating for 1 h. Gas chromatography (GC) analysis was performed on a 1 μL sample with a Varian 3800 GC equipped with an electron capture detector and a 15 m × 0.53 mm ID RTX-200 column. Standard grade λ-Cy was run at 0.02, 0.1, 0.5, 1.0, and 2.0 mg $L^{-1}$ and a calibration curve was generated before analysis of the samples. Samples were diluted to fit within the curve. The primary size and capsule morphology was examined using a FEI Quanta 600 FEG (FEI Co., Hillsboro, OR, USA) scanning electron microscope (SEM) operating at 15 kV using samples prepared by dropping 20 μL of each suspension onto a Si substrate and drying before imaging.

### 2.4. Embryonic zebrafish assay

Adult zebrafish (*D. rerio*) were maintained at the Sinnhuber Aquatic Research Laboratory at Oregon State University. Zebrafish embryos were collected from group spawns of wild-type 5D fish. To eliminate possible exposure differences from the pores of the chorionic barrier, at 6 h post fertilization (hpf) the embryos were dechorinated with