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Microencapsulated BioBullets for the Control of Biofouling Zebra Mussels

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The widespread invasion of freshwaters by the zebra mussel, *Dreissena polymorpha*, during the last 2 decades has made it one of the world's most economically and ecologically important pests. Since arriving in the North American Great Lakes in the 1980s, zebra mussels have become a major biofouler, blocking the raw water cooling systems of power stations and water treatment works and costing U.S. $1−5 billion per year. Despite the development of numerous control methods, chlorination remains the only widespread and licensed technique. Zebra mussels are able to sense chlorine and other toxins in their surrounding environment and respond by closing their valves, thus enabling them to avoid toxic effects for up to 3 weeks. Furthermore, prolonged dosing of chlorine in raw water produces ecotoxic trihalomethanes (THMs) by reaction with organic material in the water. We have developed a novel, environmentally safe, and effective method for controlling the zebra mussel: the BioBullet. Our method uses the encapsulation of an active ingredient (KCl) in microscopic particles of edible material. The mussels' natural filtering ability then removes and concentrates the particles from the water, without stimulating the valve-closing response. By using the mussels' filtering behavior to concentrate BioBullets the absolute quantity of active ingredient added to the water can be reduced substantially. Our approach allows us to engineer the particles to break up and dissolve completely within a few hours, thus eliminating the risk of polluting the wider ecosystem. We demonstrate that the effectiveness of a toxin in the control of biofouling filter-feeders can be enhanced greatly by using our technique. This paves the way for a new approach to the control of some of the world's most important economic pests.

Introduction

The introduction of nonnative taxa into novel localities represents one of the greatest threats to the world's ecosystems and economies (*1*−*3*). One of the most well-known examples comes from the invasion of the zebra mussel, *Dreissena polymorpha*, into the Laurentian Great Lakes of North America during the 1980s (*4*). Zebra mussels are unusual among freshwater bivalves in possessing byssus which enables them to attach to hard substrates and form encrustations many individuals deep (*5*). Rapid population growth and invasion is assisted by high fecundities and the possession of planktonic veliger larvae that can disperse passively in the water column for up to 4 weeks before settling (*6*).

Zebra mussels can lead to system-level changes in invaded ecosystems and have led to local extirpation of some species of North American unionid mussels (*7*, *8*). For industry, zebra mussel biofouling of pipelines that carry raw water can be devastating. In North America, numerous power plants have experienced fouling and blockage of the heat exchange pipes, screenhouses, steam condensers, and trash bars (*9*). In Britain, the recent spread of zebra mussels (*10*) has resulted in many water treatment works experiencing blockage of microstrainers and pumps, the occlusion of pipes, and the compromising of filter bed efficiency (*11*). In Spain, where zebra mussels were discovered in the Ebro River in 2001 (*12*), many thousands of kilometers of irrigation pipeline are threatened by zebra mussel fouling (J. Insausti, Government of Aragon, Spain, 2003, personal communication). In North America alone, zebra mussels are estimated to cost industry ca. U.S. $1−5 billion ($10^9$) each year (*1*, *13*).

Considering the immense economic cost of zebra mussels, it is unsurprising that much effort has been put into developing control strategies (*6*). Physical removal, generally using high-pressure water jets, is only feasible within sections of industrial facilities where ready access is possible. Antifoulant coatings (e.g., copper-based) may offer practical preventative measures for new facilities or retrofitted screens but are difficult to apply to existing pipelines. Biological control using natural enemies offers an attractive option, and while fish and crayfish can regulate zebra mussel populations under some circumstances (*14*, *15*), there appear to be no grounds for expecting the development of a practicable biological control method in the foreseeable future. Chemical control options are favored by industry because treatment can be applied throughout the entire facility from a single dosing point. Many chemicals will kill zebra mussels given sufficient concentration and contact time, but the suitability of a particular chemical is determined by considerations of water quality (e.g., residual concentrations, byproducts), cost, and practicality. Chemicals which have been tested to some success include chloramines, chlorine dioxide, ozone, hydrogen peroxide, potassium permanganate, pH adjustment, and inorganic salts, such as KCl (*6*).

While numerous physical and chemical techniques have been proposed and tested, chlorination remains the only widespread and licensed option (*6*). However, chlorination poses a number of problems for industry and regulators. First, chlorine reacts with organic material in the water to produce trihalomethanes (THMs) which are toxic to humans and other animals. This restricts greatly the chlorine doses that can be applied to water in infested water treatment works. Second, zebra mussels respond to unfavorable environmental conditions by closing their valves for prolonged periods (*6*). This means that control agents, such a chlorine in the form of sodium hypochlorite, must be dosed continuously for up to 3 weeks to have their desired effects. Third, hypochlorite is rather expensive and hazardous to transport, store, and handle. Fourth, chlorine dosed into pipelines that exit into open ecosystems can impact deleteriously on nontarget biota in the recipient waters. Indeed, many of the chemicals used

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to control zebra mussels in the Laurentian Great Lakes have been found to have greater toxicity to nontarget organisms, such as fishes (*16*).

The economic and ecological problems associated with current control options for zebra mussels illustrate that there is urgent need for the development of improved approaches. This urgency is heightened by continued tightening of regulatory controls on the discharge of toxins into the environment (e.g., the European Water Framework Directive, directive 2000/60/EC; *17*).

We describe here a new and novel approach to zebra mussel control: the BioBullet. The approach involves the microencapsulation of an active ingredient, that is toxic to zebra mussels, with particles that are edible to zebra mussels. Use of microencapsulated particles has the potential to overcome the rejection and valve-closing response generally seen when zebra mussels are exposed to toxic substances. An adult zebra mussel will filter particles of 0.7−200 μm (*18*; D. Aldridge, personal observation) and process 1−2 L of water per day (*19*). By delivering the active ingredients in particulate form it is possible to monopolize on this filtering activity so that the zebra mussels concentrate the product from the water column. The use of microcapsules has the potential to not only reduce greatly the volumes of conventional toxins, such as sodium hypochlorite, that are required for effective kills but also opens the opportunity for using toxins which were previously unavailable for zebra mussel control, such as water soluble products. Furthermore, microencapsulation can use products that are designed to degrade rapidly and disperse before entering recipient waterbodies, thus minimizing the pollution of the wider ecosystem.

Materials and Methods

Particle Formulation. Controlled release particles were commercially manufactured using a modified spray drying process (TasteTech Ltd., Bristol, U.K.). A premix slurry was prepared containing the encapsulant and active ingredient under conditions of controlled shear. The premix was pumped into an ultrasonic atomising nozzle at the top of a cooling chamber. The atomized particles formed perfect spheres, cooling as they fell to the bottom of the chamber. Further cooling of the particles was achieved in an air-conveying system before discharge via cyclone to a fluid bed processor. The encapsulated particles were then coated with nonionic surfactant to aid dispersion in water. Further cooling in the fluid bed removed all heat of crystallization from the product prior to packaging.

The active ingredient used in this study was potassium chloride (KCl), a salt that at low doses is inert to most organisms, including fish (*16*), but which is particularly toxic to freshwater bivalves because of their low body fluid concentrations (*20*). Potassium ions interfere with membrane integrity, respiration, and ciliary activity on the gills of zebra mussels (*21*, *22*). Potassium ions can also inhibit the contraction of the posterior adductor muscle, so preventing zebra mussel valve closure. The specific toxicity of KCl to zebra mussels is unclear; Waller et al. (*16*) quote a 48 h LC50 of 150 mg/L, but McMahon (*23*) suggests application of KCl at 50 mg/L for 48 h results in 100% mortality. The toxicity of chemicals can differ considerably between conventional static tests and flow-through tests (*24*), with the greater mortality in static tests being attributed to the depletion of oxygen and the buildup of metabolic products in experimental vessels (*25*). As experiments in our study were conducted in a flow-through system, preliminary toxicity trials for KCl on zebra mussels were performed in the experimental apparatus and indicated that 12 h dosing at 300 mg/L KCl produced little or no mortality.

Composition of the particles included 30% w/w potassium chloride; >10% w/w vegetable oil and vegetable wax; <10% w/w magnesium stearate, silicon dioxide, mono- and diglycerides of fatty acids, nonionic surfactants, palmitic acid. The BioBullets had near neutral buoyancy in water.

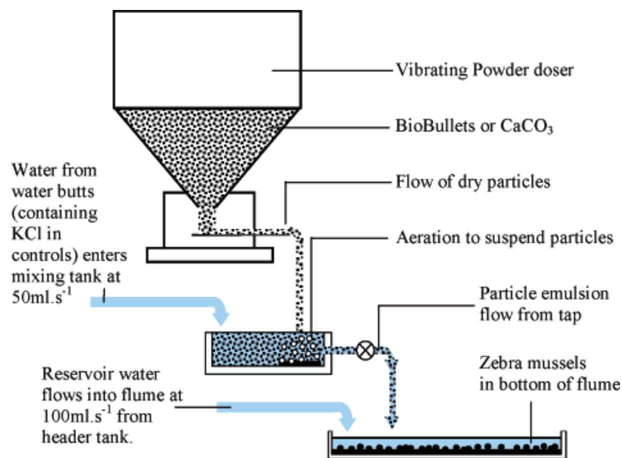

FIGURE 1. Schematic of the dosing system used to deliver BioBullets into the flume apparatus.

Particle Assessment. The BioBullets were analyzed using scanning electron microscopy. Freeze-fractured samples were prepared in order to assess internal structures by placing BioBullets in liquid nitrogen before striking with a sharp blade. The response of live zebra mussels to BioBullets was investigated by inserting a video endoscope through the inhalant siphon after the mussels had been exposed to BioBullets for ca. 5 min.

Field Trials. Zebra mussels, *D. polymorpha*, were collected from the walls of a water treatment works in north London, U.K. They were then placed into a 1 cm^2 mesh bag and washed in untreated reservoir water, removing smaller mussels and any dirt that could potentially contaminate the experiment. The mussels were allowed to infest 10 20 cm $\times$ 5 cm steel plates in the bottom of each of 10 open flow-through flumes. Mussels (mean length 20.5 $\pm$ SE 0.1 mm) were evenly placed on each steel plate and left for 2 days before the commencement of experiments. Immediately before the experiments any dead mussels were removed, leaving a density of 52.4 $\pm$ SE 1.6 mussels per plate.

Each flume consisted of a 4 m long pipeline with a basal width of 5 cm. Untreated reservoir water was pumped into the top of each flume from a 4 m^3 header tank. Water was maintained at a depth of 5 cm using a weir system and maintained at a constant flow of 100 mL/sec using regulatory taps.

Five replicate experimental flumes and five replicate control flumes were individually and concurrently dosed continually for 12 h with BioBullets or control microcapsules. BioBullets were dosed at 1 g/L, equivalent to 300 mg/L KCl. Control microcapsules comprised an inert $CaCO_3$ core with the same thickness and composition of coating material as BioBullets. Control microcapsules were also dosed at 1 g/L, along with the continuous addition of 300 mg/L KCl direct to the water.

All microcapsules were initially in powder form and so were first mixed briefly with reservoir water to aid dispersion (Figure 1). The dry particles were supplied into mixing tanks from a 40 L vibrating powder doser (Coote Vibratory Ltd., Fareham, Hants.). The particles were mixed with reservoir water, or reservoir water with KCl in controls, by placing a large airstone connected to an air pump into each tank. The resulting particle suspension then flowed by gravity from taps on the side of each mixing tank into each of five flumes at a rate of 10 mL/s. At a time of 24 and 48 h after the commencement of dosing, two steel plates were removed at random from each flume. Dead and live mussels were

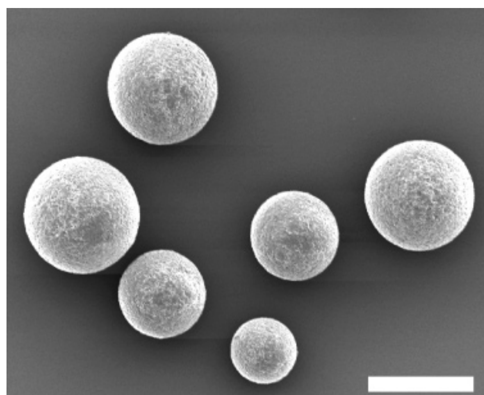

FIGURE 2. Scanning electron micrograph (SEM) of BioBullets. Scale bar is 100 μm.

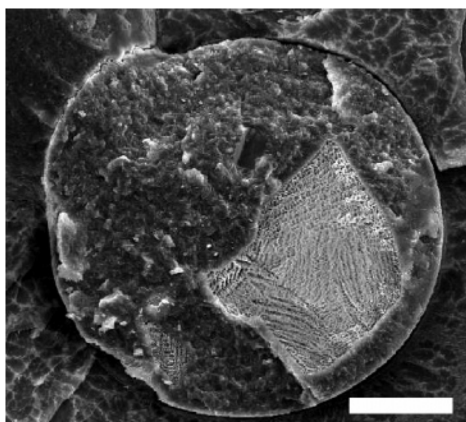

FIGURE 3. SEM of a freeze-fractured BioBullet to reveal a cubic KCl crystal within the vegetable oil matrix. Scale bar is 20 μm.

counted. The water temperature for the duration of the experiments was approximately 22 °C. Water entering the water treatment works during the experimental period had mean pH 8.6 ± 0.1 SE, turbidity 1.1 ± 0.2 FTU, conductivity 673 ± 3 μS/cm, TON 5.2 ± 0.2 mg/L, chlorophyll *a* 11.9 ± 2.5 μg/L.

Impact on Nontarget Biota. The potential impact of BioBullets (with KCl incorporated) on the biota of recipient waters was assessed by exposing the native unionid mussel, *Anodonta anatina*, to BioBullets. A total of 120 *A. anatina* (mean length 45.9 ± SE 3.2 mm) were collected from the River Great Ouse, Ely, U.K. (National Grid Reference TL553801). Twelve individually aerated 500 mL aquaria were dosed with BioBullets at the same concentration as that used in the flume trials to kill zebra mussels. A further 12 control aquaria were aerated without the addition of BioBullets. After 3 h of aeration (the time needed to allow the BioBullets to dissociate according to Figure 4) five *A. anatina* were placed into each of the 24 aerated aquaria. After 12 h of further aeration (the time for which a recipient water body would receive dissociated BioBullets following dosing at an in-service pipeline) the number of mussels in each aquarium that displayed protruding siphons was counted. The water was then exchanged for clean, aerated, dechlorinated tap water, and the survival of *A. anatina* was monitored for a further 7 days. The water temperature for the duration of the experiment ranged from 21 to 22 °C, and mussels were exposed to a 12 h light−dark cycle.

Results and Discussion

The BioBullets were spherical and had a mean diameter of 105.4 ± SD 59.6 μm, thus matching the size of particles typically removed from the water column by zebra mussels

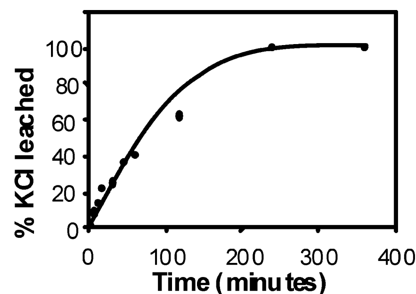

FIGURE 4. Dissociation curve for BioBullets in water. KCl loss was calculated from water conductivity, using a standard curve of mass of KCl against conductivity.

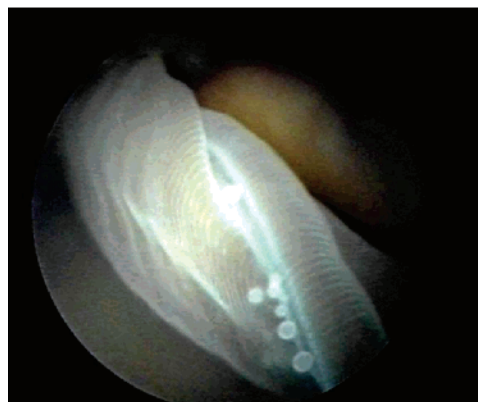

FIGURE 5. Endoscopic photograph of BioBullets being transported along the ctenidial gill of a live zebra mussel.

(*18*) (Figure 2). Freeze fracture revealed the presence of cubic KCl crystals embedded within the encapsulant matrix (Figure 4). KCl loss was calculated from water conductivity, using a standard curve of mass of KCl against conductivity. The particles leached 90% of their KCl within ca. 150 min (Figure 4), thus quickly dissolving into concentrations nontoxic to zebra mussels. Studies using video endoscopy showed that the microcapsules were transported by the zebra mussels along the ctenidial gills toward the labial palps and mouth (Figure 5). BioBullets were transported both within the marginal food groove, from which they are likely to be ingested (*26*), and also within the superficial mucous strings above the food groove, from where they are likely to be rejected (*26*). BioBullets could be seen through the visceral mass within the gut, indicating that some had been ingested. It is likely that the smallest BioBullets were those ingested because Sprung and Rose (*18*) found that zebra mussels preferentially ingest particles in the size range of 15−40 μm. We may therefore expect to increase the efficiency of BioBullets in the control of zebra mussels by refining our manufacturing process to produce particles within this smaller size range.

A significantly greater proportion of zebra mussels died in experimental flumes (59.9 ± SE 6.6%) compared with control flumes (2.6 ± 1.0%), and there was no further mortality between 24 and 48 h, by which time the BioBullets had leached all their KCl (two-way ANOVA following arcsine transformation, BioBullets vs control $F_{1,16} = 66.91$, $P < 0.001$; 24 vs 48 h $F_{1,16} = 0.14$, $P = 0.716$, interaction $F_{1,16} = 0.05$, $P = 0.823$) (Figure 6).

While a single 12 h dosing of BioBullets did not achieve a 100% kill, this is very much in line with expectations. A proportion of zebra mussels will not be feeding at a given time and so may avoid exposure during a one-off dosing period. We may therefore predict that a second dosing of BioBullets would result in a greater overall mortality (e.g., 84% compared with 60% overall mortality).

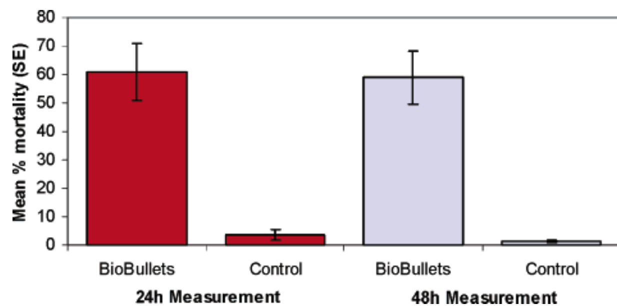

FIGURE 6. Mortality of zebra mussels in flume trials using BioBullets (dosed for 12 h at 1 g/L) and controls (dosed for 12 h at 1 g/L $CaCO_3$ particles + 300 mg/L KCl).

The BioBullets which had been allowed to dissociate for 3 h had no effect on the native unionids. After 12 h of exposure there was no difference in the number of *A. anatina* displaying extended siphons between the experimental (median 3.5/5) and control (3.0/5) aquaria (Mann−Whitney $W = 148.0$, $P = 0.926$). We therefore assume that experimental mussels were not actively avoiding the water containing dissociated BioBullets. After a further 7 days in clean water, no *A. anatina* had died in either the experimental or control aquaria. As unionid mussels are likely to be some of the most sensitive filter-feeding organisms in recipient waters (*27*) we conclude that BioBullets containing KCl and dosed at the levels used in these experiments are unlikely to impact on nontarget biota.

The impact on nontarget biota is further reduced in the formulation of BioBullets used in this study because the active ingredient (KCl) is particularly toxic to zebra mussels. Fisher et al. (*28*) found that KCl produced no mortality in mosquitofish (*Gambusia affinis*) or snails (*Helisoma* spp.) at concentrations that caused complete mortality in zebra mussels. Waller et al. (*16*) reported similarly high toxicity of KCl to zebra mussels compared with that to rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), and the threehorn wartyback unionid mussel (*Obliquaria reflexa*). While Fisher et al. (*28*) did find that the unionid *Anodonta* imbecillus was more sensitive to KCl than other nontarget taxa, our experiments suggest that by supplying KCl in microcapsules we can reduce the overall KCl doses to levels below which unionids are deleteriously affected. The overall exposure to toxins of nontarget organisms, such as unionids, is further reduced by the relatively short-term, one-off dosing regime that is possible with BioBullets.

BioBullets have the potential to offer a viable alternative to chlorination in the control of zebra mussels, with the advantages of requiring only a one-off treatment rather than continuous dosing and using a soluble nontoxic active ingredient which overcomes the problems of environmental pollution and the production of harmful THMs. By monopolizing on the natural filtering ability of zebra mussels to remove and concentrate BioBullets from the water column, we are able to reduce dramatically the concentration of any toxin we choose to encapsulate, compared with simply dosing directly into the water.

Our results clearly support our hypotheses regarding the benefits of encapsulation in the control of zebra mussels. Now that we have established proof of principle we are in a position to begin testing different active ingredients and coating products, refining our dosing regimes, and scaling up to commercial trials. A number of important considerations are needed in the future development of BioBullets. For example, microcapsules need to balance the advantages of a small size (ideally 15−40 μm) with the lower core/coating ratio of smaller particles. Different active ingredients can be trialed (e.g., proprietary molluscicides such as Clam-Trol and Calgon H130), and these will influence the appropriate coating techniques and the stability of the products. Furthermore, the specific dosing environment will affect the performance of the BioBullets and products may need to be tailored to different applications. For example, more robust particles may be required in conditions of high turbulence; particles with faster dissociation rates will be needed for shorter pipelines; higher doses may be required at lower temperatures (*29*).

It is envisaged that commercial application of BioBullets would involve a one-off end of season treatment (i.e., between September and October). End of season treatments are already used by most European facilities and many North American facilities (*6*) and assume that the plant system can tolerate one season's worth of zebra mussel fouling before treatment. Delivery of BioBullets could use existing chemical dosing points and current supply chains thus minimizing the disruption to the facility. As BioBullets will contain active ingredients which already enjoy regulatory approval for zebra mussel control, we foresee few problems with gaining necessary consents. While it is currently too early to make accurate assessments of the likely cost of supplying BioBullets, we are confident that the benefits of a relatively short dosing period, combined with the requirement for relatively little product and the absence of storage and handling hazards, will make BioBullets an economically competitive alternative to hypochlorite.

There is considerable opportunity for diversification of our technology, bringing further economic and environmental benefits. Other filter-feeding pests may be controlled, such as Asian clams (*Corbicula* spp.) (*30*) and disease-carrying blackfly larvae (*Simulium* spp.). In addition, microcapsule cores containing nutrients and growth promoters could enhance the productivity of commercially harvested bivalve beds.

Acknowledgments

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