EFFICACY OF SELECTED NATURAL AND SYNTHETIC NOVEL ORGANIC COMPOUNDS IN PREVENTION OF ZEBRA MUSSEL (*DREISSENA POLYMORPHA*) MACROFOULING BY BYSSAL ATTACHMENT INHIBITION

by

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ABSTRACT

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The invasive, freshwater, bivalve, *Dreissena polymorpha* (zebra mussel) was introduced to North America in the Great Lakes in 1986 and has since spread throughout the waterways of the eastern and central United States and southeastern Canada. This species has imposed escalating economic burdens through its macrofouling of submerged structures and raw water systems. Increasing restrictions on the chemical mitigation and control methodologies used to prevent their macrofouling, particularly organometallic or oxidizing chemicals, continue to stimulate

research efforts to develop effective yet environmentally benign antifouling agents against this species and other macrofouling organisms. The efficacy in inhibiting zebra mussel byssal thread attachment was assessed for 29 potential antifouling compounds with structural or receptor ligand similarities to the active ingredient in hot peppers, capsaicin, and its analogue, anandamide. The acute lethality effects of these natural products were also tested on the non-target freshwater crustacean, *Daphnia magna,* at varying compound concentrations.

Of nineteen compounds structurally similar to capsaicin, N-vanillylnonanamide (compound B) and N-benzoylmonoethanolamine benzoate (compound L) in addition to capsaicin itself (compound A) were shown to be potent inhibitors of mussel byssal attachment with extrapolated EC_{50} values in the micromolar range. Zebra mussel exposure to the remaining ten anandamide-like compounds demonstrated that O-2050 (compound Y), noladin ether (compound Z), CP 55,940 (compound AA), and AM630 (compound AB) significantly inhibited mussel byssal attachment, with estimated EC_{50} values also in the micromolar range. For the majority of compounds, mussels fully recovered capacity for byssal attachment 48 h post exposure. In contrast, three compounds, L, Z and AB, exhibited negative physiological impacts on zebra mussels leading to post-treatment byssal attachment inhibition and/or mortality. In general, none of the compounds deemed efficacious were lethal to *D. magna* at the minimal concentrations which inhibited mussel byssal attachment.

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CHAPTER 1

INTRODUCTION

1.1 Historical overview

Since their unintentional translocation from Europe into the American Great Lakes region by way of contaminated ballast water discharge from international ships in the 1980's (Hebert et al., 1989), zebra mussel (*Dreissena polymorpha)* populations are now well established in 22 states and seven major river systems east of the Rocky Mountains and in the Provinces of Ontario and Quebec, Canada. In a short span of approximately 9 years (1986-1995), zebra mussels spread eastward through Lakes Erie and Ontario into the St. Lawrence River from their original infestation in Lake St. Clair, MI (Hebert et al., 1989) as well as into isolated lakes in six bordering states (McMahon, 1996). Because the dispersal of *D. polymorpha* through European inland waterways has been well documented over the last 170 years, this species' explosive colonization of, and dispersal through, North American inland waterways was anticipated. However, the almost immediate invasion of waterways surrounding a major U.S. industrial hub was not predicted, as rapid zebra mussel dispersal via attachment to barge hulls was unforeseen (Morton, 1997). Although the spread of zebra mussels has greatly slowed in Europe over the last 50 years, this species continues to disperse westward through North America inland waters (McMahon and Bogan, 2001).

1.2 Zebra mussel dispersal

Dreissena polymorpha has several adaptations for dispersal, foremost being a pelagic planktonic veliger stage which can be passively carried long distances downstream on water currents. However, byssal thread drifting, in which special threads are secreted as draglines to catch the current and help carry the animal suspended in the water column, may be the primary dispersal method for post-settlement juveniles and small adults. Additionally, mussel attachment to floating debris, macrophytes or floating bubbles facilitates passive zebra mussel relocation on water currents (Carlton, 1993; Mackie and Schloesser, 1996). Human-mediated mechanisms represent a major means of intercontinental dispersion as well as between unconnected drainage systems. Mussels encrusted on the bottoms of ships or barges plying freshwaters for example, or veliger contaminated ballast water in ocean-going vessels can transport individuals over large distances (Mackie and Schloesser, 1996). Sport fishing gear, including boats on trailers, bait buckets and bait lines, may aid the spread of veligers, post-veligers, and attached adults over shorter distances (Carlton, 1993) and are a potential major vector for the dispersal of zebra mussels into the western U.S. Such dispersal by recreational boating has recently been evidenced by the introduction of the related quagga mussel (*Dreissena rostriformis bugensis*) into Lake Mead, Nevada, which is not connected to inland waterways navigable by barges (D. Britton, U.S. Fish and Wildlife Service, personal communication).

1.3 Economic impacts

The consequences of zebra mussel colonization are of great biological and economic concern, as they represent one of three top invasive molluscs in the U.S. along with the Asian clam and the shipworm (Pimentel et. al, 2005). *Dreissena polymorpha* is one of the few freshwater bivalve species with an epifaunal lifestyle, attaching to submerged solid surfaces including wood, concrete, plastic, metal and even other organisms (Claudi and Mackie, 1994), making this species an undesirable biofouler. It successfully inhabits freshwater lakes and rivers and colonizes industrial cooling ponds, irrigation systems and quarry reservoirs in massive numbers (Mackie and Schloesser, 1996). Particularly at risk to zebra mussel biofouling are the intake structures and piping of the raw water systems of utilities, water processing plants and other industries such as paper processing plants and agricultural facilities drawing water from a freshwater source. According to a recent report, zebra mussel aggregation on interior pipe walls was the cause of an Oklahoma farmer's failing field irrigation system (Everett Laney, U.S. Corps of Engineers, personal communication). Overall, it has been estimated that total annual zebra mussel damage and control costs approach a staggering $1 billion in the United States alone (Pimentel et al., 2005).

Zebra mussels are capable of achieving population densities of 15,000 adults$\cdot m^{-2}$ on artificial substrates (Garton and Haag, 1993) and upwards of 400,000$\cdot m^{-2}$ on natural substrates in lakes (MacIsaac, 1991). Massive zebra mussel fouling occludes the intake structures and piping of raw water systems in industrial, power plant (electric and

nuclear), municipal and agricultural facilities. Serious impairment of internal water flow disrupts overall plant production and endangers the proper functioning of vital raw water systems, requiring periodic plant shut downs to remove or mitigate fouling zebra mussel masses. In a similar fashion, infestation of water treatment plants results in potable water tainted by mussel feces, pseudofeces and decaying bodies. Mussel attachment to steel and iron substrates such as pipes exacerbates their corrosion, increasing maintenance and replacement costs. Even after the removal of mussels from fouled surfaces, it has been proposed that remaining attached byssal threads may disrupt laminar flow (McMahon and Tsou, 1990; Claudi and Mackie, 1994) as well as stimulate veliger settlement, form veliger attachment sites and support further substrate corrosion (Claudi and Evans, 1993). Fouling by *D. polymorpha* compromises the fuel efficiency of ships and barges due to the sheer weight of mussel colonies on their hulls and by increasing their resistance to passage through water. Mussel fouling can also negatively impact operation of commercial fishing gear and trap nets by increasing their resistance to water flow and causing them to sink (Claudi and Mackie, 1994). Mussel fouling has also been reported to cause navigation buoys to sink creating a major hazard for commercial ships and barges on inland waterways (Miller et al., 1992).

1.4 Biological impacts

The biotic impacts of zebra mussels are less costly but nonetheless ecologically destructive. Most notably, native unionid populations have been shown to decline by as much as 80 percent (University of Wisconsin Sea Grant Institute, 2000) as a result of infestation of the posterior ends of unionid shells by aggregations of zebra mussels.

Maximum infestations were reported to be 10,500 mussels per unionid potentially creating a 4-6 cm thick layer of mussels accumulating on those portions of the unionid shell exposed above the substratum (Schloesser et al., 1996). Zebra mussel-infested unionids die of starvation, predation and/or toxicant exposure as their locomotion, normal valve movements, siphon functioning, and ability to filter feed are impaired by of the accumulation of mussels on their shells (Claudi and Mackie, 1994; Schloesser, et al., 1996; Byrne et al., 1995).

Sport and commercial fisheries may eventually suffer from the disruption of fish habitats caused by zebra mussel infestation of lakes and rivers. As *D. polymorpha* dominates and enriches the benthic zone, the euphotic zone increases due to clarification of surface waters through the mussels' filtration capability which removes all particulate material including phytoplankton and bacteria from the water column (Claudi and Mackie, 1994; University of Wisconsin Sea Grant Institute, 2000). Although aesthetically pleasing, increasing water clarity leading to a deepening euphotic zone reflects a reduction in the density of the phyto-bacterioplankton community which, in turn, reduces energy flow through the pelagic community food chain eventually reducing the biomass of the stocks of large, pelagic, upper trophic level commercial and sport fish species (Claudi and Mackie, 1994). Furthermore, this physical and physiological disruption of aquatic habitats may interfere with freshwater restoration and development projects. On a smaller scale, storms and other environmental disturbances can periodically cause large mussel population losses near lake and river recreational facilities resulting in shorelines littered with sharp-edged

mussel shells, which are both unsightly and dangerous, particularly for swimmers and individuals participating in other forms of beach recreation.

1.5 Impact on human health

Overpopulation by *D. polymorpha* can also impact human health. As a consequence of their extensive filtering capacities and high body fat content mussels tend to bioaccumulate high body burdens of heavy metals and other organic pollutants. Zebra mussels can accumulate these agents to levels 10 times greater than occur in native mollusks (University of Wisconsin Sea Grant Institute, 2000), achieving tissue concentrations 300,000 times above environmental background levels (Ohio Sea Grant College Program, 2000). Thus, feeding on zebra mussels by higher trophic level predators could increase the rate of transport of toxicants through the food chain leading to restrictions on human consumption of aquatic species, notably of upper trophic level commercial and sport fish species, in certain polluted waters.

1.6 Life history

Zebra mussels have adaptations typically associated with r-selected invasive species (i.e., species with life history traits that specifically adapt them for life in unstable habitats) (McMahon, 2002; McMahon and Bogan, 2001). Among these characteristics, *D. polymorpha* reproduces by external fertilization. Adult zebra mussels exhibit a high fecundity; mature females typically produce 30,000-40,000 eggs in a year (McMahon and Bogan, 2001) with egg production increasing exponentially with age up to 1 million eggs/female by the second year of life (Griffiths, 1993). The fertilized egg produces a swimming veliger larval form, unique amongst freshwater bivalves. Other

bivalve species spawn eggs from which are hatched larger, more advanced, non-swimming larvae or juveniles resembling miniature adults. After living for two to three weeks as plankton suspended in the water column, planktonic zebra mussel veligers metamorphose into a pediveliger form which settles and attaches to solid substratum via byssal threads and develops into the juvenile (Carlton, 1993; Claudi and Mackie, 1994).

As is characteristic of r-selected invasive species, the growth rates of juvenile mussels are rapid, averaging 0.10-0.15 mm/day up to as much as 0.5 mm/day leading to early sexual maturity, usually within one year, at a relatively small size (8 mm shell length). Zebra mussels also have very short life spans in which adults rarely survive beyond three years of life (Claudi and Mackie, 1994; McMahon, 2002; McMahon and Bogan, 2001). The r-selected characteristics of a short life span, high fecundity, and rapid growth allow zebra mussels to achieve very high densities after invading a new habitat. They also allow zebra mussel populations to recover rapidly after human mediated or natural reductions in their population densities (McMahon, 2002). It is also clearly these life history characteristics which account for the zebra mussel's capacity to rapidly foul raw water systems (McMahon 1996).

1.7 Mode of attachment

The typical zebra mussel shell is triangular with a tapered dorsal aspect. This shape is an adaptation which prevents predator dislodgement from the surface to which it is attached by byssal threads (Claudi and Mackie, 1994). The flattened ventral surface accommodates close adherence to the substratum with the aid of proteinaceous byssal threads produced from a gland on the ventral side of the mussel just posterior to

the foot. A fluid byssal protein is secreted from this gland which flows down a groove on the posterior margin of the ventrally extended foot and forms an attachment plaque at the point where the tip of the foot is applied to the substratum (Clarke and McMahon, 1996). The fluid byssal protein hardens to form a strong thread. Production of multiple attachment threads in this manner allows the mussel to resist dislodgement generated by natural water currents, wave action and the prying behaviors of predators (Claudi and Mackie, 1994; Clarke and McMahon, 1996). Mussels can also release from their attachment by secreting an exogenous enzyme that dissolves byssal threads (Clarke and McMahon, 1996). Auto dislodgement and subsequent reattachment allows mussels to disperse to more favorable microenvironments within mussel masses or to leave the substratum and be carried by water currents to new environments. Such dispersal behavior is particularly prevalent in juvenile and small adult mussels (Claudi and Mackie, 1994). Thus, the byssus is an integral characteristic for the success of zebra mussels as an invasive and macrofouling species (Morton, 1993; Claudi and Mackie, 1994; McMahon and Bogan, 2002).

1.8 Chemical control methods

No control method to date has been successful in impeding the dispersal of zebra mussels in the inland waters of the United States. Nor has a method been developed for their eradication once they have become established in natural water bodies. In contrast, there have been a number of technologies developed for prevention and control of *D. polymorpha* macrofouling of raw water systems. However, no single methodology has been universally successful. For this reason, application of several

methodologies in combination is often recommended to mitigate and prevent mussel macrofouling of municipal and industrial facilities (Mussalli et al., 1992; Claudi and Evans, 1993; Claudi and Mackie, 1994; Mead and Adams, 1993). Currently, control and mitigation methods for *D. polymorpha* have been categorized as chemical (oxidizing and nonoxidizing), physical or non-chemical (for general review, see Mussalli et al., 1992; Claudi and Mackie, 1994). Overall, oxidizing chemical treatments are primarily effective in preventing the settlement of post-veliger stages, whereas non-oxidizing chemicals in combination with mechanical methods are used to periodically mitigate established adult zebra mussel fouling populations (Claudi and Mackie, 1994).

Application of oxidizing chemical agents such as chlorine has been the primary biofouling control method utilized in a majority of industrial facilities for decades (Mussalli et al., 1992). Chlorine in most forms can be used to induce adult mortality and prevent veliger settlement, making this agent the preferred short-term oxidizing agent (Mussalli et al., 1992; Benschoten, et al., 1993; Claudi and Mackie, 1994). However, remedial measures relying on chlorine additives produce carcinogenic by-products (i.e., trihalomethanes) and can have detrimental effects on non-target species (Mussalli et al., 1992). Alternatively, this category contains potential chlorination replacements, such as ozone and bromine, which pose a less severe environmental threat but are handicapped by reduced efficacy and increased cost relative to chlorine (Mussalli et al., 1992). Chlorination methods in some utilities have been replaced with less hazardous bromine application that does not produce trihalomethanes (Mussalli et

al., 1992) but, although bromination is an effective molluscicide at relatively low concentrations, particularly at a pH above 8.0 where chlorination is ineffective, it requires the implementation of complex generating equipment thereby increasing operating costs (Mussalli et al., 1992; McMahon et al., 1993). Current remedial measures utilizing oxidative compounds are far from ideal, exhibiting environmental and non-target species toxicity, substantial economic costs, reduced efficacy, exacerbation of metal corrosion or a combination thereof.

Non-oxidizing chemical strategies include the use of proprietary molluscicides or potassium salts which under specific conditions and application strategies can be more efficacious than oxidizing biocides in mitigation and control of zebra mussel macrofouling. They are not known to promote corrosion or system component damage and generally do not produce toxic by-products (McMahon et al., 1993). Furthermore, non-oxidizing agents kill mussels more quickly than oxidizing molluscicides at approved application concentrations (Claudi and Evans, 1993). For this reason, they are the preferred means of short-term chemical mitigation of zebra mussel macrofouling relative to oxidizing biocides (Mussalli et al., 1992). The potential for environmental persistence and restricted EPA approval for general use of non-oxidizing molluscicides limits the use of these compounds in zebra mussel control strategies to periodic mitigation rather than continuous application to prevent veliger settlement. Thus, they are often used in conjunction with oxidizing treatments to periodically kill settled mussels that are resistant to continuous or semi-continuous low-level application of oxidizing agents most commonly employed to minimize pediveliger settlement and

subsequent development of fouling adult populations (Mussalli et al., 1992; McMahon et al., 1993).

1.9 Antifouling coatings

Antifouling coatings represent a subcategory of chemical control methods targeting exposed structural surfaces at risk for zebra mussel attachment (for general review, see Claudi and Mackie, 1994). In use for centuries, coatings containing active ingredients such as metals, metal oxides and organometallic copper, zinc and tin combinations have been effective in the prevention of zebra mussel macrofouling (Race and Kelly, 1996). Typically, leaching of an impregnated biocide or deterrent into the surrounding water is the primary mechanism of action resulting in prevention of pediveliger settlement and, thus, development of adult zebra mussel fouling communities. In some cases, biocide leached from these coatings can have strong detrimental impacts on non-target species (Race and Kelly, 1996). For this reason, effective yet highly toxic organotin (TBT) containing coatings have been officially banned from use in Europe and Canada, and will be in the U.S by 2008 (Marine Paint, 2005; Watermann, 1999). In contrast, relatively non-toxic, irritant antifouling coatings such as those leaching capsaicin-based compounds (i.e., the irritating substance in hot peppers), have been developed in response to the need for environment-friendly antifoulant substances. However, when subjected to field testing against zebra mussel settlement, few of these coatings demonstrated both reasonable efficacy and environmental friendliness (Race and Kelly 1996). Failure of such hot pepper coatings

has been attributed to the relatively low water-solubility of capsaicin and, thus, poor leaching capabilities (Race and Kelly, 1996).

In contrast to antifouling coatings, foulant-release coatings prevent adherence of macrofouling organisms by utilizing low surface tension silicone or epoxy surfaces without release of toxic ingredients into the environment. These coatings work by reducing the strength of byssal attachment to their surface and by sloughing-off of surface material with attached macrofouling organisms. In a long term study of the effects of commercially available non-toxic coatings, including those with red pepper additives, only the soft, ablative silicone had documented efficacy (Gross, 1993). Unfortunately, the disadvantages of foulant-release coatings are significant, including the requirement for periodic manual cleaning, multiple layer application, periodic regeneration and increased material costs (Mussalli et al., 1992; Race and Kelly, 1996).

The non-chemical mitigation category encompasses many creative technologies ranging from simple sand or mechanical filters and thermal flushes, to more complex robotic or diver mediated manual removal and ultrasonic frequency treatment of mussel colonies. While physical control/prevention methodologies can be useful, they have the drawback of having to be specifically designed for, and tailored to, facilities on an individual basis.

1.10 Natural product control methods

In an effort to replace chemical and organometallic antifoulants with less toxic or nontoxic alternatives, current research efforts have focused on a wide variety of natural products, repellants and biogenic biocides for example, agents used as defensive

mechanisms by sponges, corals, algae, seaweeds and aquatic bacteria to repel or inhibit adhesion of biofouling organisms (Nandakumar and Yano, 2003; Watermann, 1999). As a result of millions of years of natural selection, biogenic antifoulants possess ideal characteristics for commercial use as they are generally efficacious, and targeted in their activity and readily biodegradable (Steinberg, 2001). Natural product antifoulants, however, are currently underutilized and underdeveloped in the majority of countries worldwide. Germany estimates that <1% of market share goes to biocide free antifouling products (Watermann, et al., 2004).

Current studies report successful reduction in biofouling using zosteric acid, an eelgrass extract that prevents bacterial adhesion (Callow and Callow, 2002; Sundberg et al., 1997), diterpenoid lipids extracted from corals which prevents barnacle attachment (Clare, 1995) and furanones, potential barnacle inhibitors, isolated from red algae (Clare, 1995). Studies investigating other non-toxic alternatives to organometallic biocides include catemine neurotransmitters, shown to inhibit barnacle larval settlement at non-lethal concentrations (Dahlstrom et al., 2000; Marine Paint, 2005) and enzymes disrupting the glue protein binding mechanism (Marine Paint, 2005). Taylor and Zheng (1995) have documented the repellant activity and latent mortality of a brown algal extract incorporated in a silicone-based coating on the marine blue mussel, *Mytilus edulis*.

Other work has demonstrated that exposure to capsaicin, the component in chili peppers generating a hot sensation (Cope et al., 1997; John A. Schetz, unpublished data, 2003), and a structural analogue of capsaicin, anandamide, inhibited zebra mussel

byssal attachment (John A. Schetz, unpublished data, 2003). Like capsaicin, anandamide is a vanilloid receptor (VR1) agonist in addition to a cannabinoid receptor agonist in humans (DiMarzo, et al., 1998, 2001) and other mammals (Elphick and Egertova, 2001). Furthermore, anandamide has been extracted from the tissues of an invertebrate deuterostomous sea urchin, *Paracentrotus lividus* (Bisogno et al., 1997). It has also been isolated from five species of marine bivalves (Sepe et al., 1998) and the leech, *Hirudo medicinalis* (Matias et al., 2001), suggesting the presence of an endocannabinoid system in protostomous as well as deuterostomous invertebrates. Based on these findings, the goal of this research was to investigate the potential efficacy of other compounds structurally related to capsaicin or with known cannabinoid receptor activity in inhibiting byssal attachment in zebra mussels and for their possible lethality towards the non-target species *Daphnia magna.*

CHAPTER 2

PREVENTION OF ZEBRA MUSSEL BYSSAL ATTACHMENT BY CAPSAICIN-LIKE ANALOGUES

2.1 Introduction

Dreissena polymorpha (Pallas, 1771), the zebra mussel, is a non-native, macrofouling, freshwater bivalve mollusc unintentionally translocated from Europe into the American Great Lakes region via ship ballast water discharge in the 1980's (Hebert et al., 1989). Zebra mussel populations are now well established in 22 US states (USGS, 2006) and continue to disperse westward through North American inland waters (McMahon and Bogan, 2001). Highly adapted for rapid dispersal (Carlton, 1993; Mackie and Schloesser, 1996), *D. polymorpha* has exhibited explosive colonization in North America, aided by their capacity for byssal attachment to the hulls of commercial ships, barges or recreational boats plying navigable freshwaters (McMahon and Bogan, 2001).

Hull fouling by *D. polymorpha* increases the resistance of ships and barges to passage through water resulting in increased fuel costs and transport time. Particularly at risk to zebra mussel biofouling are intake structures and piping of raw water systems in electrical power utilities, water processing plants and other industries utilizing freshwater sources such as paper processing plants and agricultural facilities. Zebra mussel occlusion of intake structures and piping can result in serious impairment of water flow, requiring periodic plant shut down to remove or mitigate mussel fouling. It

has been estimated that total annual zebra mussel damage and control costs approach $1 billion in the United States alone (Pimentel et al., 2005).

The byssus is an integral characteristic of zebra mussels as an invasive, macrofouling species (Morton, 1993; Claudi and Mackie, 1994; McMahon and Bogan, 2001). Mussels attach to hard surfaces by means of proteinaceous byssal threads produced from a gland on the posterior side of the proximal portion of the foot from which a fluid byssal protein is secreted down a posterior groove to harden into a strong attachment thread (Clarke and McMahon, 1996). Production of multiple byssal threads prevents dislodgement by natural water currents, wave action and prying predators (Claudi and Mackie, 1994; Clarke and McMahon, 1996).

Oxidizing and non-oxidizing chemical agents have been developed for mitigation and control of *D. polymorpha* macrofouling, however many of these chemicals have detrimental environmental impacts on non-target species, preventing continuous or long-term use (Mussalli et al., 1992; Claudi and Mackie, 1994; Mead and Adams, 1993). Chlorine is the most frequently used oxidizing agent for short-term zebra mussel biofouling prevention (Mussalli et al., 1992; Benschoten, et al., 1993; Claudi and Mackie, 1994). However, the production of carcinogenic by-products and negative impacts on non-target species limits its general use (Mussalli et al., 1992). Similar environmental impacts can also occur with more recently developed non-oxidizing molluscicides (Mussalli et al., 1992).

Antifouling coatings leaching toxic chemicals are also used to inhibit zebra mussel macrofouling. However, their release of toxins into aquatic habitats, such as

occurs with organotin (TBT) coatings, have lead to restrictions in their use in European and North American freshwaters (Watermann, 1999). Non-toxic, foul-release coatings in which coating surface properties inhibit strong byssal attachment have the disadvantage of being expensive and having relatively short operational life spans (Mussalli et al., 1992; Race and Kelly, 1996). As an alternative strategy, relatively non-toxic, irritant antifouling coatings such as those leaching capsaicin-based compounds (i.e., the hot substance of chili peppers), have been developed as more environment-friendly antifoulant applications. However, when field tested against zebra mussel settlement, none of these alternative coatings demonstrated both reasonable efficacy and environmental friendliness. The observed failure of capsaicin-containing coatings to inhibit zebra mussel byssal attachment resulted from the inability of capsaicin molecules embedded in such coatings to expose their active moiety or moieties to settling zebra mussels at the coating surface (Race and Kelly, 1996).

Yet recent studies have demonstrated that laboratory exposure to capsaicin solutions inhibited byssal attachment in zebra mussels (Cope et al., 1997; John A. Schetz, unpublished data, 2003). Based on this finding, this study investigated 18 other compounds structurally related to capsaicin for potential efficacy in inhibiting zebra mussel byssal attachment. The lethality of three compounds found to be efficacious in inhibiting byssal attachment was also tested against a non-target fresh water species, *Daphnia magna* Straus, 1820.

2.2 Materials and methods

2.2.1 Collection and maintenance of test animals

Specimens of zebra mussels (*Dreissenia polymorpha*) were collected periodically over 2004-2006 from the Rose Bud Marina on Lake Oologah, Roger County, Oklahoma, (36.4203°N, 95.6665°W) and immediately transported attached to original substrata to the laboratory at The University of Texas at Arlington in insulated containers under cool, moist conditions. In the laboratory, mussels were held in 946 L circular fiberglass tanks filled with continuously aerated and filtered dechlorinated City of Arlington tap water (DTW). Tank water was initially at the temperature of collection and subsequently lowered to 5 °C over 24-48 h. Mussels were thereafter held unfed at 5°C for a maximum of 6 months prior to experimentation with minimal loss of condition (Chase-Off, 1996; Cope, et al., 1997). Prior to testing, mussels were habituated to the 15°C experimental temperature in DTW for a minimum of 48 h in a constant temperature incubator.

Table 2.1 Zebra mussel holding tank physical parameters.

PARAMETER	**RANGE**
Ammonia (ppm)	0-0.25
Nitrate (ppm)	20-40
Nitrite (ppm)	0-0.5
Hardness (ppm)	120-250
Alkalinity (ppm)	80-120
pH	6.8-8.1
O_2 Saturation (%)	89.7-100.6
O_2 Saturation (mg/L)	11.36-12.60
Temperature (◦C)	5.0-7.0

Table 2.2 Nineteen compounds structurally similar to capsaicin utilized for evaluation of anti-fouling efficacy in zebra mussels, (*Dreissena polymorpha)* and lethality to *Daphnia magna*. Compounds (Cmp) are identified by letter designations in the body of the text. Some compounds were not assigned (N/A) CAS registry numbers.

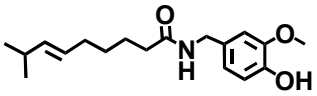
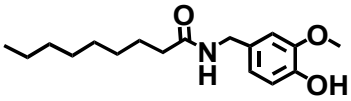
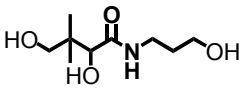
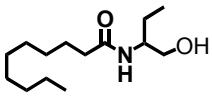
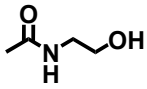
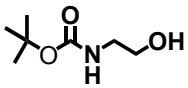
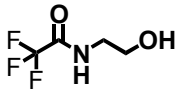
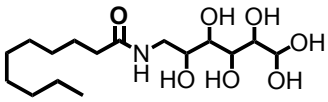
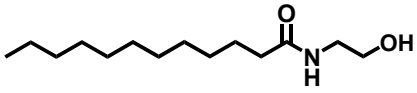
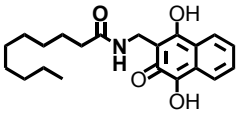
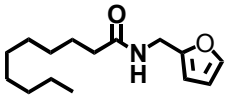
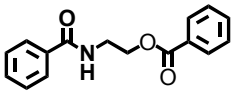
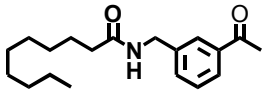
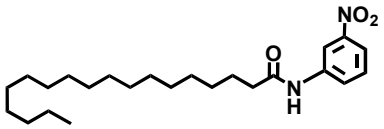
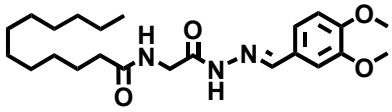
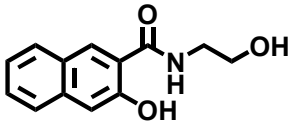
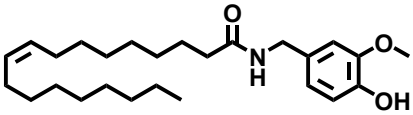
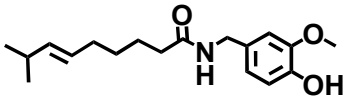
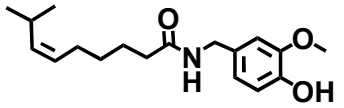
Cmp	Chemical Name	Chemical Structure	Comments
A	Capsaicin (8-Methyl-N-vanillyl-trans-6-nonenamide) purified from a natural product source *CAS: 404-86-4, MW: 305.42*		Active component of hot sauce. VR1 receptor agonist.
B	N-vanillylnonanamide *CAS: 2444-46-4, MW: 293.4*		Pseudocapsaic in: Synthetic derivative. VR1 agonist. Weak CB1 agonist
C	D-panthenol *CAS: 81-13-0, MW: 205.25*		Alkyldihydroxyl Potential CB2 receptor agonist.
D	N-(1-(hydroxymethyl)propyl) decanamide *CAS: 23054-74-2, MW: 243.39*		Shorter alkyl. Interposed ethyl.
E	N-acetylethanolamine *CAS: 142-26-7, MW: 103.12*		No lipophilic portion
F	N-Boc-ethanolamine *CAS: 26690-80-2, MW: 161.2*		Short branched alkyl.
G	Trifluoroethanolamine *CAS: 6974-29-4, MW: 157.09*		Short bulky lipophilic.
H	N-Decanoyl-N-methylglucamine *CAS: 85261-20-7, MW: 349.46*		Shorter alkyl. Multiple hydoxyls.

Table 2.2 Continued

Cmp	Chemical Name	Chemical Structure	Comments
I	N-acetylethanolamine (NAE 12:0) *CAS: 142-26-7, MW: 103.12*		Short unsaturated chain similar to pseudocapsaicin
J	N-(4-hydroxy-2-oxo-1,2-dihydro-3-quinolinyl)octanamide *CAS: 184536-28-5, MW: 302.38*		Shorter alkyl. 2,4,5 electronegative.
K	N-(2-furylmethyl)decanamide *CAS: N/A, MW: 251.37*		Shorter alkyl. Cyclic ether.
L	N-benzoylmonoethanolamine benzoate *CAS: N/A, MW: 269.3*		Benzyl. Benzoate.
M	N-(3-acetylphenyl)decanamide *CAS: 549484-38-0, MW: 289.42*		Shorter alkyl. Acetylbenzyl.
N	N-(3-nitrophenyl)octadecanamide *CAS: 143269-81-2, MW: 404.6*		Shorter alkyl. Nitrophenyl.
0	N-(2-(2-(3,4-dimethoxybenzylidene)hydrazine)-2-oxoethyl)dodecanamide *CAS: N/A, MW: 419.57*		Does not appear to have “stable” activity
P	2-hydroxy-3-naphthoic acid ethanolamide *CAS: 92-80-8, MW: 231.25*		
Q	Olvanil *CAS: 58493-49-5, MW: 417.62*		Synthetic cmpd. VR1 agonist. Weak CB1 agonist. Inhibits cmpd T degradation.
R	Capsaicin (E-isomer), trans-isomer *CAS: 404-86-4, MW: 305.41*		Bioactive isomer at VR1
S	Capsaicin (Z-isomer), cis-isomer *CAS: 25775-90-0, MW: 305.41*		Inactive isomer at VR1

Water conditions in holding tanks were tested weekly by colorimetric methods (Mardel Freshwater Test Kit®, Virbac Animal Health, Fort Worth, Texas) for assessing standard water quality parameters including nitrate, nitrite, ammonia, pH, hardness, and alkalinity. Dissolved O_2 concentrations were determined with a YSI Model 58 Oxygen Meter® (Yellow Springs Instrument Company, Yellow Springs, Ohio). Physical holding parameters were well within published tolerance limits for *D. polymorpha* (McMahon, 1996; Table 2.1).

Specimens of *D. magna* utilized in lethality testing were laboratory cultured at 20-23°C in DTW. They were fed a suspension of dried brewer's yeast and *Spirulina* fish food twice weekly (Clare, 2002). Specimens were fed a minimum of 2 h prior to use in lethality testing bioassays (USEPA, 2002).

2.2.2 Test chemical preparation

Due to their partial lipophilic nature, all experimental compounds required solubility testing. Compounds not directly dissolving in DTW test medium at a concentration of 30 μM, were tested for solubility in a range of non-aqueous solvents in the following order: methanol (MeOH); dimethylsulfoxide (DMSO); dimethylformamide (DMF); ethanol (EtOH); 95%:5% DMSO:1 N HCl; and 95%:5% DMSO:1 N NaOH. Once dissolution was achieved, the solution was diluted with DTW to a final test concentration of 30 μM where possible. Only compounds remaining fully in solution after dilution in DTW, as evidenced by lack of precipitation, were used for testing. Tested compounds and their chemical structures are listed in Table 2.2.

2.2.3 *Byssal attachment screening*

Immediately following habituation to 15°C, eight to ten groups of 16 mussels <20 mm in shell length (defined as the maximal distance from the tip of the umbos to the posterior shell margin) were severed from their byssal attachments with a scalpel. Each group of 16 mussels was equally divided between two new 240 ml Ziploc® polypropylene containers and subsequently exposed to 200 ml of a 30 μM concentration of each test compound. For some relatively insoluble compounds, exposure concentrations were less than 30 μM (Table 2.1, compounds J = 3.3 μM and N = 9 μM). This procedure was repeated in triplicate, for a total of 48 individuals tested for each compound.

All test chambers along with an untreated DTW control and non-aqueous solvent control were maintained at 15°C (±0.5°C) in a refrigerated constant temperature incubator. Following a 48-h exposure, the numbers of mussels byssally attached, living but unattached, or dead were recorded. Individuals byssally connected to each other or the walls of the container were considered attached. Of mussels remaining unattached, those with open valves not closing when gently prodded with the bristles of a fine tipped brush were considered dead (Matthews and McMahon, 1999). The shells of unattached living mussels were marked with a single dot of fast-drying enamel paint from a hobby paint pen (Krylon® Paint Pen). Attached mussels were cut from their byssus with a scalpel, after which all previously attached and unattached living individuals were placed in a new container with 200 ml of untreated DTW that was held at 15°C for a further 48-h post-exposure period. After the 48-h post-exposure period,

the number of byssally reattached, living unattached or dead mussels were determined as described above.

Compound efficacies were assessed in terms of the following parameter: p_{c1} = probability of mussels being unattached given that they were alive after exposure to a compound "c" during the initial exposure period (1) which was estimated by the ratio of the number of unattached (and alive) animals divided by the number of alive unattached or attached animals after 48 h of exposure to 30 μM of the compound or less if the compound was not soluble at 30 μM. The p_{c1} parameters were compared across the compounds with a one-way-ANOVA-like analysis using a Wald statistic (Koch et al., 1985) to test the H_o of no difference in p_{c1} across all compounds. Post hoc pair-wise comparisons using a Scheffe-type procedure (Koch et al., 1985) were used for pair-wise comparison of p_{c1} across the set of tested compounds. Compound residual effects, estimated by the parameter, $p_{c2,}$ were defined as in $p_{c1,}$ but for the 48-h post-exposure period (2) and estimated with the same analysis used for p_{c1}.

Comparisons of the exposure vs. post-exposure periods (i.e. of p_{c1} versus p_{c2}) involved the same individuals, requiring repeated-measures to be incorporated into the analysis. More specifically, the correlation in the exposure and post-exposure responses by individuals had to be accounted for. Details of this analysis are provided in Appendix A.

2.2.4 Concentration- response curves

Concentration-response curves were developed for compounds shown in prior screening (see above) to inhibit byssal attachment. The selected compounds were

Capsaicin (A), N-vanillylnonanamide (B), and N-benzoylmonethanolamine benzoate (L) (Table 2.2). In these tests, mussels were exposed to 8-9 treatment concentrations ranging from that known to inhibit attachment based on initial screening to concentrations as low as 0.01μM (i.e., 0.01-150 μM). Methodology at each tested concentration was that described for the initial screening tests above.

Concentration response curve data were used to estimate the effective concentrations for 50% and 90% sample byssal attachment inhibition (i.e., EC_{50} and EC_{90}) for each compound. The same method was also used to estimate lethal concentrations for 50% and 90% sample mortality (i.e., LC_{50} and LC_{90}) for compound L which induced greater than 50% sample mortality at higher exposure concentrations. Specifically, for a given q varying between 0 and 100, the EC_q value (termed "effective concentration") for a compound was the concentration of that compound required to produce q% unattachment after a 48-h exposure. An LC_q value (termed "lethal concentration") for a compound refers to the compound concentration resulting in q% mortality after a 48-h exposure.

In the EC analysis, the target parameters were as follows: $p_1(c, d)$ = probability of being unattached if alive on exposure to compound c at concentration = d, during phase 1 of the experiment, which was estimated, for the given compound "c" and concentration "d", by the ratio of the number of individuals unattached (but alive) after a 48-h exposure to the compound, divided by the total number of both living, attached and unattached individuals. Probit regression analysis (Fahrmeir and Tutz, 1994) was used to model the probit transform (inverse of the standard normal distribution function)

of $p_1(c, d)$ as a linear function of $\log_{10}$ (concentration), allowing the EC_q values to be determined by a standard technique. For example, the EC_{50} value for compound "c" is the value of "d" at which $p_1(c, EC_{50}) = ½$, assuming that the linear probit model gives: probit $p_1(c, EC_{50}) = b_0 + b_1 * \log_{10}(EC_{50})$. Since $p_1(c, EC_{50}) = ½$ and probit $(½) = 0$, the EC_{50} was determined by the following equation: $\log_{10}(EC_{50}) = -b_0/b_1$.

The LC analysis was similarly performed using these defined target parameters: $p_1(c, d)$ = probability of dying during exposure to compound c at concentration = d, during phase 1 of the experiment, which was estimated, for the given compound "c" and concentration "d", by the ratio of the number of dead individuals after a 48-h exposure to the compound, divided by the total number of living and dead individuals; $p_2(c, d)$ = conditional probability of dying during exposure to compound c at concentration = d during phase 2 of the experiment provided individuals survived phase 1, which was estimated for the given compound "c" with concentration "d", by the ratio of the number of dead individuals after 48-h withdrawal of the compound, divided by the total number of living individuals surviving phase 1.

The maximum likelihood estimates of b_0 and b_1 were generated by the GENMOD procedure in the SAS package, and plugged into the equation, $\log_{10}(EC_{50}$ [or $LC_{50}]) = -b_0/b_1$, to yield the EC_{50} [or LC_{50}] estimate. The delta method (Serfling, 1980) was used to compute the standard error of the EC_{50} and LC_{50} estimates. Identical methods yielded the EC_{90} and LC_{90} estimates.

Once the set of ECq estimates were obtained for compounds in this study, where q = 50 or 90, a Wald test was undertaken to compare ECq (and LCq) for each fixed q,

across the compounds (Serfling, 1980). Post-test attachment recovery (i.e., comparison of pre- versus post-test probability of not forming a byssal attachment in living individuals) was analyzed by categorical modeling using SAS procedure CATMOD. Post-test mortality (i.e., comparison of pre- versus post-test probability of individuals dying) was analyzed by the same method.

2.2.5 Daphnia magna lethality screen

The three compounds subjected to concentration response testing were also evaluated for lethality to the freshwater cladoceran crustacean, *Daphnia magna,* a commonly used toxicity test species with a relatively high sensitivity to a wide range of toxic chemicals (USEPA, 2002). Three replicates of 20 adult individuals of *D. magna* were exposed to eight to nine concentrations of efficacious compounds A, B and L (Table 2.2) identical to those used in byssal attachment concentration response testing. For lethality testing, specimens of *D. magna* were maintained for 72 h in 40 ml of test medium at 15°C under the same conditions and in the same containers described above for mussel byssal attachment testing. Numbers of alive and dead individuals were recorded every 24h and dead individuals were removed at each observation. Death was defined as lack of limb movement and heart beat as observed under a binocular light microscope.

Daphnia magna survival probabilities at 24, 48 and 72 h were estimated by Probit Regression (Fahrmeir and Tutz, 1994) for each compound at each tested concentration. Lethal concentration values (i.e., LC_{50} and LC_{90}) were estimated using the same methods employed in the mussel byssal attachment response versus compound

concentration studies described above. Probit Regression (Fahrmeir and Tutz, 1994) with both concentration and compound type as predictors was utilized to compare survival probabilities by concentration across tested efficacious compounds. A multiple logit model (Koch et al., 1985) was used to compare the survival probabilities at 24, 48 and 72 h for the same compound and exposure concentration.

2.3 Results

2.3.1 Byssal attachment screening

Wald test analysis of byssal unattachment screening data revealed that there was no statistical difference ($p > 0.05$) between the probability of byssal unattachment in mussels held in water and vehicle control treatments after the initial 48-h treatment, after the subsequent 48-h recovery period, or between these two periods. Thus, the impacts of tested compounds on byssal attachment were only compared to results for water controls.

Scheffé pair wise comparison testing demonstrated that when mussels were exposed to a single high concentration (≤ 30 μM) of the tested chemical for 48 h, the p_{1c} parameter (i.e., probability of byssal unattachment) was statistically indistinguishable ($p > 0.05$) between the water controls and compounds C, E, F, G, H, J, K, M, N, O, P, and Q (Fig. 2.1A-C). Differences between p_{1c} (exposure period) versus p_{2c} (recovery period) were also statistically insignificant for these compounds (Fig. 2.1A-C), thus they were deemed non-efficacious. Significant differences in the probability (p_{1c}) of byssal unattachment ($p < 0.05$) relative to water controls were recorded for compounds A (Capsaicin: 8-Methyl-N-vanillyl-trans-6-nonenamide), B (N-vanillylnonanamide), D

(N-(1-(hydroxymethyl) propyl) decanamide), L (N-benzoylmonoethanolamine benzoate), R (E or trans-isomer of Capsaicin), and S (Z or cis-isomer of Capsaicin). For these six compounds, probability (p_{2c}) of byssal unattachment during the subsequent 48-h recovery period was not statistically different ($p < 0.05$) than that of water controls suggesting that compound exposure had no lasting impact on byssal attachment ability (Fig. 2.1A-C).

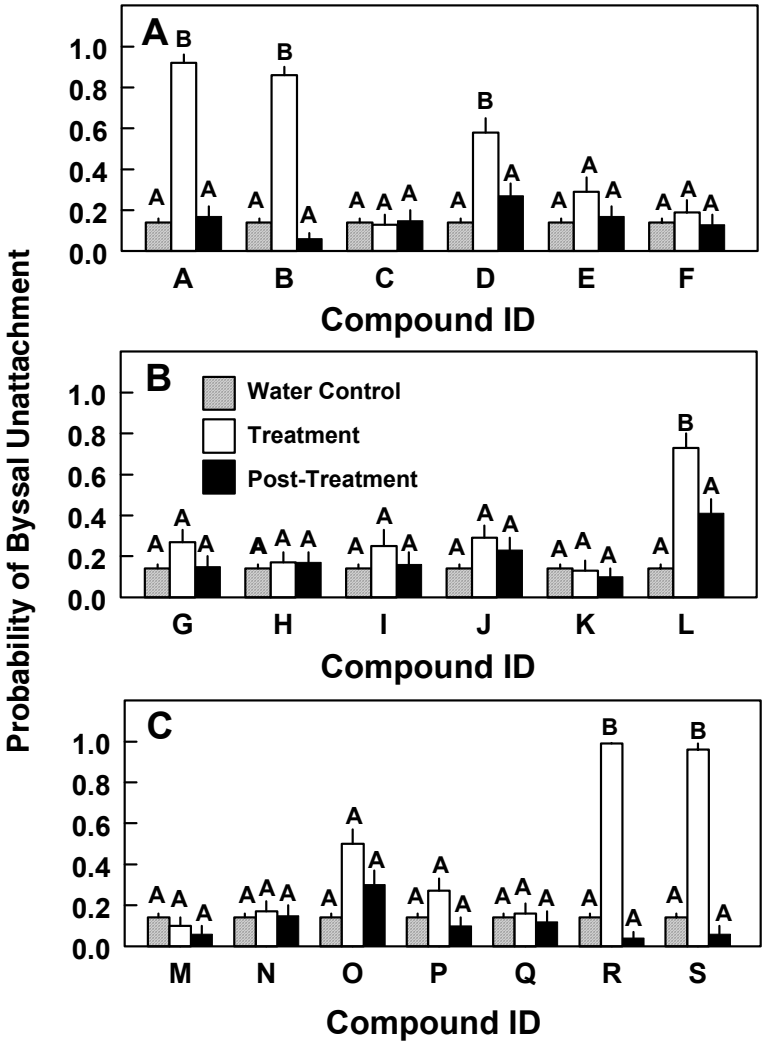


Figure 2.1A-C Probability of byssal unattachment after 48 h of exposure to 19 capsaicin-like compounds at concentrations of 30μM or less (depending on solubility), and after a subsequent 48-h recovery period in zebra mussels (*Dreissena polymorpha*). Compound letter designations (Table 2.2) are listed on the horizontal axis and probability of inducing byssal unattachment on the vertical axis. Cross-hatched bars represent water controls while open and solid bars represent unattachment probabilities following exposure and recovery periods, respectively. Unattachment probabilities for any one compound not significantly different ($p > 0.05$) from water controls are indicated with an "A" above the bar while those significantly different ($p < 0.05$) from water controls are indicated with a "B". **A**. Data for compounds A-F. **B**. Data for compounds G-L. **C**. Data for compounds M-S. Vertical lines above columns represent the standard error of the mean.

Very little mortality was recorded in either the treatment or post-treatment phases for the majority of screened compounds. For all but four of the tested compounds, no mortality occurred during either the treatment or post-treatment testing periods. Mortality was observed only in the treatment phase for compounds F, N and O, but was minimal at ≤ 2.13%. Increased mortality was observed for compound L at 8.33% (s.e. = ±5.5) in the treatment phase and 15.91% (s.e. = ±5.9) in the post-treatment phase for a total treatment plus post-treatment sample mortality of 22.9 (s.e. = ±8.3)%. Among the six compounds found to inhibit mussel byssal attachment, compound D was the least effective (Fig. 2.1A-C) and hence, eliminated from further investigation because its probability of inducing byssal unattachment (0.58, s.e. = ±0.07) was not significantly different ($p > 0.05$) from that of other compounds shown to have unattachment probabilities equal to water controls. By contrast, the p_{1c} estimate for compound A was 0.92 (s.e. = ±0.04), for compound B, 0.86 (s.e. = ±0.04), for compound L, 0.73 (s.e. = ±0.07), for compound R, 0.99 (s.e. = ±0.003) and for compound S, 0.96 (s.e. = ±0.03). Compounds R and S were eliminated from further investigation because they were isomers of compound A and their capacity to inhibit byssal attachment was not significantly different from compound A.

2.3.2 Concentration-response analysis

Scheffé pairwise comparison testing of concentration response data for compounds A, B and L indicated that there was no statistical difference ($p > 0.05$) in the probability of byssal unattachment among the water and vehicle controls after either the 48-h treatment (p_{c1}) or 48-h post-treatment experimental phases (p_{c2}), or across these

phases (Fig. 2.2A-C) allowing probability of byssal unattachment for compound treatments to be compared only against water controls.

For compound A (Fig. 2.2A), there was, at concentrations of 0.01–10 μM, no significant difference in the probability of unattachment after the 48-h exposure phase relative to water controls ($p > 0.05$). However, for compound A above 20 μM, probabilities of unattachment were >0.9 and significantly greater ($p < 0.05$) than water controls (Fig. 2.2A). Byssal unattachment probabilities after the 48-h recovery period, across all tested concentrations of A, were not statistically different from water controls ($p > 0.05$), suggesting that mussels fully recovered from byssal attachment inhibition induced by the prior 48-h exposure to this compound. Recovery from the effects of compound A was further demonstrated by the probability of byssal unattachment after the recovery period being significantly less ($p < 0.05$) than that recorded after the initial treatment period at concentrations ≥20 μM (Fig. 2.2A).

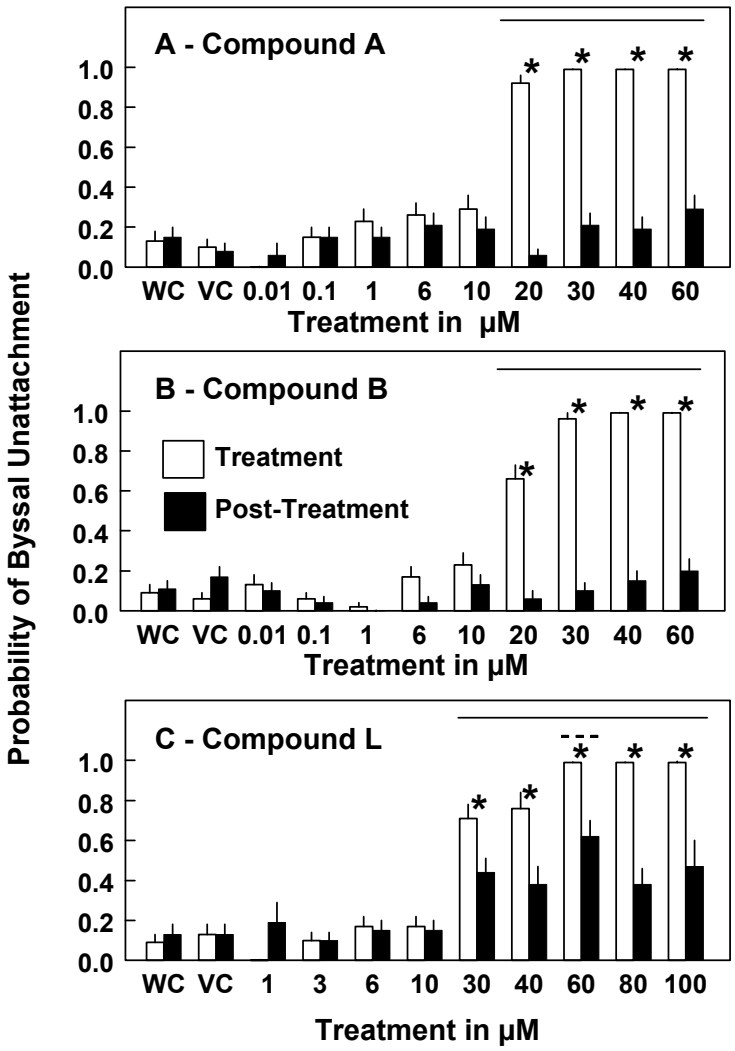


Figure 2.2A-C. Probability of byssal unattachment in zebra mussels, *Dreissena polymorpha*, after a 48-h exposure to varying concentrations of capsaicin-like compounds A, B and L (Table 2.1) and after a subsequent 48-h recovery period relative to water (WC) and vehicle (VC) controls. Exposure concentrations in µM are given on the horizontal axis and probability of inducing byssal unattachment on the vertical axis. Open and solid bars represent probabilities following 48-h exposure and recovery periods, respectively. Unattachment probabilities significantly different ($p < 0.05$) from water controls in the initial 48-h treatment period are indicated by a solid line above the bars and in the subsequent 48-h post-treatment period, by dashed lines. Significant differences ($p < 0.05$) between treatment and post treatment periods for a particular compound concentration are indicated by an asterisk above the bars. **A**. Data for compound A. **B**. Data for compound B. **C**. Data for compound L. Vertical lines above columns represent the standard error of the mean.

For compound B, there was, at concentrations of 0.01-10 μM, no significant difference in the probabilities of byssal unattachment relative to water controls after the 48-h exposure phase ($p > 0.05$), but they were statistically different ($p < 0.05$) at concentrations ≥20 μM (Fig. 2.2B). Probability of byssal unattachment after the 48-h treatment phase was 0.66 at 20 μM and >0.96 at 30-60 μM (Fig. 2.2B). After the 48-h recovery period, probabilities of byssal unattachment across all tested concentrations of compound B were not statistically different ($p > 0.05$) from water controls, indicative of full recovery of byssal attachment ability. At ≥20 μM, byssal unattachment probabilities following the 48-h treatment period were statistically greater than recorded at the end of the 48-h recovery period (Fig. 2.2B).

For compound L, there was, at concentrations ranging from 1-10 μM, no significant difference in the probability of byssal unattachment relative to that of water controls at the end of the initial 48-h exposure phase ($p > 0.05$), but at concentrations ≥30 μM the unattachment probabilities were significantly greater ($p < 0.05$) than that of water controls (Fig. 2.2C). Probability of byssal unattachment after initial exposure was 0.71 at 30 μM, 0.76 at 40 μM and ≥0.99 for 60-100 μM (Fig. 2.2C). Probabilities of byssal unattachment across all tested concentrations of compound L after the 48-h recovery period were not statistically different ($p > 0.05$) from water controls, except at 60 μM ($p < 0.05$), suggesting relatively good recovery of byssal attachment following treatment withdrawal (Fig. 2.2C). After exposure to 60 μM, byssal unattachment probabilities at the end of the 48-h recovery period were statistically greater ($p < 0.5$) than that for recoveries from exposures of 3, 6 and 10 μM, also indicative of a potential

latent negative impact of compound L on the capacity of mussels for byssal attachment (Fig. 2.2C).

Values of EC_{50} and EC_{90} as estimated by Probit Regression for byssal unattachment, were 10.33 µM (s.e. = ±0.62) and 20.09 µM (s.e. = ±1.77) for compound A, 13.16 µM (s.e. = ±0.81) and 27.80 µM (s.e. = ±2.40) for compound B and 16.02 µM (s.e. = ±1.36) and 53.28 µM (s.e. = ±6.76) for compound L, respectively (Fig. 2.3). The EC_{50} value for compound A was significantly less ($p < 0.05$) than that of compounds B and L which were not significantly different from each other. The EC_{90} values for compounds A and B were not significantly different ($p > 0.05$), but both were significantly less ($p < 0.05$) than that of compound L.

Mortality during the 48-h exposure period, the 48-h post-exposure period and across both periods among mussels exposed to concentrations of 0.01 to 60 µM of compounds A and B was less than 6.25% and generally comparable to that of water controls which did not exceed 2.08%. Such extremely low mortality precluded generation of reliable LC estimates by Probit Analysis. The highest percentage of mussel deaths observed occurred during the initial 48-h exposure period to 60 µM of compound A (6.25%) and 40 µM of compound B (4.17%). Mortality over the recovery period was virtually zero for both these compounds.

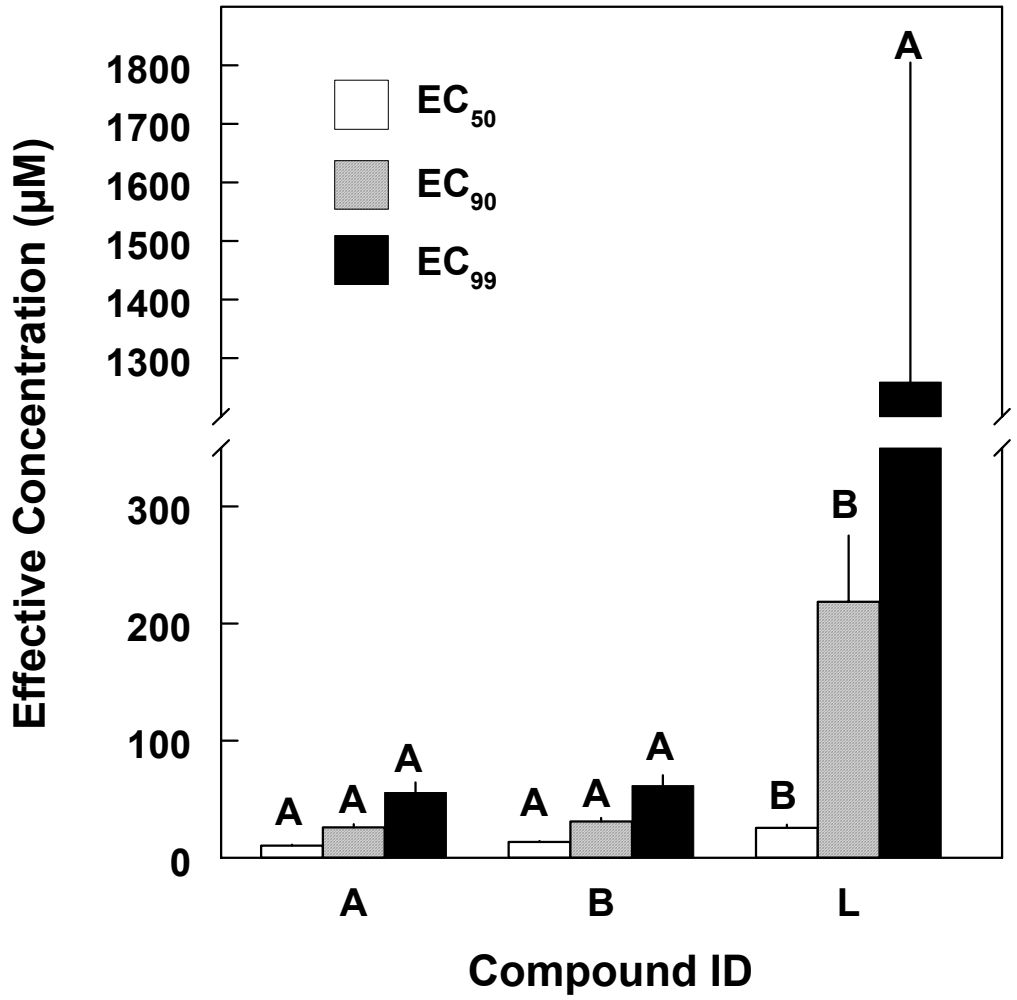


Figure 2.3 Effective concentrations for prevention of byssal attachment by zebra mussels (*Dreissena polymorpha*) determined from concentration-response curve data for capsaicin-like compounds A, B and L presented in Figs. 2.2A-C. Compound letter designation (Table 2.2) is given on the horizontal axis and estimated effective concentrations (EC) for 50% (open bars) and 90% (cross-hatched bars) are represented on the vertical axis in µM. Differing letters above EC_{50} and EC_{90} bars indicate significant differences ($p < 0.05$) across compounds. Vertical lines above columns represent the standard error of the mean.

In contrast, compound L induced much greater mussel mortality during the exposure and post-exposure periods than either compounds A or B. While no mortality occurred at exposures to ≤20 μM of compound L, exposure to ≥30 μM induced mortality which progressively increased with increasing compound concentration to the maximum tested concentration of 100 μM (Fig. 2.4). Mean mortality on exposure to 100 μM, was 53.13% (s.e. ±2.55) in the 48-h exposure period and 46.67% (s.e. = ±13.85) in the 48-h post exposure period. Pair-wise comparisons across concentrations indicated that there was no significant difference ($p > 0.05$) between number of deaths occurring during the exposure or post-exposure periods, with an exception at 80 μM. Mean total mortality for compound L across both periods ranged from 2.08% (s.e. = ±2.08) at 30 μM to 75% (s.e. = ±5.10) at 100 μM (Fig. 2.4). The overall calculated LC_{50} value for compound L was 72.0 μM (s.e. = ±3.6), inclusive of both the initial exposure and post-exposure periods. However, individual LC_{50} estimates for each experimental phase were somewhat higher at 103.6 μM (s.e. ±10.3) following the initial 48-h treatment period and 93.1 μM (s.e. ±9.1) following the post-exposure period (Table 2.3). Results of the Wald test showed no significant difference between the exposure and post-exposure LC estimates (LC_{50} and LC_{90}).

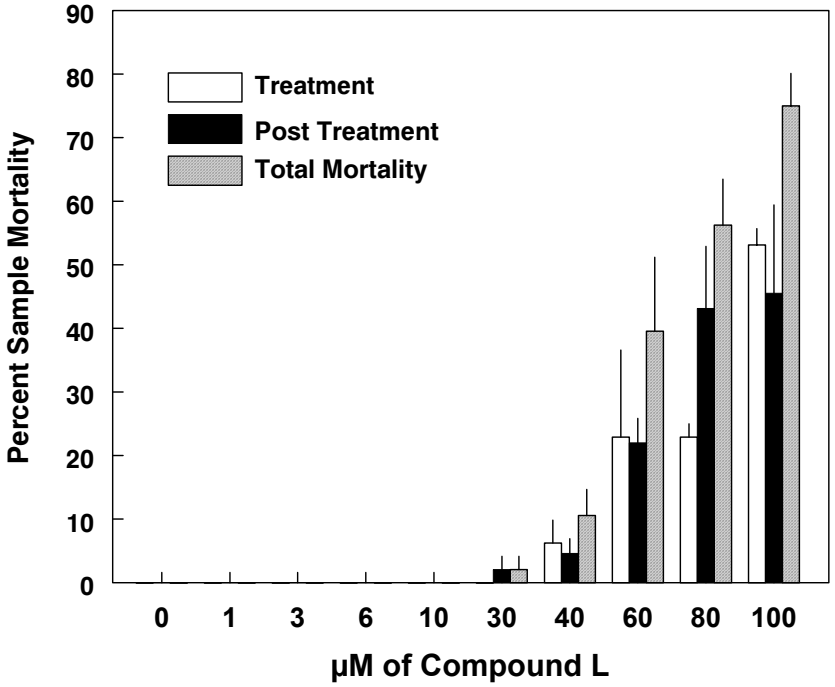


Figure 2.4 Mean mortality of specimens of *Dreissena polymorpha* comparing death during treatment exposure, post-treatment and both experimental periods for varying concentrations of compound L (Table 2.2). Compound exposure concentrations are given on the horizontal axis in µM and percent mortality on the vertical axis. Open, solid and cross-hatched bars represent percent mortality values after 48 h exposure, 48 h post-exposure and total mortality following both exposure and post-exposure periods, respectively. Water control mortality was zero. Vertical lines above columns represent the standard error of the mean.

Table 2.3 Lethal concentration values in µM for *Dreissena polymorpha* exposed to compound L during 48 h exposure, 48 h post-exposure and cumulative 96 h following both exposure and post-exposure periods.

Treatment	LC_{50} (µM)	s.e. (µM)	LC_{90} (µM)	s.e. (µM)
48 h Exposure	103.8	±10.3	217.0	±47.7
48-h Post-exposure	93.1	±9.1	186.5	±39.3
Overall 96-h	72.0	±3.6	131.5	±14.0

2.3.3 Daphnia lethality analysis

Raw cumulative *Daphnia* mortality data for all tested compounds generally displayed, at concentrations above 6-10 μM, a tendency for increased mortality with increasing concentration relative to the water controls (Fig. 2.5A-C). At higher concentrations, there was also a tendency for mortality to increase with exposure time.

For compound A, the highest level of *Daphnia* mortality (20%, s.e. ±0.001) relative to water controls occurred in 30 μM at 72-h exposure (Fig. 2.5A). For compound B, the highest *Daphnia* mortality (50%, s.e. ±0.001) was recorded after a 72-h exposure to 90 μM, followed by mortalities of 28.3% (s.e. ±10.9) and 31.7% (s.e. ±1.7) at 60 and 40 μM, respectively (Fig. 2.5B). In contrast, for compound L, *Daphnia* mortality remained relatively constant over a concentration range of 3-100 μM and was greatest (13.3%, s.e. ±10.9) at both 30 and 100 μM (Fig. 2.5C).

Scheffé pair-wise comparison testing of *D. magna* survival for compounds A, B and L indicated no statistical differences ($p > 0.05$) among the water and vehicle controls (Fig. 2.6A-C), allowing the impacts of tested compounds on *D. magna* survival to be compared only to water controls.

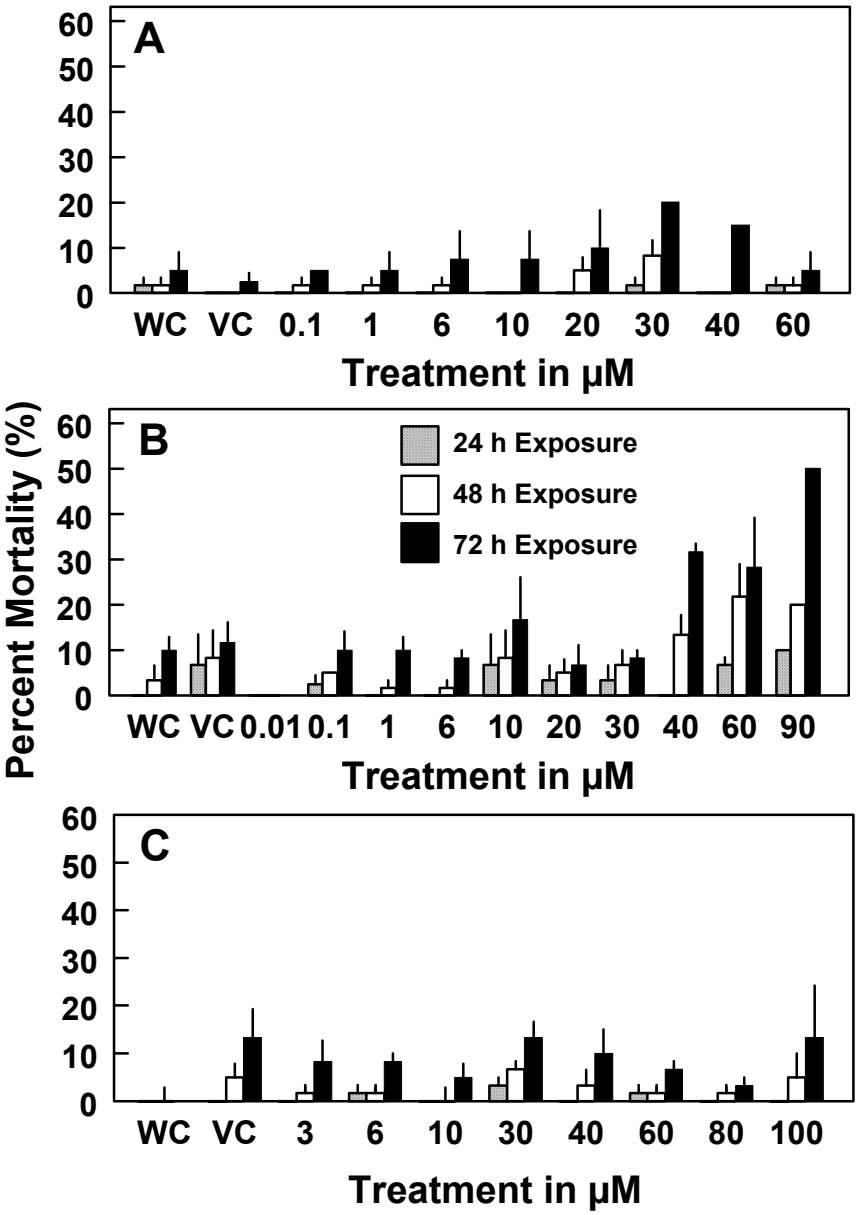
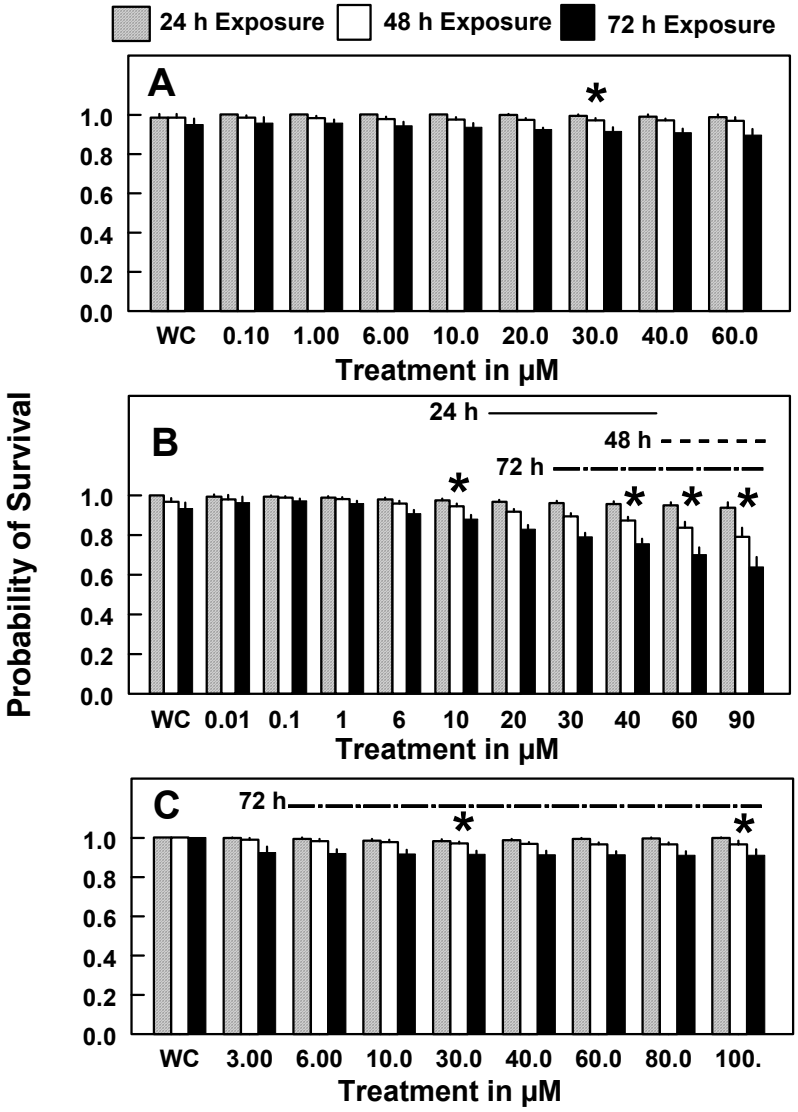


Figure 2.5A-C Mean raw cumulative mortality of *Daphnia magna* during exposure to varying concentrations of capsaicin-like compounds A, B and L (Table 2.2) relative to water (WC) and vehicle (VC) controls. Compound exposure concentrations are given on the horizontal axis in µM and percent mortality on the vertical axis. Cross-hatched, open and solid bars represent percent cumulative mortality values after 24, 48 and 72 h of exposure, respectively. Vertical lines above columns represent the standard error of the mean.

For compound A, the *D. magna* 24-h, 48-h and 72-h estimated survival probabilities did not significantly vary across tested concentrations, the lowest of these estimates being 0.89 (s.e. ± 0.03) at 60 μM after 72 h exposure (Fig. 2.6A). Also, for a fixed concentration, there were no significant differences ($p > 0.05$) in survival probabilities across the 24-, 48- and 72-h exposure intervals excepting the 30 μM concentration for which probability of survival decreased with exposure time (Fig. 2.6A).

For compound B, the 24-h survival probability of *D. magna* was significantly lower ($p < 0.05$) at 20-40 μM than in the water control (Fig. 2.6B). The 48-h survival probability was significantly lower than that of the water control at concentrations of 60-90 μM while the 72-h survival probability was significantly lower than that of the water control at concentrations of 30-90 μM (Fig. 2.6B). The lowest survival probability for compound B was 0.64 (s.e. ±0.05) at the 72-h exposure to 90 μM (Fig. 2.6B). Excepting the 10, 40, 60 and 90 μM concentrations of compound B, there were no significant differences in *D. magna* survival probabilities ($p > 0.05$) across the 24-, 48- and 72-h exposure intervals. At these four concentrations, probability of survival decreased with exposure time (Fig. 2.6B).



Figures 2.6A-C Estimated survival probabilities for specimens of *Daphnia magna* over time following exposure to varying concentrations of capsaicin-like compounds, A, B and L (Table 2.2), relative to water (WC) controls. Exposure concentrations are presented on the horizontal axis in µM and survival probability on the vertical axis. Cross-hatched, open and solid bars represent survival probabilities after 24, 48 and 72 h of exposure, respectively. Survival probabilities significantly different ($p<0.05$) from water controls at 24 h, 48 h and 72 h are indicated by solid, dashed and dot-dashed lines, respectively, above the bars. Significant ($p<0.05$) within-treatment differences for specific compound concentrations are indicated by an asterisk above the relevant bars. **A**. Data for compound A. **B**. Data for compound B. **C**. Data for compound L. Vertical lines above columns represent the standard error of the mean.

For compound L, the *D. magna* 24-h and 48-h survival probabilities did not significantly differ ($p > 0.05$), at any tested concentration (i.e., 3-100 μM), from the water control (Fig. 2.6C). However, the 72-h survival probabilities were significantly different ($p < 0.05$) from water controls at concentrations of 6-100 μM. The lowest survival probability for compound L was 0.90 (s.e. ±0.03) at 72-h exposure to 100 μM (Fig. 2.6C). Excepting the 30 and 100 μM concentrations, there were no significant differences ($P > 0.05$) in the 24-h, 48-h and 72-h survival probabilities. For these two concentrations, the probability of survival decreased significantly ($p < 0.05$) with exposure time (Fig. 2.6C). Mean mortality for all three compounds did not approach 50% precluding estimation of reliable LC values by Probit analysis.

2.4 Discussion

Significant zebra mussel byssal attachment inhibition by capsaicin solutions, as shown in previously published work, was reconfirmed in this study. Of the three compounds identified as efficacious in inhibiting byssal reattachment at 30 μM, capsaicin (compound A) induced the highest level of attachment inhibition in 92% of sampled individuals (Fig. 2.1A). In contrast, compounds B and L at 30 μM inhibited 86% and 67% of sampled individuals, respectively (Figs. 2.1A-B). All three compounds showed significant byssal attachment inhibition at low concentrations following 48 h exposure (Fig. 2.3), with compound A being most potent (EC_{50} = 10.33 μM, s.e. ±0.62, EC_{90} = 20.09 μM, s.e. ±1.77), followed by compound B (EC_{50} = 13.66 μM, s.e. ±0.81, EC_{90} = 27.80 μM, s.e. ±2.40) and compound L (EC_{50} = 16.02 μM , s.e.

±1.36, EC_{90} = 53.28 µM, s.e. ±6.76). Similar EC_{50} and EC_{90} values for capsaicin (compound A) of 16.04 µM and 25.9 µM have been reported by Cope et al. (1997).

In the current study, compound inhibition of byssal attachment was reversible after exposure for 48 h to 30 µM for all three compounds (Fig. 2.1A-C), however compound B had the highest recovery rate (94%) (Fig. 2.1B) compared to compound A (83%) (Fig. 2.1A) and compound L (59%) (Fig. 2.1C). Ability to reattach following exposure to 30 µM of both compounds A and B (Fig. 2.1A) was not shown to be statistically different from that of water controls. In contrast, exposure to 30 µM of compound L, which also induced initial byssal unattachment, resulted in significant levels of post-treatment byssal unattachment, suggesting that this compound had latent negative impacts on exposed individuals (Fig. 2.1B).

Exposure to compounds A and B resulted in virtually no mussel mortality during or up to 48 h following treatment exposure suggesting the relative lack of lethality of these compounds to zebra mussels. Compound L, however, induced significant mussel mortality in both the exposure and recovery periods, indicating latency of lethal effects during the 48-h post-exposure (Fig. 2.4). Since there was little difference between mussel mortality in the exposure and recovery phases across concentrations, mortality appeared to be just as likely to occur during the 48-h recovery phase as it did during the prior 48-h exposure to compound L. The lack of significant difference between LC_{50} and LC_{90} values with regard to the exposure and post-exposure periods also supported this finding. The general trend for decreasing LC values between exposure and post-exposure periods suggests that the lethal impacts of exposure to compound L continued

to be expressed for an extended period time after initial exposure. Thus, the data suggested that the observed effectiveness of compound L in inhibiting byssal attachment was a result of its general molluscicidal characteristics rather than a specific ability to interfere with the byssal attachment mechanism as appears to be the case for compounds A and B.

Mortality and significant inhibition of byssal attachment were both observed at concentrations of compound L ≥30 µM (Figs. 2.2C and 2.4). The ability of this compound to induce relatively high levels of mussel mortality after a single 48-h exposure to concentrations ≥60 µM (Fig. 2.4) suggests that it may have efficacy as a general molluscicide for control of zebra mussels and other macrofouling molluscs particularly as it was not lethal to the non-target species, *D. magna* (Fig. 2.5). Thus, efficacy of compound L for mitigation and control of zebra mussel and other macrofouling molluscs appears to warrant further investigation.

Actual *D. magna* mortality did not exceed 50% at all tested concentrations and exposure durations of compounds A, B and L preventing accurate estimation of lethal concentration values (Fig. 2.5A-C). At 72 h exposure, across all concentrations of the three compounds, the probability of *D. magna* survival was high, being minimally 90% for compound A (60 µM for 72 h), 64% for compound B (90 µM for 72 h) and 90 % for compound L (100 µM for 72 h) (Fig. 2.5A-C). In contrast, compound concentrations which significantly inhibited *D. polymorpha* byssal attachment were much lower, being 20 µM (6.11mg/L) for compound A, 20 µM (5.87mg/L) for compound B, and 30 µM (8.08 mg/L) for compound L (Fig 2.2A-C). These concentrations were not lethal to *D.*

magna during a 48-h exposure to compounds A and L and only negligibly lethal to *D. magna* for compound B (Fig. 2.6A-C). In comparison, published *D. magna* 48-h LC_{50} values for currently preferred zebra mussel biocides such as $CuSO_4$ and $ZnSO_4$ are 0.0826 mg/L and 4.03 mg/L, respectively (Guilhermino, et al., 2000). Sano and Landrum (2005) report a *D. magna* LC_{90} estimate of 0.7 mg/L for hypochlorite following a 24-h exposure.

In addition to proof of concept, some interesting findings suggesting potential structure-function relationships among the compounds were noted in this research. The tested compounds exhibited a LASEN structural motif in common consisting of a lipophilic (L) segment, followed in succession by an amino group (A), carbon spacer segment (S) and a terminal electronegative group (EN). Compounds A (capsaicin) and B (N-vanillylnonanamide) share a longer, non-bulky lipophilic acyl chain end and a cyclic benzyl aromatic electronegative end containing a hydroxyl group. In compound L (N-benzoylmonethanolamine benzoate), a single aromatic structure occurred on both the electronegative and lipophilic ends of the molecule (Table 2.2) which suggested that an aromatic structure might successfully replace a long chain lipophilic segment without decreasing the molecule's inhibitory action. In contrast, lack of inhibition of mussel attachment by compounds D, H, I, J, K, M, and N despite similar lipophilic chains to capsaicin and compound B may be attributed to the structural differences in the electronegative end: lack of a benzyl structure and/or hydroxyl group, substitution of hydroxyl group with side group such as $–NO_2$, double aromatic or a cyclic ether (Table 2.2).

Significant efficacy of both types of capsaicin isomers (i.e. compounds R and S), which differed only in the aliphatic chain structure, suggested that the spatial configuration of the lipophilic portion of LASEN molecules did not impact byssal reattachment inhibition. Furthermore, this result suggested that vanilloid 1 receptor (VR1) activity may not be involved in the mechanism inhibiting byssal reattachment, as the bioactive isomer at the VR1 receptor site (compound R) was equally effective as the inactive isomer (compound S) (Ralevic et al., 2001).

Use of capsaicin-like agents as natural mussel attachment inhibitors represents a novel approach to prevention of mussel settlement relative to conventional molluscicides. This approach features a non-lethal, reversible inhibitory effect on mussel attachment. Observations indicated that mussels did not produce byssal threads when exposed to compounds A, B and L. Hypotheses for inability to produce byssal threads during exposure to compounds A, B, and L include: impairment of the byssal manufacturing processes or closing of the valves in response to irritating effects of the compounds, preventing pedal extension to the substratum surface.

2.5 Conclusions

The results of this study indicated that compounds A and B exhibited pronounced byssal attachment inhibition efficacies with minimal impacts on post-exposure capacity for byssal reattachment. Compound A, although highly efficacious at inhibiting byssal attachment and non-lethal to *D. magna,* is expensive and presents manufacturing challenges as it must be extracted and purified from natural plant material. Compound B is more economical and readily available as a synthesized

compound, but was also the most lethal of the three efficacious compounds to *D. magna* at concentrations greater than 30 μM. However, the EC_{90} for inhibition of byssal attachment for compound B was estimated to be 27.80 μM relative to an estimated 0.89 survival probability for *D. magna* after 48 h of exposure to this compound at 30 μM, suggesting that compound B could be an efficacious antifouling agent against zebra mussels with minimal impacts on non-target species, especially if settlement competent pediveligers prove more sensitive than adults to its capacity to inhibit byssal attachment.

Compound L, while being nonlethal to *D. magna*, was the least effective of the three compounds shown to inhibit zebra mussel byssal attachment. The estimated effective concentration of this synthetic compound for 90% byssal attachment inhibition was 53.28 μM relative to an estimated 0.97 survival probability for individuals of *D. magna* after a 48-h exposure to 60 μM of this compound. This result suggested that compound L could be an efficacious zebra mussel antifouling agent at slightly higher concentrations than compound B with almost no negative impact on the non-target species *D. magna*. Given the current need for effective, nontoxic, environment friendly agents for prevention of zebra mussel biofouling that are reasonably priced and suitable for mass production, compounds B and L and, perhaps, other structurally similar molecules, appear to be potential candidates for future research as antifouling agents based on the results of this study.

CHAPTER 3

PREVENTION OF ZEBRA MUSSEL BYSSAL ATTACHMENT BY COMPOUNDS WITH CANNABINOID RECEPTOR ACTIVITY

3.1 Introduction

Dreissena polymorpha (Pallus, 1771), the zebra mussel, is a well established invasive macrofouling freshwater mollusc in North America that has serious economic impacts on raw water utilizing facilities (See Chapter 1). Structures at high risk of colonization include the intake structures and piping of raw water systems of electrical power plants and water processing facilities. Estimated annual costs for periodic mitigation of established zebra mussel populations and repair of structural damage caused by their fouling approach $1 billion in the US alone (Pimentel et al., 2005). *Dreissenia polymorpha* populations are now well established in 22 states and seven major river systems in the United States. Although zebra mussels have not fulfilled predictions of in-land water habituation west of the Mississippi River to date, recent sightings of the closely related nonindigenous, invasive, quagga mussel, *Dreissena bugensis,* in Lake Mead, Nevada, forewarn that zebra mussel infestation of the western US waterways may be imminent (Stokstad et al., 2007).

Similar to other mussel species, zebra mussel attachment to submerged hard surfaces is achieved by the secretion of a liquid proteinaceous glue material from a gland at the base of the foot. Such cementation with proteinaceous glues also occurs in

other sedentary marine invertebrates such as barnacles (Callow and Callow, 2002; Lindner, 1984). In mussels, this material hardens into a byssal thread which, when applied to a solid surface, provides attachment (Clarke and McMahon, 1996). Production of numerous byssal threads increases attachment strength, allowing mussels to withstand dislodgement due to high flow velocities and the prying activity of predators (Claudi and Mackie, 1994; Clarke and McMahon, 1996).

Byssal attachment of masses of zebra mussels causes obstruction, and at times near total occlusion, of the water intake piping and internal structures of such industries as potable water and sewage treatment plants, electrical utilities, paper processing plants (Ulrich, 2004) and agricultural irrigation systems (Everette Laney, personal communication). Costly plant shut downs for manual removal of mussels from surfaces, biocide treatment or thermal flushing are means of zebra mussel mitigation and remediation. Severe corrosion of metal surfaces beneath points of byssal thread attachment can also necessitate expensive structural repairs. Additionally, massive zebra mussel macrofouling of boat and barge hulls also affects shipping by increasing hydrodynamic drag and hence fuel costs, of ships plying navigatable waters as well as increasing the need for frequent cleaning and repeated treatment of boat and barge hulls. Increased weight due to mussel fouling also causes sinking of navigation buoys and floating marina structures (Mussalli, et al., 1992; Claudi and Mackie, 1994).

Current methods to prevent zebra mussel settlement and induce adult mussel mortality in closed systems include commonly used oxidizing chemicals such as sodium hypochlorite, which although inexpensive and effective (Benschoten et al., 1993; Claudi

and Mackie, 1994; Mussalli et al., 1992), have significant negative environmental impacts such as inducing corrosion and the production of toxic, cancer inducing trihalomethane compounds (Mussalli et al., 1992). Similarly, application of protective paints or coatings to surfaces at risk for byssal attachment, although effective, have the potential to leach toxic metals and biocides into the environment. Organic biocides, heavy metals and organo-metallic combinations such as organotin (TBT) are common ingredients embedded in commercially available coatings (Gross, 1993; Race and Kelly, 1996; Watermann, 1999; Omae, 2003). Due to their environmental persistence, induced reproductive failures in molluscs and bioaccumulation, organotin coatings have been severely restricted and will be permanently banned worldwide by 2008 (Nandakumar and Yano, 2003; Watermann, 1999; Xu et al., 2005). As alternative strategies to organotin based paints and coatings, formulations containing copper or an organic biocide (i.e., Irgarol 1051 and Diuron) exhibit many of the same side effects as organotin compounds (Omae, 2003; Watermann, 1999; Xu et al., 2005; Diers et al., 2006) and are considered by some as strictly interim solutions (Watermann, 1999). Furthermore, metals including copper and zinc have been restricted in their use as additives in antifouling preparations (Marine Paint, 2005; Voulvoulis et al., 2002).

In an effort to replace organometallic antifoulants with less or nontoxic alternatives, current research efforts have focused on a wide variety of natural products (repellants and biogenic biocides), particularly those used as defensive mechanisms by some aquatic organisms including bacteria, sponges, corals, algae, and seaweeds to repel or inhibit adhesion of biofouling organisms (Nandakumar and Yano, 2003;

Watermann, 1999). For example, Taylor and Zheng (1995) documented the repellant activity and latent mortality of a brown algal extract incorporated in a silicone-based coating on settlement by the marine blue mussel, *Mytilus edulis*. Of the natural antifouling products (NAP) currently under investigation as countermeasures for zebra mussel macrofouling, ingestion of the bacterium *Pseudomonas fluorescens* resulted in species specific mortality (Molloy, 2001). Furthermore, exposure to aaptamines, an antifoulant secreted by sponges, has been shown to inhibit zebra mussel byssal attachment (Diers et al., 2006).

This dissertation research has reconfirmed similar nontoxic inhibitory effects of capsaicin, the natural extract of chili peppers, on mussel byssal attachment (see Chapter 2) reported in previous studies (Cope et al., 1997; John Schetz, unpublished data, 2003). Remarkably, treatment with anandamide, a structural analogue of capsaicin and a cannabinoid (CB) receptor agonist in humans (DiMarzo, et al., 1998 and 2001) and other mammals (Elphick and Egertova, 2001) proved effective in preventing zebra mussel byssal attachment in vivo (John A. Schetz, unpublished data, 2003). Studies indicate that the CB receptor (subtype CB1) was well conserved amongst humans, mice, amphibians and fish (Lutz, 2002). Furthermore, isolation of CB1 genes was achieved in 62 placental mammalian species (Lutz, 2002). Isolation of anandamide in tissues of the sea urchin *Paracentrotus lividus* (Bisogno et al., 1997), the leech *Hirudo medicinalis* (Matias et al., 2001) and five species of marine bivalves (Sepe et al., 1998) suggested the presence of an endocannabinoid system in invertebrates as well. Based on these findings, this chapter reports the results of an investigation of the antifouling activity on

zebra mussels, *Dreissena polymorpha,* of anandamide and nine other compounds similar to anandamide in structure or cannabinoid or vanilloid receptor activity.

3.2 Materials and methods

3.2.1 Collection and maintenance of test animals

As described in Chapter 2, specimens of zebra mussels (*Dreissenia polymorpha*) were collected periodically over 2004-2006 from the Rose Bud Marina on Lake Oolagah, Roger County, Oklahoma, (36.4203°N, 95.6665°W). They were immediately transported attached to original substrata to the laboratory at The University of Texas at Arlington in insulated containers under cool, moist conditions. In the laboratory, mussels were held in 946 L circular fiberglass tanks filled with continuously aerated and filtered dechlorinated City of Arlington tap water (DTW) (see table 2.1 for holding tank physical parameters). Tank water was initially at the ambient water temperature of collection and subsequently lowered to 5°C over 24-48 h. Thereafter, mussels were held unfed at 5°C for a maximum of 6 months prior to experimentation with minimal condition loss (Chase-Off, 1996; Cope, et al., 1997). Mussels were habituated to the 15°C experimental temperature in DTW for a minimum of 48 h in a constant temperature incubator prior to testing.

Initially, specimens of *D. magna* utilized in lethality testing were purchased from Carolina Biological Supply Company®. These specimens formed the basis for a laboratory culture maintained at 20-23°C in DTW and fed a suspension of dried brewer's yeast and *Spirulina* fish food twice weekly (Clare, 2002). Specimens were fed a minimum of 2 h prior to use in toxicity testing bioassays (USEPA, 2002).

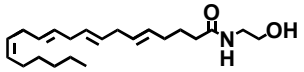
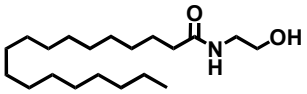
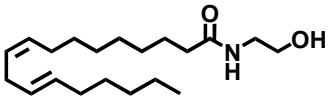
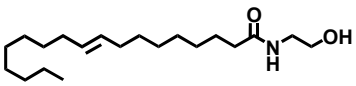
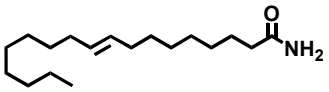
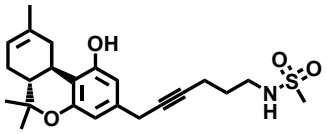
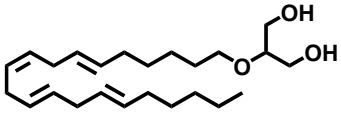
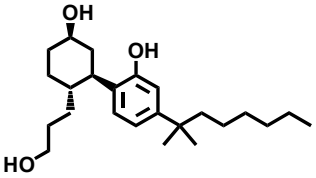
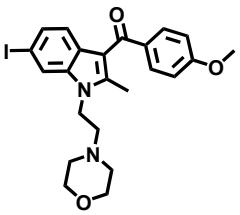
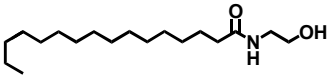
3.2.2 Test chemical preparation

Tested compounds were characterized as having cannabinoid receptor activity. Due to their partial lipophilic nature, all tested compounds required solubility testing. Compounds not directly dissolving in DTW at a concentration of 30 μM, were tested for solubility in a range of non-aqueous solvents in the following order: methanol (MeOH); dimethylsulfoxide (DMSO); dimethylformamide (DMF); ethanol (EtOH); 95%:5% DMSO:1 N HCl; and 95%:5% DMSO:1 N NaOH. Once dissolution was achieved, the solution was diluted with DTW to a final test concentration of 30 μM where possible. Only compounds remaining fully in solution after dilution with DTW were used for testing. Tested compounds and their chemical structures are listed in Table 3.1.

3.2.3 Byssal attachment screening

As described in Chapter 2, immediately following habituation to 15°C, eight to ten groups of 16 mussels <20 mm in shell length (defined as the maximal distance from the tip of the umbos to the posterior shell margin) were severed from their byssal attachments with a scalpel. Each group of 16 mussels was equally divided between two new 240 ml Ziploc® polypropylene containers and subsequently exposed to 200 ml of a 30 μM concentration of each test compound. For some relatively insoluble compounds, exposure concentrations were less than 30 μM (Table 3.1, compounds U = 15 μM and AB = 20 μM). This procedure was repeated in triplicate, for a total of 48 individuals tested for each compound.

Table 3.1 Ten anandamide-like compounds utilized for evaluation of anti-fouling efficacy in zebra mussels, (*Dreissena polymorpha)* and lethality to *Daphnia magna*. Compounds (Cmp) are identified by letter designations in the body of the text. CAS numbers and molecular weights (MW) are provided.

Cmp	Chemical Name	Chemical Structure	Comments
T	Anandamide (Arachidonylethanolamide) *CAS: 94421-68-8* *MW: 347.5*		Endocannabinoid. Partial CB1 and CB2 receptor agonist; Potent VR1 agonist.
U	Stearoyl Ethanolamide *CAS: 111-57-9* *MW: 327.55*		Endogenous cannabinoid
V	Linoleyl ethanolamide *CAS: 68171-52-8* *MW: 323.5*		Endogenous cannabinoid. Weak CB1 and CB2 agonist. Inhibits Cmp T uptake by AMT.
W	N-oleoylethanolamine *CAS: 111-58-0, MW: 325.53*		Endogenous cannabinoid. Inhibits Cmp T uptake by AMT.
X	Oleamide *CAS: 301-02-0* *MW: 281.48*		Shorter alkyl and No alkylhydroxyl. Disputable CB1 receptor agonist. Inhibits Cmp T degradation by FAAH.
Y	O-2050 *CAS: 667419-91-2* *MW: 417.56*		Silent CB1 receptor antagonist.
Z	Noladin ether *CAS: 222723-55-9* *MW: 364.6*		CB1 receptor agonist.
AA	CP 55,940 *CAS: 83002-04-4* *MW:376.6*		CB1 & CB2 receptor agonist.
AB	AM630 *CAS: 164178-33-0* *MW: 504.4*		CB1 and CB2 receptor antagonist. Some CB1 and CB2 agonistic properties.
AC	Palmitylethanolamine *CAS: 544-31-0* *MW: 299.4*		Shorter alkyl. Cogener of CmpT. Potentiates Cmp T by inhibiting degradation by FAAH.

All test chambers along with an untreated DTW control and non-aqueous solvent control were maintained at 15°C (±0.5°C) in a refrigerated constant temperature incubator. Following 48 h exposure, the numbers of mussels byssally attached, living but unattached, or dead were recorded. Individuals byssally connected to each other or the walls of the container were considered attached. Of mussels remaining unattached, those with open valves not closing when gently prodded with the bristles of a fine tipped brush were considered dead (Matthews and McMahon, 1999). The shells of unattached living mussels were marked with a single dot of fast-drying enamel paint from a Krylon® paint pen. Attached mussels were cut from their byssus with a scalpel, after which all attached and unattached living individuals were placed in a new container with 200 ml of untreated DTW and held at 15°C for a further 48-h post-exposure period. After the 48-h post-exposure period, the number of byssally reattached, living unattached or dead mussels were determined as described above.

As in Chapter 2, compound efficacies were assessed in terms of the following parameter: p_{c1} = probability of mussels being unattached given that they were alive after exposure to a compound "c" during the initial exposure period (1) which was estimated by the ratio of the number of unattached (and alive) animals divided by the number of live unattached or attached mussels after 48 h of exposure to 30 μM of the compound or less if the compound was not soluble at 30 μM. The p_{c1} parameters were compared across the compounds with a one-way-ANOVA-like analysis using a Wald statistic (Koch et al., 1985) to test the H_o of no difference in p_{c1} across all compounds. Post hoc comparisons using a Scheffe-type procedure (Koch et al., 1985) were used for pair-wise

comparison of p_{c1} across the set of tested compounds. Compound residual effects, estimated by the parameter, $p_{c2,}$ were defined as in $p_{c1,}$ but for the 48-h post-exposure period (2) and estimated with the same analysis used for p_{c1}.

Comparisons of outcomes in the exposure vs. post-exposure periods (i.e. of p_{c1} versus p_{c2}) involved the same individuals, requiring repeated-measures to be incorporated into the analysis. More specifically, correlation in the exposure and post-exposure responses by individuals had to be accounted for. Details of this analysis are provided in Appendix I.

3.2.4 Concentration-response curves

Concentration response curves were developed for compounds shown in prior screening (see above) to have induced a greater than 0.90 probability of inhibition of byssal attachment. The selected compounds were Anandamide (T), Linoleyl ethanolamide (V), O-2050 (Y), Noladin ether (Z), CP 55,940 (AA) and AM630 (AB) (Table 1). In these tests, mussels were exposed to 8-9 treatment concentrations ranging from that known to inhibit attachment based on initial screening to concentrations as low as 0.01μM (i.e., 0.01-150 μM). Methodology at each tested concentration was that described for the initial screening tests above.

Concentration response curve data were used to estimate the effective concentrations for 50% and 90% sample byssal attachment inhibition (i.e., EC_{50} and EC_{90}) for each compound. These data were also used to estimate lethal concentrations for 50% and 90% sample mortality (i.e., LC_{50} and LC_{90}) for the compound AB which induced significant levels of mortality. Specifically, for a given q varying between 0

and 100, the EC_q value (termed "effective concentration") for a compound is the concentration of that compound required to produce q% unattachment after a 48-h exposure. An LC_q value (termed "lethal concentration") for a compound refers to the compound concentration resulting in q% mortality after a 48-h exposure.

In the EC analysis, the target parameters were as follows: $p_1(c, d)$ = probability of being unattached if alive on exposure to compound c at concentration = d, during phase 1 of the experiment, which was estimated, for the given compound "c" and concentration "d", by the ratio of the number of individuals unattached (but alive) after a 48-h exposure to the compound, divided by the number of living attached and unattached individuals. Probit regression analysis (Fahrmeir and Tutz, 1994) was used to model the probit transform (inverse of the standard normal distribution function) of $p_1(c, d)$ as a linear function of $\log_{10}$ (concentration), allowing the EC_q values to be determined by a standard technique. For example, the EC_{50} value for compound "c" is the value of "d" at which $p_1(c, EC_{50}) = ½$, assuming that the linear probit model gives: probit $p_1(c, EC_{50}) = b_0 + b_1 * \log_{10}(EC_{50})$. Since $p_1(c, EC_{50}) = ½$ and probit (½) = 0, the EC_{50} is determined by the following equation: $\log_{10}(EC_{50}) = -b_0/b_1$.

The LC analysis was similarly performed using these defined target parameters: $p_1(c, d)$ = probability of dying during exposure to compound c at concentration = d, during phase 1 of the experiment, which was estimated, for the given compound "c" and concentration "d", by the ratio of the number of dead individuals after a 48-h exposure to the compound, divided by the total number living and dead individuals; $p_2(c, d)$ = conditional probability of dying during exposure to compound c at concentration = d

during phase 2 of the experiment provided individuals survived phase 1, which was estimated for the given compound "c" with concentration "d", by the ratio of the number of dead individuals after 48-h withdrawal of the compound, divided by the total number of living individuals surviving phase 1.

The maximum likelihood estimates of b_0 and b_1 were generated by the GENMOD procedure in the SAS package, and plugged into the equation, $\log_{10}(EC_{50}$ [or $LC_{50}]) = -b_0/b_1$, to yield the EC_{50} [or LC_{50}] estimate. The delta method (Serfling, 1980) was used to compute the standard error of the EC_{50} and LC_{50} estimates. Almost identical methods yielded the EC_{90} and LC_{90} estimates.

Once the set of ECq estimates were obtained for compounds in this study, where q = 50 or 90, a Wald test was undertaken to compare ECq (and LCq) for each fixed q, across the compounds (Serfling, 1980). Post-test attachment recovery (i.e., comparison of pre- versus post-test probability of not forming a byssal attachment in living individuals) was analyzed by categorical modeling using SAS procedure CATMOD. Post-test mortality (i.e., comparison of pre- versus post-test probability of individuals dying) was analyzed by the same method.

3.2.5 Daphnia magna lethality screen

The six compounds subjected to concentration response testing were also evaluated for toxicity to the freshwater cladoceran crustacean, *Daphnia magna,* a commonly used toxicity test species with relatively high sensitivity to a wide range of toxic chemicals (USEPA, 2002). Three replicates of 20 adult individuals of *D. magna* were exposed to 8-9 concentrations of efficacious compounds T, V, Y, Z, AA and AB

(Table 3.1) identical to those used in byssal attachment dose response testing. For lethality testing, specimens of *D. magna* were maintained for 72 h in 40 ml of test medium at 15°C under the same conditions and in the same containers described above for mussel byssal attachment testing. Numbers of alive and dead individuals were recorded every 24 h. Death was defined as lack of limb movement and heart beat, and dead individuals were removed at each observation.

Daphnia magna survival probabilities at 24, 48 and 72 h were estimated by Probit Regression (Fahrmeir and Tutz, 1994) for each compound at each tested concentration. Lethal concentration values (i.e., LC_{50}, LC_{90} and LC_{99}) were estimated using the same methods employed in the mussel byssal attachment response versus compound concentration studies described above. Probit Regression (Fahrmeir and Tutz, 1994) with both concentration and compound type as predictors was utilized to compare survival probabilities by concentration across tested efficacious compounds. A multiple logit model (Koch et al., 1985) was used to compare the survival probabilities at 24, 48 and 72 h for the same compound and exposure concentration.

3.3 Results

3.3.1 Byssal attachment screening

Data analyses of single dose byssal attachment inhibition experiments using the Wald test indicated that no statistical difference ($p > 0.05$) existed between the probability of byssal unattachment in mussels held in water and vehicle control treatments after the initial 48-h treatment, after the subsequent 48-h recovery period, or

between these two periods. Hence, compound treatment effects were only compared to water controls.

A Wald test also indicated that when mussels were exposed to a single high concentration (≤30μM) of the tested compound for 48 h, the parameter p_{1c} (probability of byssal unattachment) was not significantly different ($p > 0.05$) between water controls and compounds U, W, X and AC (Fig. 3.1A-B). Because differences between p_{1c} (treatment period) and p_{2c} (recovery period) were also not statistically significant ($p > 0.05$) for these compounds (Fig. 3.1A-B), they were deemed non-efficacious in inhibiting byssal attachment. In contrast, T (Anandamide), V (Linoleyl ethanolamide), Y (O-2050: (6a*R,* 10a*R*)-3-(1-Methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetahydro-6,6,9-trimethyl-6*H*-dibenzo [b,d]pyran), Z (Noladin ether), AA (CP 55,940: (-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol) and AB (AM630: [(6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl)(4-methoxyphenyl) methanone]) (Table 3.1) induced statistically greater inhibition of byssal attachment (p_{1c}) ($p < 0.05$) relative to water controls (Fig. 3.1A-B). For all but compounds Y and AB, post-treatment recovery period p_{c2} was not statistically different ($p > 0.5$) from water controls suggesting that the effects of compounds T, U, V, Z and AA were short term and did not extend beyond the exposure period (Fig. 3.1A-B). The probabilities of byssal unattachment during the 48-h recovery (p_{c2}) for compounds Y and AB were significantly greater than water controls ($p < 0.05$) at 0.53 (s.e. = ± 0.07) and 0.63 (s.e. = ±0.07), respectively (Fig. 3.1A-B).

Overall, minimal mussel mortality was recorded for all screened compounds during both the 48-h exposure and post-exposure periods. Zero mortality resulted from treatment with compounds T, U, V, W, X, and AC and less than 5% mortality for compounds Y and AB. Higher mortality was associated with compound Z at 4.17% and 10.87% in the treatment and post-treatment phases, respectively. A maximal mortality was observed following exposure to compound AA, with 16.7% of deaths occurring during the treatment period and 5.0% in the post-treatment phase. Total sample mortality spanning both experimental periods was 14.58% for compound Z and 20.83% for compound AA.

Compounds Y, Z, and AA were most efficacious of the six compounds that induced >90% byssal unattachment. Of the six compounds inducing greater than 90% probability of byssal unattachment (p_{1c}) , the p_{1c} estimate for compound T was 0.94 (s.e. = ±0.03), compound V, 0.94 (s.e. = ±0.03), compound Y, 0.99 (s.e. = ±0.003), compound Z, 0.99 (s.e. = ±0.003), compound AA, 0.99 (s.e. = ±0.004) and compound AB, 0.93 (s.e. = ±0.04) (Fig. 3.1A-B).

3.3.2 Concentration-response analysis

Scheffé Pair-wise Comparison testing of concentration response data for efficacious compounds T, V, Y, Z, AA and AB indicated that there was no statistical difference in the probability of byssal unattachment among the water and vehicle controls after either the 48-h treatment (p_{c1}) or 48-h post-treatment experimental phases (p_{c2}), or across these phases (Figs. 3.2A-C and 3.3D-F), allowing probability of byssal unattachment for compound treatments to be compared only against water controls.

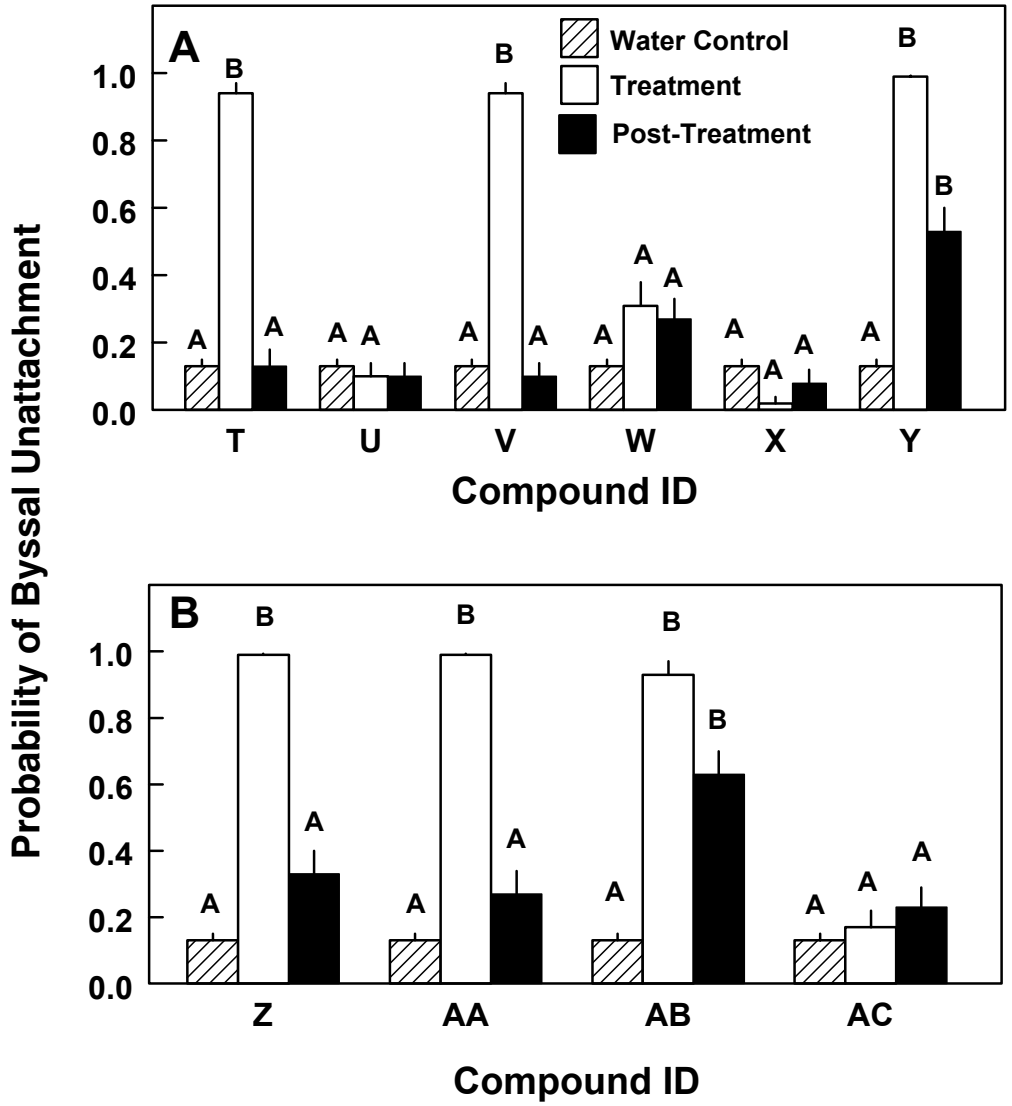


Figure 3.1A-B. Probability of byssal unattachment after 48 h of exposure to 10 anandamide-like compounds at concentrations of 30μM or less (depending on solubility), and after a subsequent 48-h recovery period in zebra mussels (*Dreissena polymorpha*). Compound letter designations (Table 3.1) are listed on the horizontal axis and probability of inducing byssal unattachment on the vertical axis. Cross-hatched bars represent water controls while open and solid bars represent unattachment probabilities following exposure and recovery periods, respectively. Unattachment probabilities for any one compound not significantly different ($p > 0.05$) from water controls are indicated with an "A" above the bar while those significantly different ($p < 0.05$) from water controls are indicated with a "B". **A**. Data for compounds T-Y. **B**. Data for compounds Z-AC. Vertical lines above columns represent the standard error of the mean.

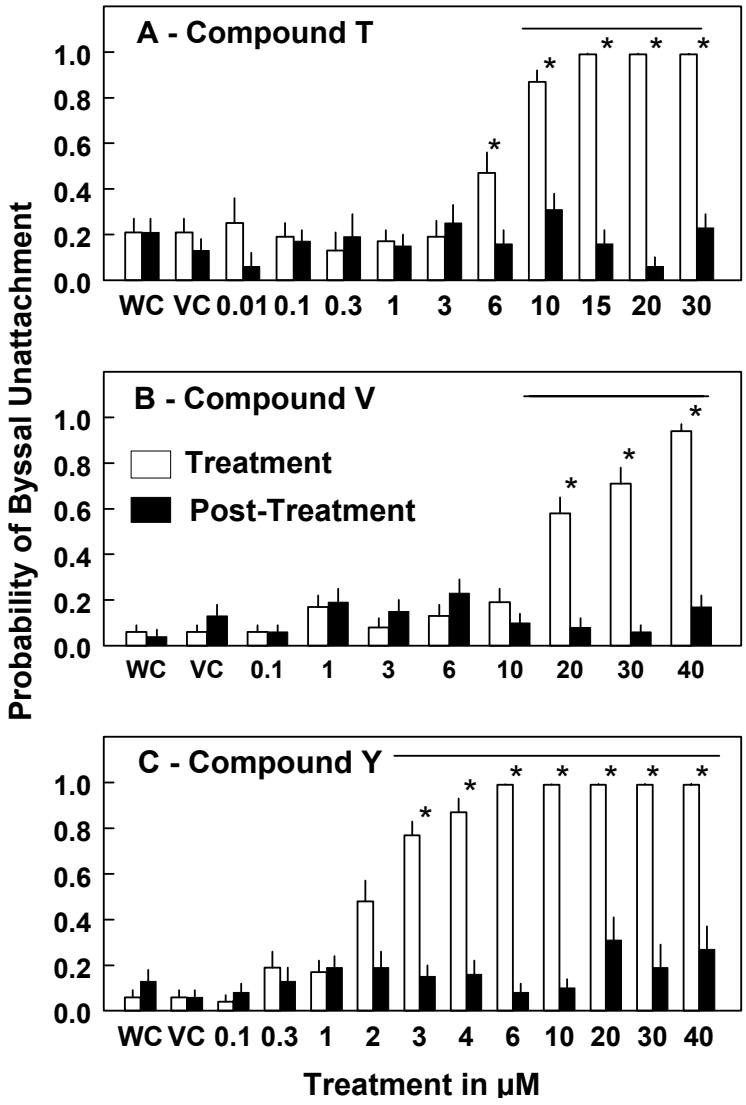


Figure 3.2A-C. Probability of byssal unattachment in zebra mussels, *Dreissena polymorpha*, after a 48-h exposure to varying concentrations of anandamide-like compounds T, V, Y, Z, AA and AB (Table 3.1) and after a subsequent 48-h recovery period relative to water (WC) and vehicle (VC) controls. Exposure concentrations in µM are given on the horizontal axis and probability of inducing byssal unattachment on the vertical axis. Open and solid bars represent probabilities following 48-h exposure and recovery periods, respectively. Treatment and post-treatment unattachment probabilities significantly different ($p < 0.05$) from water controls are indicated by a solid and dashed line above the bars, respectively. Significant differences ($p < 0.05$) between treatment and post treatment periods for a particular compound concentration are indicated by an asterisk above the bars. **A**. Data for compound T. **B**. Data for compound V. **C**. Data for compound Y. Vertical lines above columns represent the standard error of the mean.

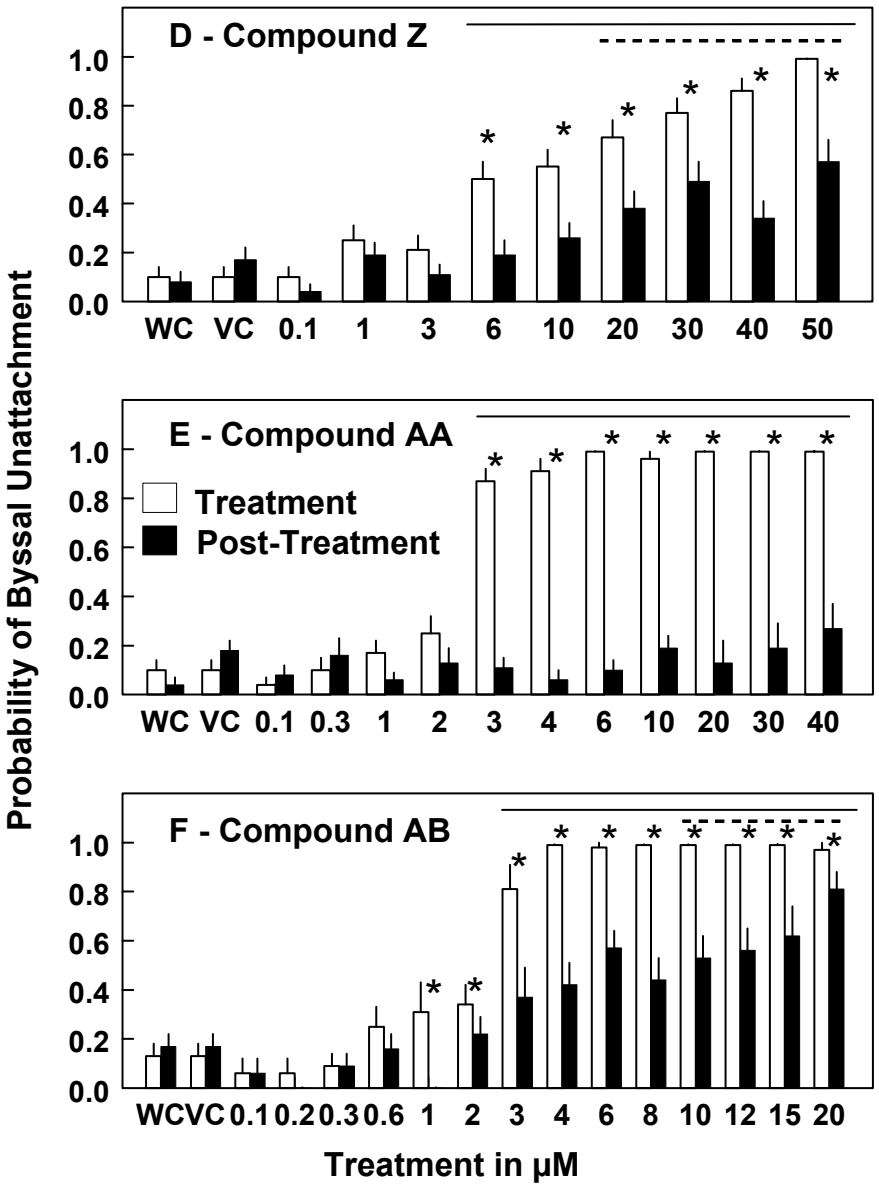


Figure 3.3D-F. Figure 3.2 continued. **D.** Data for compound Z. **E.** Data for compound AA. **F.** Data for compound AB.

For compound T at concentrations of 0.01-6 μM (Fig. 3.2A), probabilities of byssal unattachment following 48-h treatment exposure were not statistically different ($p > 0.05$) from water controls. At concentrations above 10 μM, however, probabilities of unattachment for compound T were >0.87 and significantly greater ($p < 0.05$) than water controls. Byssal unattachment probabilities after the 48-h post-exposure period were not significantly different relative to water controls ($p > 0.05$) across all tested concentrations of compound T, suggesting that mussels fully recovered from byssal attachment inhibition. Further support for this finding was demonstrated by byssal attachment inhibition probabilities following treatment recovery being significantly less ($p < 0.05$) than those recorded for the previous 48-h treatment phase at concentrations ≥6 μM.

For compound V at concentrations of 0.1-10 μM (Fig. 3.2B), probabilities of byssal unattachment following 48-h treatment exposure were not statistically different ($p > 0.05$) from water controls. However, at concentrations ≥20 μM, probabilities of unattachment for compound V were >0.58 and significantly greater ($p < 0.05$) than that of water controls. Byssal unattachment probabilities after the 48-h post-exposure period were not significantly different relative to water controls ($p > 0.05$) across all tested concentrations of V, indicating that mussels fully recovered from inhibition of byssal attachment. At ≥20 μM, byssal unattachment probabilities following the 48-h exposure period were statistically greater than recorded at the end of the 48-h post-exposure period (Fig. 3.2B). Furthermore, the probabilities of byssal unattachment were not

statistically different ($p > 0.05$) between compounds V and U following 48-h exposure to 15 μM, the highest concentration at which compound U was completely soluble.

For compound Y at concentrations of 0.1- 2 μM (Fig. 3.2C), probabilities of byssal unattachment at the end of the 48-h treatment exposure period were not statistically different ($p > 0.05$) from water controls but they were significantly different ($p < 0.05$) at concentrations ≥3 μM. The probabilities of unattachment following exposure to compound Y were 0.77 (s.e. = ±0.06) at 3 μM, 0.87 (s.e. = ±0.06) at 4 μM, and 0.99 (s.e. = ±0.002) at 6-40 μM. Probabilities of byssal unattachment across all tested concentrations of compound Y after the 48-h post-exposure period were not significantly different ($p > 0.05$) from water controls, suggesting full recovery of byssal attachment capabilities. Statistically greater ($p < 0.05$) mussel byssal inhibition probabilities were observed after the 48-h exposure phase at concentrations ≥3 μM in comparison to the subsequent 48-h recovery period (Fig. 3.2C).

For compound Z at concentrations of 0.1- 3 μM (Fig. 3.3D), probabilities of byssal unattachment at the end of the 48-h treatment exposure period were not statistically different ($p > 0.05$) from water controls, but were significantly greater ($p < 0.05$) relative to water controls at concentrations ≥6 μM. At 6 μM, the probability of unattachment was 0.50 (s.e. = ±0.07) , compared to 0.55 (s.e. = ±0.07) at 10 μM, 0.67 (s.e. = ±0.07) at 20 μM, 0.77 (s.e. = ±0.06) at 30 μM, 0.86 (s.e. = ±0.05) at 40 μM, and 0.99 (s.e. = ±0.003) at 50 μM (Fig. 3.3D). Probabilities of byssal unattachment across all tested concentrations of compound Y after the 48-h recovery period were not significantly different ($p > 0.05$) from water controls at concentrations <20 μM, but were

different ($p < 0.05$) at concentrations ranging from 20-50 μM (Fig. 3.3 D), suggesting latent negative impacts of compound Z on mussel attachment abilities at these higher concentrations (Fig. 3.3D).

For compound AA at concentrations of 0.1- 2 μM (Fig. 3.3E), probabilities of byssal unattachment at the end of the 48-h treatment exposure period were not statistically different ($p > 0.05$) from water controls but were significantly different ($p < 0.05$) at concentrations ≥3 μM. The probabilities of unattachment following exposure to compound AA were 0.87 (s.e = ±0.05) at 3 μM, 0.91 (s.e = ±0.05) at 4 μM, 0.99 (s.e = ±0.002) at 6 μM, 0.96 (s.e = ±0.03) at 10 μM, and 0.99 (s.e = ±0.004) at 20-40 μM. Probabilities of byssal unattachment across all tested concentrations of compound AA after the 48-h recovery period were not statistically different ($p > 0.05$) relative to water controls, indicative of a full recovery of byssal attachment abilities. In comparison to the 48-h recovery period, statistically greater ($p < 0.05$) mussel byssal inhibition probabilities were observed after the 48-h exposure phase at concentrations ≥3 μM (Fig. 3.3E).

For compound AB at concentrations of 0.1- 2 μM (Fig. 3.3F), probabilities of byssal unattachment at the end of the 48-h treatment exposure period were not statistically different ($p > 0.05$) from water controls. However, byssal unattachment probabilities were significantly greater ($p < 0.05$) relative to water controls at concentrations ≥3 μM. Probabilities of unattachment were 0.81 at 3 μM, 0.99 (s.e = ±0.003) at 4μM, 0.98 (s.e = ±0.02) at 6 μM, 0.99 (s.e = ±0.003) at 8-12 μM, 0.99 (s.e = ±0.004) at 15μM and 0.97 (s.e = ±0.03) at 20 μM. Probabilities of byssal unattachment

across all tested concentrations of compound AB were not significantly different ($p > 0.05$) from water controls after the 48-h recovery period at concentrations <10 μM, but were statistically different ($p < 0.05$) at concentrations ranging from 10-20 μM, suggesting incomplete recovery of byssal attachment abilities due latent negative effects (Fig. 3.3F).

Probit Regression estimates of EC_{50} and EC_{90} values, respectively, for byssal unattachment were 5.34 (s.e. = ±0.34) and 10.66 (s.e. = ±0.96) for compound T, 17.01 (s.e. = ±1.2) and 44.63 (s.e. = ±5.56) for compound V, 1.89 (s.e. = ±0.13) and 4.00 (s.e. = ±0.36) for compound Y, 6.51 (s.e. = ±0.91) and 67.84 (s.e. = ±17.8) for compound Z, 1.97 (s.e. = ±0.14) and 4.40 (s.e. = ±0.40) for compound AA, and 1.78 (s.e. = ±0.19) and 4.32 (s.e. =± 0.44) for compound AB (Fig. 3.4). The EC_{50} value of compounds T and Z were not significantly different ($p > 0.05$) from each other but both were significantly less ($p < 0.05$) than that of compound V. The EC_{50} estimates of compounds Y, AA and AB were also not statistically different ($p > 0.05$) from each other but they were significantly less ($p < 0.05$) than those of compounds T, V and Z. The EC_{90} values for compound T were significantly less ($p < 0.05$) than estimates for compound V but statistically greater ($p < 0.05$) than those of compounds Y, AA and AB. There was no significant difference ($p > 0.05$) in EC_{90} values found between compounds T and Z and V and Z, or among compounds Y, AA and AB (Fig. 3.4).

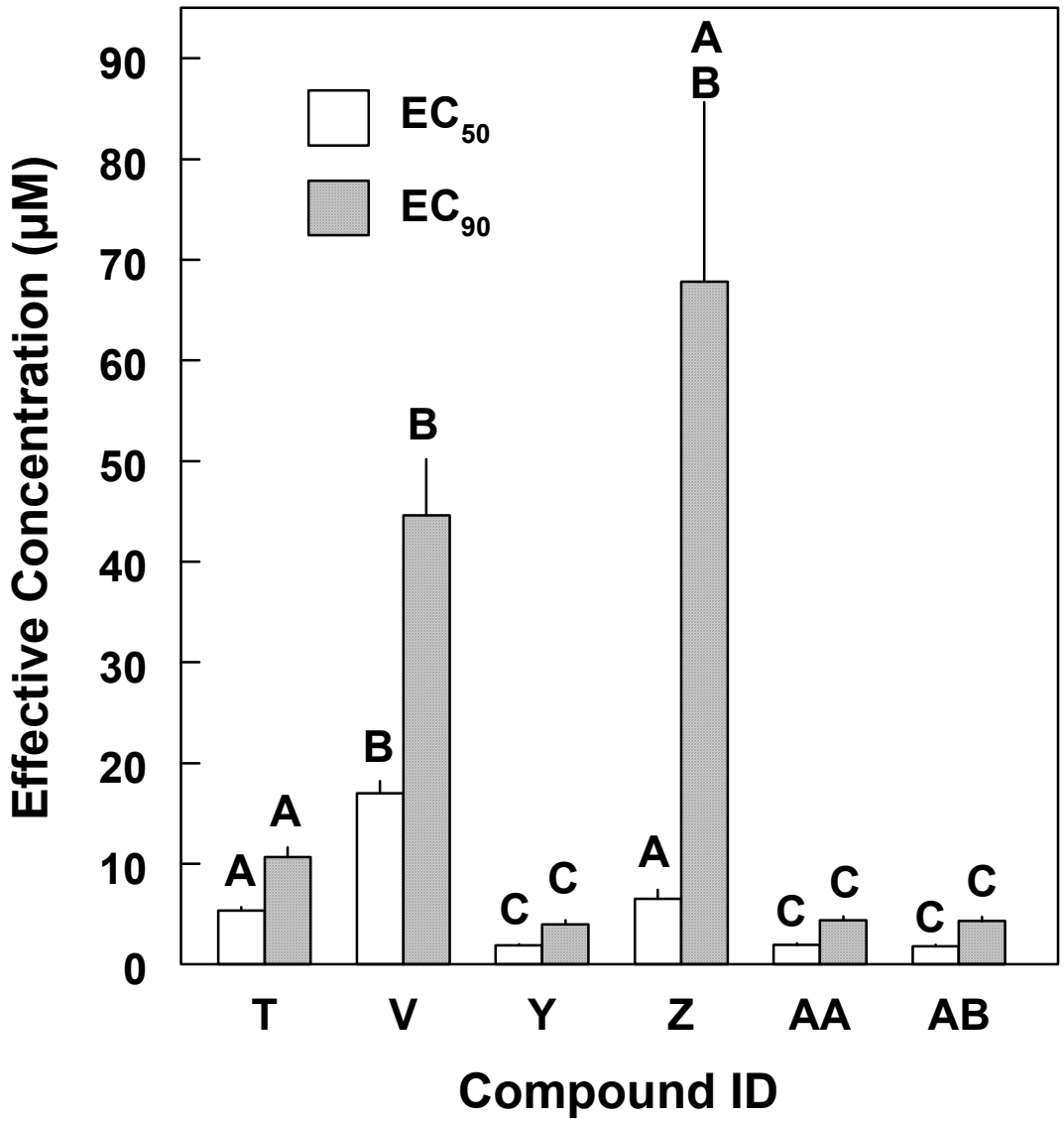


Figure 3.4. Effective concentrations for prevention of byssal attachment by zebra mussels (*Dreissena polymorpha*) determined from concentration-response curve data for anandamide-like compounds T, V, Y, Z, AA and AB presented in Figs. 3.2A-C and 3.3D-F. Compound letter designation (Table 3.1) is given on the horizontal axis and estimated effective concentrations (EC) for 50% (open bars) and 90% (cross-hatched bars) are represented on the vertical axis in μM. Differing letters at the top of the bars indicate the statistically significant differences ($p < 0.05$) in EC_{50} or EC_{90} values among compounds. Vertical lines above columns represent the standard error of the mean.

Mortality during the 48-h exposure phase, the 48-h post-exposure phase and across both periods among mussels exposed to concentrations ranging from 0.01-40 μM of compounds T, V and Y did not exceed 4.17% and was always 0% in water controls. Exposure to compound V resulted in no sample mortality. Because mortality in these three compounds was negligible, generation of reliable LC estimates by Probit analysis was precluded. Slightly higher mortality across both periods was recorded for compound AA when exposed to concentrations in the 20-40 μM range with 18.75% maximal mortality recorded at 20 μM. At 20 μM of AA, greater than twice the sample mortality was observed at end of the 48-h post-exposure period (13.33%) than at the end of the treatment exposure period (6.25%), suggesting the presence of latent negative effects on mussel survival at these higher concentrations.

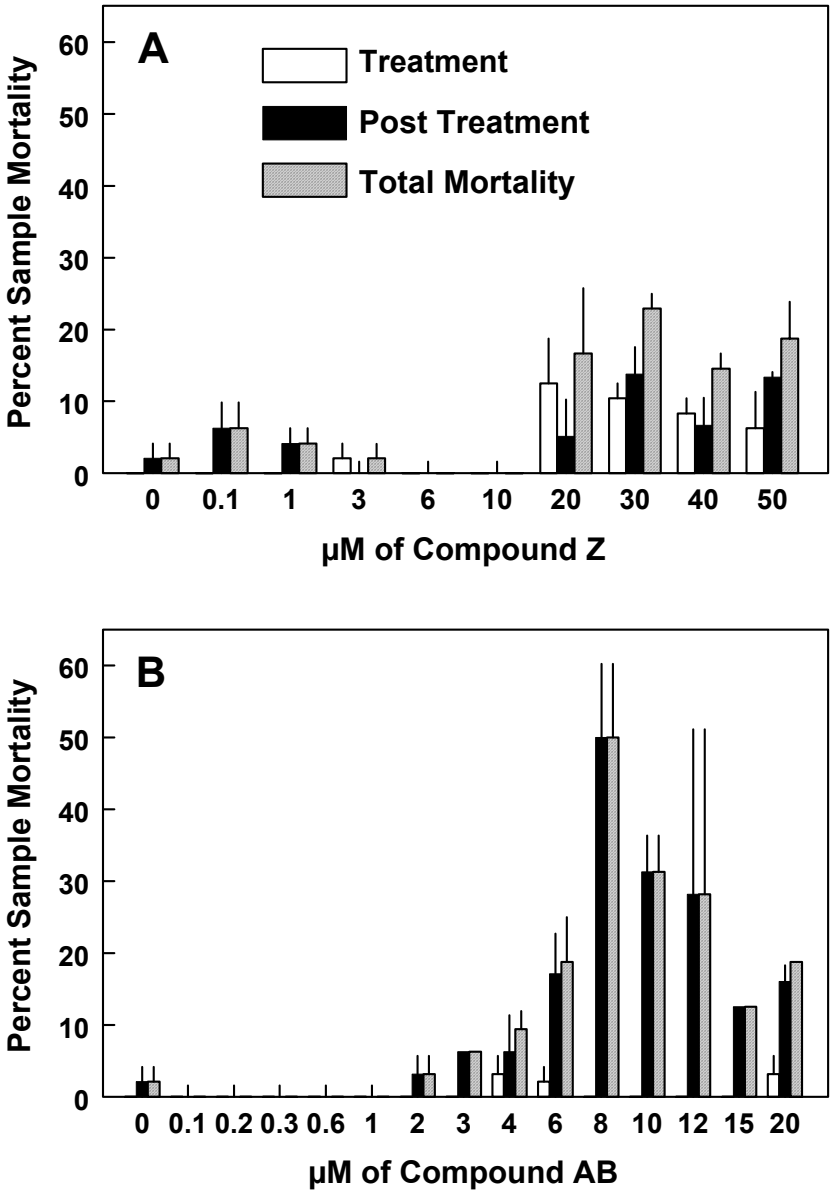


Figure 3.5A-B. Mean mortality of *Dreissena polymorpha* comparing death during treatment exposure, post-treatment and both experimental periods for varying concentrations of compounds Z and AB (Table 3.1). Compound exposure concentrations are given on the horizontal axis in µM and percent mortality on the vertical axis. Open, solid and cross-hatched bars represent percent mortality values after 48 h exposure, 48 h post-exposure and total mortality following both exposure and post-exposure periods, respectively. Water control mortality was zero. **A.** Data for compound Z. **B.** Data for compound AB. Vertical lines above columns represent the standard error of the mean.

In contrast, compound Z and AB exhibited the highest total mortality among mussels of all six efficacious compounds tested. Although mean mortality during a 48-h exposure to compound Z did not exceed 2.08% (s.e. = ±2.80) at ≤10 μM, greater mortality was recorded at exposures to concentrations of 20-50 μM, with 12.25% (s.e. = ±6.25) maximal mortality occurring at 20 μM (Fig. 3.5A). For the 48-h post-exposure period, mortality was minimal at concentrations of compound Z of ≤20 μM and increased at concentrations ≥ 30 μM but never exceeded 13.95% (s.e. = ±3.72). Total mortality was highest in a concentration of 30 μM at 22.9% (s.e. = ±2.08). Generation of reliable LC estimates for compound Z by Probit analysis was precluded as the mortality rate, although higher than recorded for compounds T, V, Y, and AA did not approach 50%. In comparison, compound AB exhibited minimal mortality ≤3.13% (s.e. = ±2.55) across all tested concentrations (0.1 – 20 μM) in the 48-h exposure phase, but high levels of mortality were recorded after the 48-h post-exposure period, particularly at concentrations ≥6 μM (Fig. 3.5B). Maximal mortality after a 48-h post-exposure to compound AB was 50.0% (s.e. = ±10.21) at 8 μM. Across the exposure and post-exposure periods, the overall LC_{50} value was 40.4 μM (s.e. = ±18.4).

3.3.3 Daphnia lethality analysis

Raw cumulative *Daphnia magna* mortality results for all tested compounds showed, at concentrations above 6 μM, a general tendency for increasing mortality with increasing treatment concentration relative to water controls. At higher concentrations, there was also a tendency for increased *D. magna* mortality with increasing exposure time (Figs. 3.6A-C and 3.7D-F).

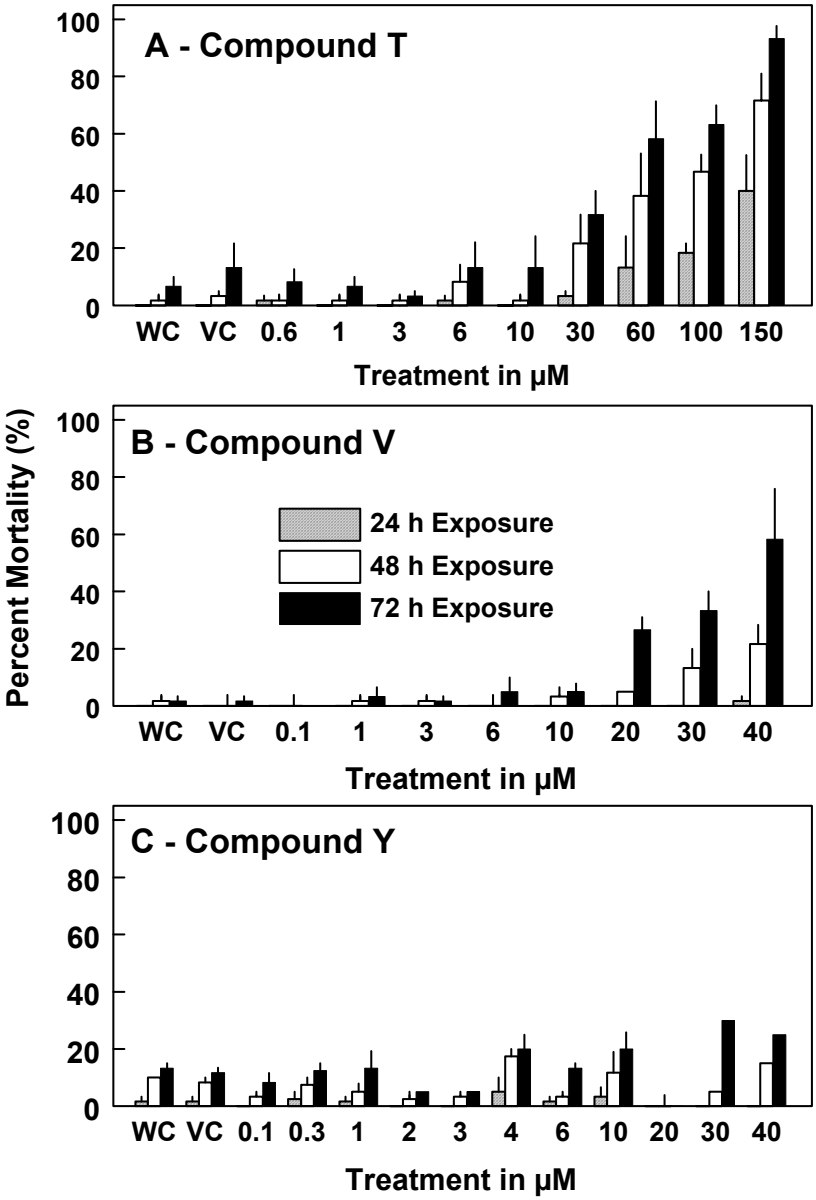


Figure 3.6A-C. Mean cumulative mortality of *Daphnia magna* during exposure to varying concentrations of anandamide-like compounds T, V, Y, Z, AA and AB (Table 3.1) relative to water (WC) controls. Compound exposure concentrations are given on the horizontal axis in µM and percent mortality on the vertical axis. Cross-hatched, open and solid bars represent percent cumulative mortality values after 24, 48 and 72 h of exposure, respectively. **A**. Data for compound T. **B**. Data for compound V. **C**. Data for compound Y. Vertical lines above columns represent the standard error of the mean.

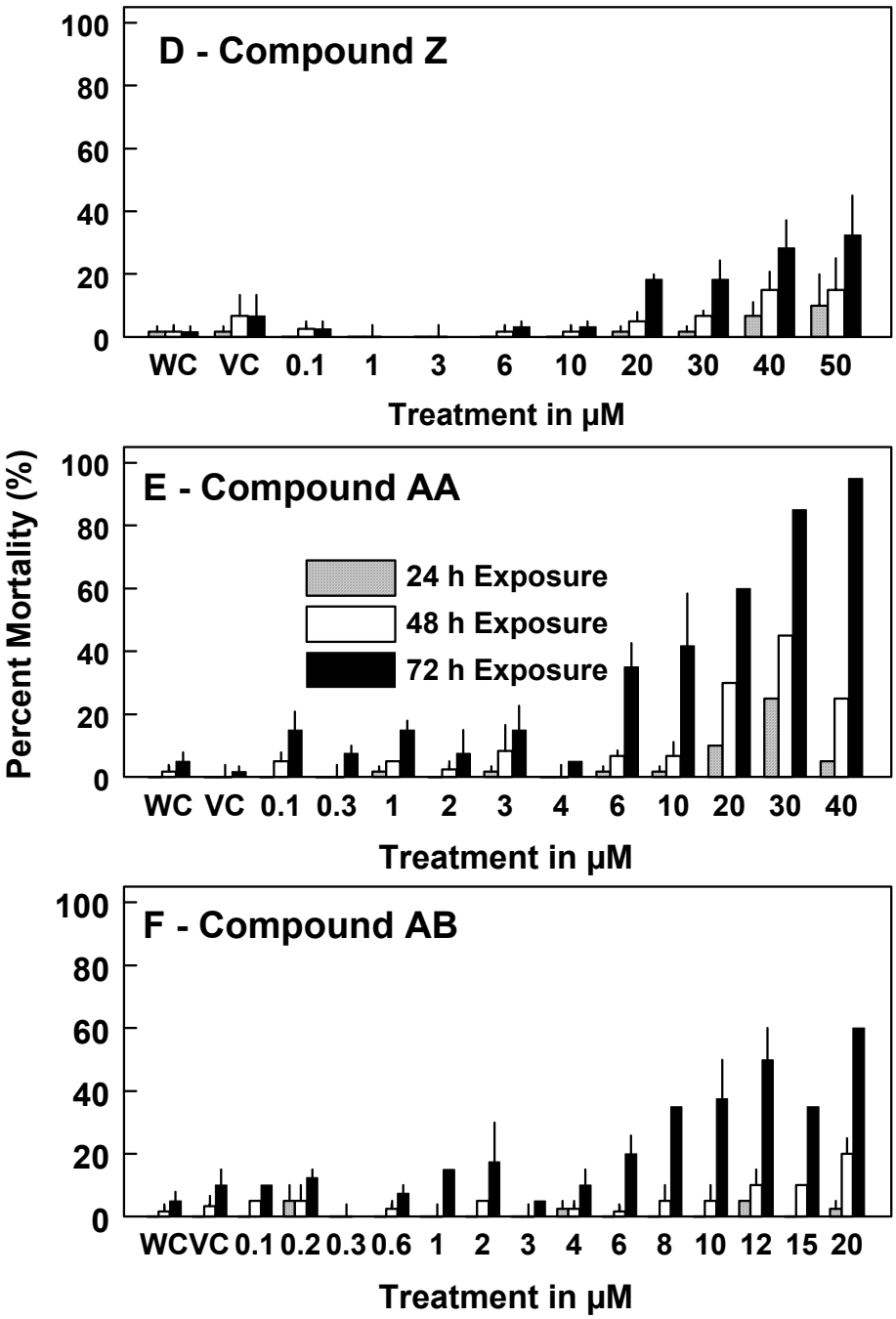


Figure 3.7D-F. Figure 3.6 continued. **D.** Data for compound Z. **E.** Data for compound AA. **F.** Data for compound AB.

For compound T, the greatest *D. magna* mortality (93.3%, s.e. = ±4.4) was recorded in 150 μM at 72-h exposure, followed by 63.3% (s.e. = ±6.7), 58.3% (s.e. = ±13.0) and 31.7% (s.e. = ±8.3) after a 72-h exposure to 100, 60 and 30 μM, respectively (Fig. 3.6A). For compound V, maximal mortality was 58.3% (s.e. = ±17.6) following 72-h exposure to 40 μM, followed by 33.3% (s.e. = ±6.7) and 26.7% (s.e. = ±4.4) after a 72-h exposure in the 30 and 20 μM treatments (Fig. 3.6B). For compound Y, maximal *D. magna* mortality was 30% (s.e. = ±0.0) in 30 μM after 72-h exposure (Fig. 3.6C). For compound Z, the highest recorded mortality was 32.5% (s.e. = ±12.5) at 50 μM after a 72-h exposure, followed by mortalities of 28.3% (s.e. = ±8.8), 18.3% (s.e. = ±6.0), 18.3% (s.e. = ±1.7) at 72-h exposures to 40, 30 and 20 μM, respectively (Fig. 3.7D). For compound AA, maximal *D. magna* mortality of 95.5% (s.e. = ±8.8), was recorded after a 72-h exposure to 40 μM, and 85.0% (s.e. = ±0.0), 60.0% (s.e. = ±0.0), 41.7% (s.e. = ±8.8) and 35.0% (s.e. = ±7.6) following a 72-h exposure to concentrations of 30, 20, 10 and 6 μM, respectively (Fig. 3.7E). For compound AB, 60.0% (s.e. = ±0.0) maximal mortality occurred after a 72-h exposure to 20 μM (s.e. = ±0.0), followed by mortalities of 35.0% (s.e. = ±0.0), 50.0% (s.e. = ±10.0), 37.5% (s.e. = ±12.5), and 20.0% (s.e. = ±5.8) after a 72-h exposure to concentrations of 15, 12, 10, 8 and 6 μM, respectively (Fig. 3.7F).

Scheffe´ pair-wise comparison testing of *D. magna* survival for compounds T, V, Y, Z, AA and AB showed no statistical difference ($p > 0.05$) between water and vehicle controls (Figs. 3.8A-C and 3.9D-F), allowing the impacts of tested compounds on *D. magna* survival to be compared to water controls only. For compound T, the 24-h

survival probability of specimens of *D. magna* was significantly lower ($p < 0.05$) than that of water controls at concentrations ranging from 60-150 μM. The 48-h and the 72-h survival probabilities were significantly lower ($p < 0.05$) relative to water controls at concentrations of 30-150 μM (Fig. 3.8A). The lowest survival probability for compound T was 0.13 (s.e. = ± 0.03) at the 72-h exposure to 150 μM. With the exceptions of the 10, 30, 60, 100 and 150 μM concentrations of compound T, there were no significant differences in *D. magna* survival probabilities ($p > 0.05$) within any test concentration across the 24-, 48- and 72-h exposure intervals. At 10, 30, 60, 100 and 150 μM, probability of survival significantly decreased ($p < 0.05$) with exposure time (Fig. 3.8A).

For compound V, the *D. magna* survival probabilities did not significantly differ ($p > 0.05$) from water controls at any tested concentration (i.e. 0.01-40 μM) (Fig. 3.8B). In contrast, survival probabilities at 72 h were significantly lower ($p < 0.05$) than that of water controls at concentrations ranging from 20-40 μM. The lowest survival probability for compound V was 0.48 (s.e. = ±0.03) after a 72-h exposure to 40 μM. Excepting the 20, 30 and 40 μM concentrations, there were no significant differences in the 24-, 48- and 72-h survival probabilities within any one test concentration. 20, 30 and 40 μM, the probability of survival significantly decreased ($p < 0.05$) with increased exposure time (Fig. 3.8B).

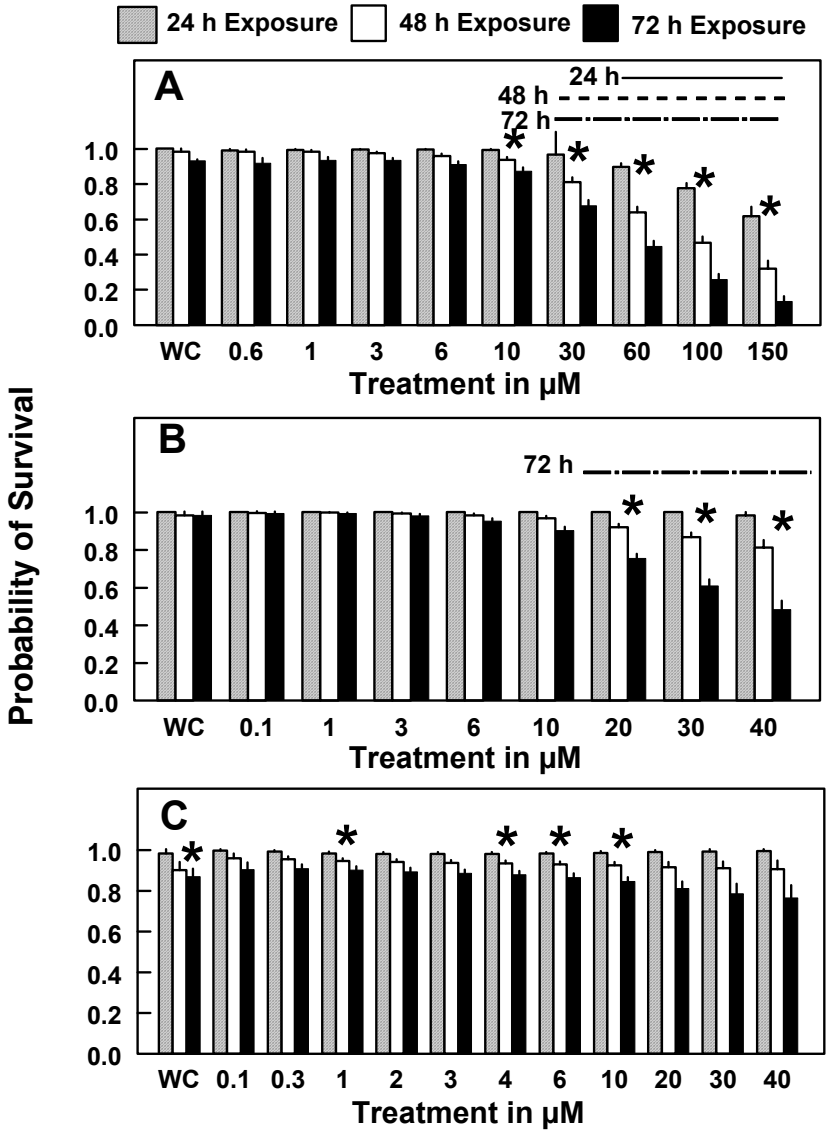


Figure 3.8A-C. Survival probabilities for *Daphnia magna* over time following exposure to varying concentrations of anandamide-like compounds, T, V, Y, Z, AA and AB (Table 3.1), relative to water (WC) controls. Exposure concentrations are presented on the horizontal axis in µM and survival probability on the vertical axis. Cross-hatched, open and solid bars represent survival probabilities after 24, 48 and 72 h of exposure, respectively. Survival probabilities significantly different ($p < 0.05$) from water controls at 24 h, 48 h and 72 h exposure are indicated by a solid, dashed and a broken line, respectively, above the bars. Significant ($p < 0.05$) within treatment differences for specific compound concentrations are indicated by an asterisk above the relevant bars. **A**. Data for compound T. **B**. Data for compound V. **C**. Data for compound Y. Vertical lines above columns represent the standard error of the mean.

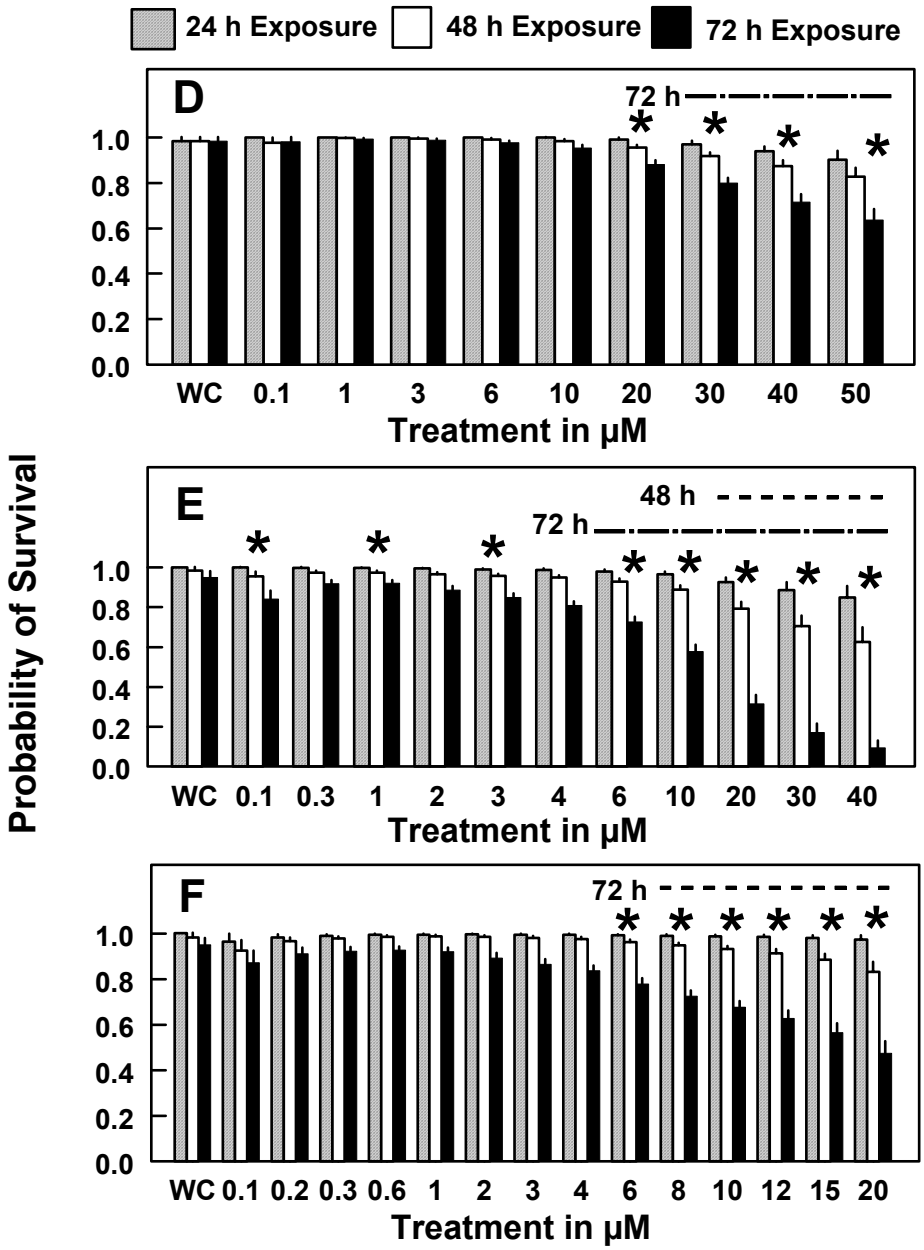


Figure 3.9D-F. Figure 3.8 continued. **D.** Data for compound Z. **E.** Data for compound AA. **F.** Data for compound AB.

For compound Y, there were no statistical differences ($p > 0.05$) in the 24-, 48-, or 72-h survival probabilities relative to water controls at any tested concentration (i.e. 0.1-40 μM) (Fig. 3.8C). The lowest probability of *D. magna* survival was 0.76 (s.e. = ±0.06) following a 72-h exposure to 40 μM. Excepting the 1, 4, 6, and 10 μM concentrations, 24-, 48- and 72-h survival probabilities did not significantly differ ($p > 0.05$) within a test concentration. At 1, 4, 6, and 10 μM, the probability of survival significantly decreased ($p < 0.05$) with increased exposure time (Fig. 3.8C).

For compound Z, the *D. magna* 24-h and 48-h survival probabilities did not significantly differ ($p > 0.05$) from that of water controls at any tested concentrations (i.e. 0.2-50 μM (Fig. 3.9D). However, the 72-h survival probabilities were significantly lower than for water controls at concentrations >30 μM. The probability of survival was lowest at 0.64 (s.e. = ±0.05) following a 72-h exposure to 50 μM. With the exception of 20, 30, 40 and 50 μM treatments, there were no significant differences ($p > 0.05$) in *D. magna* survival probabilities across the 24-, 48- and 72-h exposure periods within a treatment concentration (Fig. 3.9D). At 20, 30, 40 and 50 μM, the probability of survival significantly decreased ($p < 0.05$) with increased exposure time (Fig. 3.9D).

For compound AA, the 24-h probability of survival for *D. magna* was not statistically different ($p > 0.05$) relative to water controls across all tested concentrations, while the survival probability at 48-h was significantly different ($p < 0.05$) than the water control at concentrations ranging from 20-40 μM (Fig. 3.9E). The 72-h survival probabilities also were significantly different ($p < 0.05$) from the water control at concentrations ranging from 6-40 μM. The lowest probability of *D. magna* survival for

compound AA was 0.09 (s.e. = ±0.03) following a 72-h exposure to 40 μM. Survival probabilities across 24-, 48- and 72-h exposure intervals did not significantly differ ($p > 0.05$) except at concentrations of 0.1, 1, 3, 6, 10, 20, 30 and 40 μM in which probability of survival significantly decreased ($p < 0.05$) with increased exposure time (Fig. 3.9E).

For compound AB, neither the 24-h or 48-h survival probabilities for *D. magna* were significantly different ($p > 0.05$) from that of water controls across all tested concentrations (i.e. 0.1-20 μM) (Fig. 3.9F). The 72-h survival probabilities, however, were significantly lower than that of the water control at concentrations ≥8 μM, with the lowest survivorship estimate being 0.47 (s.e. = ±0.05) in the 20 μM treatment. Excepting the 6, 8, 10, 12, 15 and 20 μM concentrations, there was no significant difference ($p > 0.05$) in the 24-, 48- and 72-h survival probabilities within a particular test concentration. For these six concentrations, the probability of survival significantly decreased ($p < 0.05$) with increasing exposure time (Fig. 3.9F).

3.4 Discussion

Anandamide and nine other compounds similar to anandamide in structure or cannabinoid receptor activity were investigated for their antifouling effects, latent effects and lethality. Six compounds were shown to be efficacious inhibitors of byssal attachment in zebra mussels. Of these six agents, compounds Y, Z and AA were most effective, inhibiting 99% of sample byssal attachment at a fixed 30 μM concentration, followed by compounds T, V and AB which inhibited byssal attachment among 94%, 94% and 93% of sampled individuals, respectively (Fig. 3.1A-B). Concentration

response testing revealed that compounds AB (EC_{50} = 1.78 μM, s.e. = ±0.19; EC_{90} = 4.32 μM, s.e. = ±0.44), Y (EC_{50} = 1.89 μM, s.e. = ±0.13; EC_{90} = 4.00 μM, s.e. = ±0.36) and AA (EC_{50} = 1.97 μM, s.e. = ±0.14; EC_{90} = 4.40 μM, s.e. = ±0.40) were the most potent at inhibiting byssal attachment although all six compounds significantly inhibited byssal attachment at low concentrations following a 48-h exposure (Fig. 3.4). Lower potencies were recorded for compounds T (EC_{50} = 5.34 μM, s.e. = ±0.34; EC_{90} = 10.66 μM, s.e. = ±0.96), Z (EC_{50} = 6.51 μM, s.e. = ±0.91; EC_{90} = 67.84 μM, s.e. = ±17.8) and V (EC_{50} = 17.01 μM, s.e. = ±1.2; EC_{90} = 44.63 μM, s.e. = ±5.56) (Fig. 3.4).

Mussels exposed to all efficacious compounds showed full recovery during the 48-h post-treatment period with the exception of those exposed to compounds Y and AB (Fig. 3.1A-B). Compound V had the highest recovery rate (90%), followed by compounds T (87%), AA (73%) and Z (69%) at 30 μM. Ability to byssally reattach following exposure to 30 μM of compounds V, T, AA and Z was not significantly different from water controls, unlike compounds AB and Y in which the probability of unattachment was 0.63 (s.e. = ±0.07) and 0.53 (s.e. = ±0.07), respectively (Fig. 3.1A-B). These results suggested that exposure to compounds AB and Y had latent negative effects on mussel reattachment abilities.

Extremely low mussel mortality upon exposure to all tested concentrations of compounds T, V, Y and AA during, or even up to 48 h after exposure, was indicative of the relative lack of lethality of these compounds. However, compound Z induced much higher mortality during exposure as well as post-exposure periods at concentrations ≥20 μM, suggesting that it had molluscicidal properties (Fig. 3.5A). In contrast, despite

demonstrating <3% mortality after a 48-h exposure across all tested concentrations, high mortality in the 48 h post-treatment period after exposures to ≥6 μM led to the greatest total mortality being documented for compound AB than any of the other five tested compounds. This finding suggested that the majority of mortality to compound AB was latent, occurring during the 48-h post treatment period rather than during the preceding 48-h treatment period. The inability to estimate 48-h exposure LC values for compound AB also supported the conclusion that lethality was much more likely to occur after exposure (Fig 3.5B). The data for both compound Z and especially AB suggest their general efficaciousness in inhibiting byssal attachment was a result of their general molluscicidal characteristics in comparison to the non-lethal inhibitory effects on the byssal attachment mechanism characteristic of compounds T, V, Y and AA.

Mortality and significant inhibition of byssal attachment were both observed at concentrations of compound AB ≥6 μM (Figs. 3.3F and 3.5B). The ability of this compound to induce relatively high levels of mussel byssal unattachment after a single 48-h exposure to concentrations ≥4 μM (Fig. 3.3F) suggested that it may have efficacy as a semi-continuous treatment for control of zebra mussels particularly as it was not lethal to the non-target species, *D. magna* (Fig. 3.9F) when applied for a period of 48 h or less. Thus, the potential efficacy of compound AB for mitigation and control of zebra mussels and other macrofouling molluscs appears to warrant further investigation.

Overall, the probability of *D. magna* survival was high at 48 h of exposure across all tested concentrations of the six compounds with the exception of compound T (31% in 150 μM at 48 h). Probability of survival at 48 h of exposure at the highest

tested concentrations was 81% for compound V (40 μM at 48 h), 90% for compound Y (40 μM at 48 h), 90% for compound Z (50 μM at 48 h), 85% for compound AA (40 μM at 48 h) and 97% for compound AB (20 μM at 48 h) (Figs. 3.8A-C and 3.9D-F). There was a significant decrease in *D. magna* survivorship with increasing time of exposure in five of the six tested compounds. At 72 h of exposure, survivorship was reduced to 62% for compound T (150 μM), 48% for compound V (40 μM), 64% for compound Z (50 μM), 9% for compound AA (40 μM) and 47% for compound AB (20μM) (Figs. 3.8A-B and 3.9D-F). In contrast, there was no significant impact of exposure time on *D. magna* mortality after 72 h of exposure to 40 μM of compound Y at which a survivorship of 76% was recorded (Fig. 3.8C). Thus, compounds T and V, with a 13% and 9% probability of *D. magna* survivorship, respectively, following a 72-h exposure were the most lethal of all the tested efficacious compounds to this species (Figs. 3.8A-C and 3.9D-F).

Much lower concentrations than those inducing *D. magna* lethality were shown to significantly inhibit mussel byssal attachment, being 6 μM (2.09 mg/L) for compound T, 20 μM (6.47 mg/L) for compound V, 3 μM (1.25 mg/L) for compound Y, 6 μM (2.19 mg/L) for compound Z, 3 μM (1.13 mg/L) for compound AA and 3 μM (1.51 mg/L) for compound AB (Figs. 3.2A-C and 3.3D-F). In comparison, published *D. magna* 48-h LC_{50} values for currently preferred zebra mussel biocides such as $CuSO_4$ and $ZnSO_4$ are 0.0826 mg/L and 4.03 mg/L, respectively (Guilhermino, et al., 2000). Sano and Landrum (2005) report a *D. magna* LC_{90} estimate of 0.7 mg/L for hypochlorite following a 24-h exposure. Furthermore, Hernando et al. (2003) document

concentrations of $1x10^{-6}$ mg/L, 7.3 mg/L and 8.6 mg/L to induce 48-h immobility of neonatal *D. magna* for the commercial molluscides TBT, Diuron and Irgarol 1051, respectively.

Proposed hypotheses for the mode of action of natural product antifouling include: 1) acute toxicity, 2) avoidance of (or repellence by) the substratum surface and 3) nontoxic inhibition of adhesion to the substratum by altering cell surface properties such as surface hydrophobicity and target receptors necessary for attachment, or alteration of the actual adhesive glues (Sundberg et al., 1997). In this research, surviving zebra mussels exposed to the ten compounds investigated generally exhibited avoidance behavior, manifested as valve closure without pedal extension, with production of few if any byssal threads. A small percentage of surviving zebra mussels exposed to compounds T, V, Y, Z and AB did, although sporadically and infrequently, produce unattached byssal threads that remained in contact with the closed animal, suggesting that compound exposure could also have resulted in interference with the byssal plaque adhesion mechanism. Compounds Z and AB were also shown to be potentially lethal to *D. polymorpha* at concentrations inducing maximal byssal attachment inhibition, supporting acute toxicity as a leading mode of action for these two compounds.

Although all of the compounds tested interact with mammalian cannabinoid receptors (subtypes CB1, CB2 or both, see Table 3.1), the role of cannabinoid receptors in zebra mussel byssal inhibition remains elusive. Thus, any conclusions drawn in this study regarding the mode of action of the tested efficacious compounds were based on

the known properties of these and other compounds primarily in the endocannabinoid systems of mammals but also in other vertebrate species. The pronounced attachment inhibition demonstrated by compounds Z and AA, both CB1 receptor agonists (Lutz, 2002) (Fig. 3.3D-F), and compounds T and V, both weak CB1 and CB2 receptor agonists (Lutz, 2002) (Fig. 3.2A-C), suggested some involvement of cannabinoid receptors. In contrast, strong inhibition of mussel byssal attachment by compound Y, a CB1 silent receptor antagonist (Tocris, 2006), and a conflicting lack of response to compound X, a disputable CB1 agonist that also potentiates compound T binding to CB1 receptors by decreasing fatty acid anandamide hydrolase (FAAH) degradation of same (Reggio and Traore, 2000), indicated that the CB1 receptor may not be of importance in these compounds' byssal attachment inhibition mechanism (Fig. 3.1A). Furthermore, a significant byssal inhibitory effect upon mussel exposure to compound AB, a putative partial CB1 receptor agonist with documented mixed agonistic and antagonistic properties at this receptor site (Pertwee, 2001), is difficult to interpret in terms of mussel CB1 activity based on the compound's varied behavior in mammalian systems. Likewise, the significant response to compound AB, also a CB2 receptor antagonist with reported inverse agonistic properties (Pertwee, 2001), did not indicate a clear role for the CB2 receptor in the byssal attachment inhibition mechanism (Fig. 3.1B).

Endocannabinoids, compounds participating in the complex and not yet fully understood endocannabinoid signaling system, do not all function as primary ligands for CB1 or CB2 receptors. Endocannabinoids may alternatively modulate (i.e. inhibit or

enhance) the anandamide molecule transporter (AMT), inhibition of which may attenuate the cannabinoid activity of anandamide, and/or modulate the cannabinoid degradation enzyme, fatty acid amide hydrolase (FAAH) (Razdan and Mahadevan, 2002). Inhibition of FAAH potentially attenuates the action of compounds recognized and targeted for destruction by this enzyme such as anandamide (Lutz, 2002; Reggio and Traore, 2000). The presence of compounds T, AC, their endocannabinoid precursors and an FAAH-like enzyme shown to degrade compound T has been confirmed in the tissues of several molluscs, suggesting a cannabinomimetic role for these and other similar compounds in these species (Sepe et al., 1998). CB receptor activation may not be the only mode of action of the tested endogenously synthesized compounds V, Y, Z, AA and AB, especially those with previously determined weak or mixed CB receptor agonistic and antagonistic behavior in mammals (Table 3.1). Furthermore, Razdan and Mahadevan (2002) reported that different endogenous endocannabinoids, such as the widely researched anandamide and 2-arachidonoyl-glycerol (2-Ara-Gl), have been shown to interact differently, and to different degrees, with the same cannabinoid receptors, particularly CB1, often resulting in different outcomes depending on the cell type.

Previous studies have documented that some endocannabinoids show little or no cannabinoid effect when tested individually, but due to a proposed entourage effect, can express an effect in the presence of other endocannabinoid compounds (Razdan and Mahadevan, 2002). For example, in a study by DiMarzo et al. (2001), the effects of anandamide (compound T) were enhanced in the presence of palmitoyl ethanolamide

(compound AC), speculated to have cannabinoid activity although not shown to activate CB1 or CB2 receptors alone. Therefore, compounds U, W, X and AC, endocannabinoids with little or no capacity to inhibit mussel byssal attachment, could potentially become efficacious in the presence of another cannabinoid related compound. In mammalian systems, compounds W and X enhance the activity of compound T at CB1 receptors by inhibiting its intracellular transport via AMT activity (DiMarzo, 1998) and inhibiting its degradation by FAAH (Reggio and Traore, 2000), respectively.

Interestingly, the structure-activity relationships for mammalian endocannabinoids and the compounds tested in this research for inhibition of mussel attachment were highly similar. Efficacious mussel byssal attachment inhibitors, compounds T, V, Y, Z, AA and AB possessed one or more of the following structural criterion for recognition of the compounds by the mammalian endocannabinoid system, particularly the CB receptor (Reggio and Traore, 2000; Razdan and Mahadevan, 2002): a free hydroxyl group (all except AB); an oxygen molecule within the main linkage (Z); a minimum of two *cis* double bonds in the acyl chain (T, V, Y, and Z) and a minimum of 18 carbons in the acyl chain (T, V, and Z). These similarities suggest that some interaction with a putative molluscan endocannabinoid system may be involved with these compounds' capacity to inhibit mussel byssal attachment. Furthermore, the efficacious compound AA, due to its unusual structure-activity relationship is often referred to as a non-classical cannabinoid agonist in the mammals (Pertwee, 2001). It does not have the structural characteristics of the other compounds, yet was also found

to be efficacious in inhibiting zebra mussel attachment which is further evidence for involvement of the endocannabinoid receptor signaling system in the capacity of these compounds to inhibit zebra mussel byssal attachment.

Statistically different efficacies were recorded between efficacious compound V and U which was not efficacious. Both compounds are endocannabinoids of identical structure with the exception that compound U possessed no double bonds whereas two were present in V (Table 3.1). Thus, the differences in their efficacies might be attributable to the hypotheses that ligand recognition by the CB receptor requires at least two or more *cis* double bonds. Similarly, the failure of compounds U, W and X to inhibit mussel attachment could be associated with the lack of appropriate double bonds in the acyl chain (U, W, X), shorter acyl chain lengths of <18 carbons (W, X), or the lack of a hydroxyl group on the electronegative end (X). Overall, it appears that the identified efficacious compounds had some structure-activity similarities particularly in their ability to interact with the mammalian endocannobinoid system; however, a clear understanding of their modes of action in inhibiting mussel byssal attachment will require further experimental investigation.

3.5 Conclusions

In conclusion, this study identified six compounds that may have potential efficacy as zebra mussel antifouling control agents, four of which, compounds T, V, Y and AA, induced pronounced and reversible byssal attachment inhibition. Similarly, compounds Z and AB were shown to be strong inhibitors of mussel byssal attachment but with latent lethal effects during the post exposure period. All compounds were non-

lethal to the non-target species, *D. magna*, following 24-h and 48-h exposures at concentrations inhibiting mussel byssal attachment.

Overall, compound Y, the most potent of all the identified efficacious compounds with an estimated EC_{90} of 4.00μM, exhibited the best combination of characteristics for a zebra mussel antifouling control agent. It induced a maximal 99% probability of mussel unattachment at ≥6 μM with negligible lethal effects on either *D. polymorpha* or *D. magna*. Similar results were observed for compounds V and AA, except compound V was less potent (EC_{90} = 44.63 μM) than Y (EC_{90} = 4.0 μM) and exposure to compound AA resulted in an 18.75% maximal mussel mortality at 20 μM. In contrast, compounds Z and AB, with maximal byssal unattachment probabilities of 99% at 50 μM and ≥4 μM, respectively, were not lethal to *D. magna* at any tested concentrations (0.1-50 μM and 0.1-20 μM, respectively) for up to 48 h, but had general molluscicidal characteristics against zebra mussels (Fig. 3.5A-B). Of these two compounds, AB (EC_{90} = 1.78 μM) was much more potent than Z (EC_{90} = 67.84 μM) at inhibiting mussel byssal attachment, but also induced higher levels of post-treatment mussel mortality 48 h following treatment. Hence, compound AB may have potential as a general molluscicide applied for treatment durations as short as 48 h.

Based on these results alone, it would be difficult to determine which of these efficacious compounds would be the best candidates for future commercial development as zebra mussel antifouling agents. Ranking effective candidate compounds would require further analyses focusing not only on relative costs of compound mass production, but also on projected costs for their incorporation into a

commercial product, such as an antifouling coating or paint. Thus, determination of the utility of these compounds in prevention and control of zebra mussel macrofouling will require further research and development.

CHAPTER 4

CONCLUSIONS

4.1 Overview

This study identified nine novel natural compounds or their synthetic derivatives as potentially effective inhibitors of zebra mussel byssal attachment under laboratory conditions. Of these, compounds Y, AA, T and A were shown to be the most potent byssal attachment inhibitors and the least lethal to *D. magna* and zebra mussels with EC_{90} values and relative *D. magna* survivorships after a 48-h exposure period being 4.00 μM and 93% at 4.00 μM for compound Y, 4.40 μM and 93% at 6 μM for compound AA, 10.66 μM and 93% at 10 μM for compound T and 20.09 μM and 92% at 20 μM for compound A. Compound AB, another potent inhibitor of mussel byssal attachment with an estimated 48-h EC_{90} of 4.32 μM and a corresponding *D. magna* survivorship of 96% at 6 μM resulted in the highest mussel mortality of all effective compounds due to its apparent general molluscicidal characteristics. Compound B was characterized by intermediate potency with an EC_{90} of 27.80 μM and 89% *D. magna* survivorship after exposure to 30 μM for 48 h.

In contrast to the most effective compounds described above, compounds V, L and Z had lower byssal attachment inhibition potencies and induced greater zebra mussel mortality even though they had negligible lethal effects on *D. magna*. The EC_{90}

values and relative *D. magna* survivorship after 48-h exposure for these three compounds were 67.84 μM and 83% at 50 μM of compound V, 53.28 μM and 93% at 10 μM of compound L and 20.09 μM and 92% at 20 μM of compound Z. Like compound AB, general molluscicidal effects were documented for compound L following a 48-h exposure to concentrations higher than 60 μM.

4.2 Compound modes of action

The identified efficacious compounds appeared to have a number of similar structure-activity relationships. Compounds A, B and L, effective inhibitors of mussel attachment characterized by a LASEN structural motif, possessed one or more of the following traits: a non-bulky acyl (lipophilic) chain of moderate length, a hydroxyl group on a benzyl aromatic electronegative end and/or a substitution of the lipophilic acyl chain with an aromatic benzyl structure. The structural dissimilarities between the electronegative ends in compounds A, B and L, and those LASEN compounds shown to be non-efficacious may be associated with their reduced capacities to inhibit byssal attachment despite similar lipophilic ends. Structural modifications in the electronegative end, namely to the hydroxyl group attached to the benzyl ring or the aromatic structure itself, may potentially decrease these compounds' inhibitory activity on the byssal attachment mechanism. Furthermore, significant inhibitory effects on mussel byssal attachment displayed by both the bioactive and nonactive stereoisomers of compound A at the VR1 receptor site suggest that involvement of this receptor is unlikely.

Similarly, structure-activity relationships were identified among the mammalian endocannabinoid compounds tested for inhibition of mussel byssal attachment. The mammalian CB1 receptor agonistic compounds T, V, Z and AB were the most efficacious at inhibiting mussel byssal attachment, indicating a putative active role for the cannabinoid receptor in inhibition of mussel byssal attachment. The majority of these compounds, T, V and Z, had similar structures characterized by one or more of the following: a long unsaturated 18-20 carbon acyl chain containing two or more *cis* double bonds, a terminal hydroxyl group, and an amide or oxygen molecule in the main molecular carbon backbone. Decreased mussel byssal inhibition was noted in compounds U, O, Q, W, X, and AC with shorter, more saturated acyl chains or substitution of the terminal hydroxyl group.

Byssal attachment inhibition induced by exposure to the CB1 receptor agonist, compound AB which was structurally unrelated to the other tested efficacious compounds, suggested that cannabinoid receptor binding may be more important than compound structure in the byssal attachment inhibition process. Because the vanilloid (VR1) compounds B and Q of the tested LASEN group also express mammalian cannabinoid characteristics (see Table 2.2), their capacity to inhibit mussel byssal attachment may be based on their endocannabinoid structure-activity relationships as described above and in Chapter 3. Compound B exhibits a weak affinity for the mammalian CB1 receptor whereas compound Q inhibits mammalian intracellular transport of compound T, thereby enhancing its CB1 agonistic effects (DiMarzo et al., 1998). As hypothesized for compound W, compound Q may need to be in the presence

of other endocannabinoid compounds to be effective (i.e. the entourage effect) and hence, was not an effective byssal attachment inhibitor in this study. Furthermore, the vanilloid compound A (capsaicin) with binding affinities strictly for the VR1 receptor (DiMarzo et al., 1998), could potentially inhibit mussel byssal attachment independently of the endocannabinoid system. Interestingly, Cochereau et al. (1996) has documented competitive inhibition of the tyrosine translational enzyme, tyrosyl-tRNA synthetase, by compound A in rat astrocytes. Since tyrosine is a major constituent of byssal thread proteins (Rzepecki and Waite, 1993), this finding suggests that compound A could potentially interfere with the production of structurally normal byssal threads.

The byssal attachment inhibition efficacy of compounds, L and AB, whose structures most deviated from the LASEN or endocannabinoid requisites discussed above (Tables 2.2 and 3.1), appeared to be primarily associated with their general molluscidal qualities. Thus, this study has identified three potential modes of action for the compounds that were efficacious at inducing inhibition of byssal attachment including behavior modification, interference with byssal thread production/adhesion mechanisms and general toxicity or a synergistic combination thereof.

4.3 Future research

It is clear from the results of this study that much is yet to be learned about the existence of an endocannabinoid signaling system in molluscs. Additional research will be required to determine the mode of action of the nine compounds shown to be effective mussel byssal attachment inhibitors. In order to test their effect on the byssal

production mechanism, studies quantifying the number of byssal threads, their relative strength and the amount of glue protein present at the point of byssal thread adhesion to a hard substrate (the plaque), is warranted.

Examination of the behavioral impacts of the effective compounds on zebra mussel byssal thread production are also warranted. Co-exposure to commercially available, highly selective CB1 and CB2 receptor agonists and antagonists including WIN 55, 212-2 (CB1, CB2 agonist), SR141716A (CB1 antagonist); capsazepine (VR1 antagonist) may support the presence, and elucidate the function of, a molluscan endocannabinoid system in zebra mussels. Furthermore, co-exposure of zebra mussels to more than one mammalian endocannabinoid may provide support for a synergistic entourage effect amongst the cannabinomimetic agents in this species. As little is known about the existence of cannabinoid or vanilloid receptors in zebra mussels or other molluscs, continuation of receptor isolation studies and cloning efforts for these receptors in particular, will be important to fully understanding the functions and impacts of the putative endocannabinoid signaling system on byssal attachment. Finally, identification of these molecules' active moiety or moieties will be critical for the development of a suitable antifouling product incorporating one or more efficacious byssal attachment inhibiting compounds identified in this study.

4.4 Application of research results

Use of the identified compounds in antifouling paints and coatings would necessitate future research on methods to embed molecules in these products in a manner that would provide effective attachment inhibition at the coating surface

without leaching molecules into the surrounding medium as occurs with most presently available commercial non-ablative toxic antifouling coatings/paints. Such a coating would prevent attachment of fouling organisms on a protected surface while having no impact on nonfouling species which do not come into direct physical contact with it. Because prolonged (48-h) exposure to a number of these compounds did not induce significant mortality in the planktonic, non-target species, *D. magna*, it is unlikely that short-term, incidental contact with such molecules would have negative impacts on nonfouling species. Future studies should be focused on the development of such coatings and the testing of their antifouling efficacies in the laboratory and field against both settlement-capable zebra mussel larval stages and adults. For broad spectrum testing, other freshwater and marine macrofouling species could be included in future studies. In addition, the toxicity of such compounds in solution should be investigated against a variety of both vertebrate and invertebrate non-target species in order to assess their environmental acceptability. Furthermore, those compounds determined to be lethal at low concentrations to zebra mussels, but non-toxic to *D. magna*, may also warrant further investigation as environmentally benign molluscicides. Such agents could be applied to intake water to control mussel macrofouling where use of presently available more toxic agents is prohibited or where particularly stringent environmental restrictions are in place.

Presently available toxin-leaching or non-toxic ablative coatings for prevention of zebra mussel and other forms of biological macrofouling have considerable post-installation costs associated with periodic coating replacement or surface renewal

(Mussalli et al., 1992). This study has identified novel, natural compounds and their derivatives with the potential to be used as effective antifouling agents and in coatings against macrofouling by zebra mussels at a time when widely-used, toxic anti-macrofouling coatings such as those based on organotins (TBT) are being greatly restricted or completely banned on an international basis (Nandakumar and Yano, 2003; Watermann, 1999; Xu et al., 2005).

APPENDIX A

DETAILS OF THE STATISTICAL ANALYSIS OF EXPOSURE AND POST-EXPOSURE BYSSAL ATTACHMENT RESPONSES OF ZEBRA MUSSELS EXPOSED TO CAPSAICIN-LIKE AND LASEN COMPOUNDS

Let Y = (Y1, Y2) denote the response vector of an animal, where Y1 is the phase 1 (48-h compound exposure) response and Y2 is the phase 2 (48-h post-compound exposure) response. Owing to the nature of the experimental design, the possible values of Y1 are: A (attached), U (unattached but alive) and D (dead). Since any dead animals were removed after phase 1, the possible values of Y2 were: A (attached), U (unattached but alive), D (dead) or N (not observed).

The possible values of Y= (Y1, Y2) are shown in the following diagram:

VALUE OF Y2

Value of Y1	A	U	D	N	Total
A	Q_{AA}	Q_{AU}	Q_{AD}	0	Q_{A*}
U	Q_{UA}	Q_{UU}	Q_{UD}	0	Q_{U*}
D	0	0	0	Q_{DN}	Q_{D*}
Total	Q_{*A}	Q_{*U}	Q_{*D}	Q_{*N}	1

In this diagram, the entries Q_{AA}, *etc*., are the probabilities of the corresponding response combination. Note that if Y1 = D, then Y2 = N is the only possible response at phase 2.

The sum of the probabilities over all (Y1, Y2) combinations where Y1 = A, U, D and Y2 = A, U, D and N will be 1. The marginal probabilities Q_{A*}, Q_{U*} and Q_{D*} define the distribution of Y1, ignoring Y2. (Note: The parameters p_{c1} above are defined as $Q_{U*}/(Q_{U*} + Q_{A*})$. The parameters p_{c2} above are given by $Q_{*U}/(Q_{*U} + Q_{*A})$.

The experimental observation process is sufficient to allow the frequencies with which Y1 and Y2 attain each possible response to determined. Further, the joint distribution of these frequencies is multinomial and governed by the probabilities shown in the above diagram. Finally, the classical weighted least squares methodology for multinomial proportions, as outlined in Koch et al. (1985) applies to allow the construction of the standard error of the estimate of $p_{c1} - p_{c2}$. These computations were carried out using the CATMOD procedure in SAS.

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