The effects of sediment organic carbon and chemical residence time on the acute toxicity of sea lice chemotherapeutants to benthic invertebrates

by

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B.Sc. (Biology), University of British Columbia, 2015

Project Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Environmental Toxicology

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY
Spring 2021

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Abstract

Chemotherapeutants are commonly used to manage sea lice outbreaks in salmonid aquaculture. Among the classes of chemotherapeutants used are avermectins; these tend to persist in the sediments underneath salmon farms and may directly impact nearby benthic fauna of marine ecosystems. The present study sought to determine how two environmental factors – namely, sediment organic carbon (OC) and chemical residence time – can modify the toxicity of emamectin benzoate (EB; formulation: Slice®) and ivermectin (IVM) in two species of benthic invertebrates: the amphipod *Eohaustorius estuarius* and the polychaete *Neanthes virens*. In both species, sediment OC significantly reduced toxicity, an effect that was more pronounced for IVM and combination exposures. Four months of chemical residence time reduced toxicity in *E. estuarius* but did not affect toxicity in *N. virens*. This research provided novel insight into the effects of two environmental factors that potentially impact avermectin toxicity in nontarget species underneath salmon farms.

Keywords: Sea lice; Avermectins; Toxicity; Organic carbon; Sediment aging; Benthic invertebrates

Acknowledgements

I would like to offer my thanks to the Department of Fisheries and Oceans Canada (DFO) for funding my research, and my supervisor Dr. Christopher Kennedy for mentoring me along the way. An enormous thanks to Geoffrey Su for all the help in the lab and for really helping me get my project up and running. Thank you to all the Kennedy and Marlatt lab members for their help and support and making the PNW-SETAC 2019 conference an unforgettable experience. My statistics consultant, Ian Bercovitz, was of tremendous help to me, and I have him to thank for navigating my way through the complex statistical analysis that I thought would never end. A big thanks to all the undergraduate students who volunteered in the lab and who helped me enormously with this project, most notably Paola, Jordan, Kevin, and Alexis; you were all fantastic. Finally, thank you Samantha Straus for helping me collect sediment, rain or shine, patiently teaching me how to use R Statistical Software, and, of course, for all your support along the way.

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List of Acronyms

ANCOVA: Analysis of covariance

ANOVA: Analysis of variance

DFO: Fisheries and Oceans Canada

DOC: Dissolved organic carbon

DOM: Dissolved organic matter

EB: Emamectin benzoate

EC50: Median effect concentration

ETMF: Exposure and toxicity modifying factor

FAO: Food and Agriculture Organization of the United Nations

GABARs: ionotropic subtypes of GABA receptors

GluClRs: glutamate-gated chloride channels

IVM: Ivermectin

LC50: Median lethal concentration

OC: Organic carbon

OECD: Organization for Economic Co-operation and Development

OM: Organic matter

OP: Organophosphate

POC: Particulate organic carbon

POM: Particulate organic matter

TOC: Total organic carbon

Chapter 1. Introduction

1.1. Aquaculture

1.1.1. Worldwide

In the past several decades, global aquaculture production has grown rapidly. In the 1980s and 1990s, the annual growth rate by tonnage of product was 10.8 and 9.5%, respectively (FAO 2018). More recently, between 2001 to 2016, annual growth rates have slowed, but continue to grow at a rate of 5.8% (FAO 2018). Meanwhile, wild capture production has not nearly expanded as quickly. The world is becoming increasingly reliant on aquaculture, relative to wild capture production; aquaculture production is now approaching wild capture production rates by tonnage (FAO 2018). For example, the global production of aquaculture grew by approximately 30% between the years 2011 and 2016 (FAO 2018). On the other hand, wild capture production remained steady with a marginal decline of approximately 1% during that same timespan (FAO 2018). The result is that aquaculture production, by tonnage, now comprises close to half of all seafood production globally.

A variety of taxa are reared globally, with finfish representing a major proportion of production (FAO 2018). In 2016, global aquaculture production was 110.2 million tons, of which 30.1 million tons were aquatic plants (FAO 2018). Finfish production was responsible for approximately 68% of aquatic animal production. The 80 million tons of animal production was comprised of 54.1 million tons of finfish, 17.1 million tons of molluscs, and 7.9 million tons of crustaceans (FAO 2018). The remaining 938,500 tons were other groups of animals such as turtles, sea cucumbers, sea urchins, frogs, and edible jellyfish (FAO 2018). As of 2016, the FAO (2018) has recorded 598 species to have been reared in aquaculture; this includes 369 finfish, 109 molluscs, 64 crustaceans, 7 amphibians, 9 aquatic invertebrates, and 40 aquatic algae (FAO 2018). Of the finfish, three species of carp (*Ctenopharyngodon idellus*, *Hypophthalmichthys molitrix*, and *Cyprinus carpio*) were the top three finfish produced globally in 2016, responsible for 29% of all finfish production (FAO 2018). Atlantic salmon (*Salmo salar*) produced the ninth largest quantity of finfish, comprising 4% of all finfish produced globally (FAO 2018).

The largest producer of aquaculture is China. It has accounted for more tonnage of aquaculture production than the rest of the world combined since 1991 (FAO 2018). From 1995 to 2016, it has contributed to over 60% of global aquaculture production, by tonnage (FAO 2018). This makes up a large percentage of Asia's production, which has been responsible for close to 90% of global aquaculture production from 1995 to 2016 (FAO 2018). In 2016, the Americas, Europe, Africa, and Oceania, were responsible for 4.2, 3.7, 2.5, and 0.3% of global aquaculture production by tonnage, respectively. Although the America's and Oceania's contributions have remained relatively constant between the years of 1995 to 2016, Africa's contribution has been rising steadily (FAO 2018). Meanwhile Europe's production has almost halved during these years (FAO 2018).

1.1.2. Canadian aquaculture

Aquaculture contributes significantly to Canada's economy and has been expanding rapidly in recent decades. Between the years 2000 and 2018, the tonnage of production by aquaculture in Canada has grown by 50% (DFO 2018). This generates approximately 20% of total seafood production in Canada, contributing to one third of Canada's total fisheries value (DFO 2018). In 2018, aquaculture produced 191,259 tons of product, corresponding to a monetary value of $1.4 billion CAD (DFO 2018). In addition, it is an important employer. In 2010, it was directly responsible for approximately $190 million CAD of the annual labor income, and indirectly for over $500 million CAD (DFO 2013).

Aquaculture operations can be found in all provinces, and in the Yukon. However, there are five provinces that contribute to 95% of Canada's aquaculture production: British Columbia, New Brunswick, Prince Edward Island, New Foundland, and Nova Scotia (DFO 2018). These provinces contributed to approximately 51%, 16.5%, 13%, 9.4%, and 5.3%, respectively, of total annual production in 2018 (DFO 2018).

Forty-five aquatic species are reared in aquaculture operations across Canada (DFO 2018). Of these, finfish, shellfish, and algae make up 26, 16, and 3 species, respectively (DFO 2018). Finfish production plays the most significant role in Canadian aquaculture. It alone constitutes most of this industry's production, accounting for close to 80% of Canada's annual production, by tonnage, over the past ten years (DFO 2018). Salmonid species are the major

contributor to finfish production, representing a crucial resource for the Canada's aquaculture industry.

1.1.3. Salmonid aquaculture in Canada

Canada is the fourth largest producer of farmed salmonids (Family: Salmonidae) worldwide, behind Chile, Scotland, and Norway (Burridge et al. 2010, DFO 2018). A total of 9 salmonid species are farmed in Canada including those of the genus *Salvelinus*, *Oncorhynchus*, and *Salmo* (DFO 2018). Of these, the three major species farmed are Atlantic Salmon (*Salmo salar*), Chinook Salmon (*Oncorhynchus tshawytscha*), and Coho Salmon (*Oncorhynchus kisutch*). Salmonids represent an overwhelming majority of total finfish production in Canada. In 2018, salmon and trout accounted for close to 90% of finfish production, by tonnage (DFO 2018). Three provinces account for essentially all salmon production in Canada: British Columbia, New Brunswick, and Nova Scotia; in 2018, these provinces comprised 70.6, 23, and 6.4% of all salmon production in Canada by tonnage, respectively (DFO 2018).

1.1.4. Salmonid aquaculture in BC

Well over half of all salmonid aquaculture production originates in BC. Approximately 130 marine finfish aquaculture operations exist in BC (DFO 2011a). These operations are primarily situated along the east and west coasts of Vancouver Island, in coastal areas near locations including Tofino, Campbell River, and Port Hardy (DFO 2011a). These facilities are almost exclusively devoted to salmonid farming, especially Atlantic salmon (DFO 2011a). In 2018, by weight, salmonid aquaculture was responsible for approximately 99.4% of all finfish aquaculture production and 90.3% of all aquaculture production in BC. Furthermore, around 80 freshwater finfish facilities also exist in BC, typically using pond culture of rainbow trout, or functioning as a hatchery for sturgeon, Coho salmon, and sockeye salmon (DFO 2011a).

Marine and freshwater salmonid aquaculture implements a variety of aquatic farming techniques. Among these, open net pens are a common method for farming salmonids in Canada. On the coast of BC, over 100 open net pen salmon farms are active in marine waters (DFO 2020). Open net pens are permeable enclosures that are placed directly in coastal waters. In this way,

they can take advantage of conditions of natural aquatic environments. For example, water temperature, salinity, and dissolved oxygen, can all be regulated in open net pens by continuous exchange with the surrounding aquatic environment. Additionally, waste products are prevented from building up in open net pens since they can disperse into the surrounding marine environment.

The operational efficiencies of open net pens come with drawbacks. Coastal open net pens can negatively impact the surrounding marine ecosystem (Burridge et al. 2010, Burridge and Van Geest 2014, Krkosek et al. 2011, Haya et al. 2001, Morton and Routeledge 2016). One reason for this is that the run-off from farms, including waste-products and chemical treatments, can disperse into the surrounding environment, polluting the ecosystem (Burridge et al. 2010, Burridge and Van Geest 2014, Haya et al. 2001). Another reason is that these densely populated pens provide an ideal environment for the propagation of pathogens (Krkosek et al. 2011, Morton and Routeledge 2016). Pathogens such as viruses, bacteria, and parasites can proliferate on salmon farms, then disperse into the environment (Krkosek et al. 2011, Morton and Routeledge 2016). Sea lice are one example of a pathogen that can thrive on salmon farms.

1.2. Sea lice

Sea lice are ectoparasitic copepods of the family Caligidae, with over 500 species belonging to 37 genera (Boxaspen 2006, Ahyong et al. 2011). They can be found in both brackish and marine environments. Their life cycle can be summarized in the following stages: (1) free-living planktonic (2) juvenile, (3) pre-adult, and (4) adult (Figure 1). The number of moulting events that occur at each of these stages depends on the species of sea lice (Boxaspen 2006). Furthermore, not all species have a pre-adult phase (Costello 2006). Immediately following hatching, the free-living planktonic stage, also called the naupliar stage, disperses with water currents (Boxaspen 2006). This precedes the juvenile stage when it begins to feed on the host (Boxaspen 2006). At the beginning of this juvenile stage, when it is referred to as a copepodid, the sea louse uses photo-, mechano-, and chemoreceptors to locate its host (Costello 2006, Thorstad et al. 2015); this is the infectious stage. Using specialized filamentous mouthparts, the copepodid attaches to the epithelium of the host, and begins feeding on the mucus, underlying tissue, and blood (Costello 2006). After attachment, the copepodid moults into a chalimus. During its juvenile stage it is sessile (Thorstad et al. 2015). Once it progresses to the pre-adult and adult

stages, it is mobile, continuing to feed with the additional ability of swimming in the water column for short periods (Thorstad et al. 2015). In this mobile phase, it has the potential to transfer from one host to another (Thorstad et al. 2015). The mobile phase is the most damaging to the host (Torrissen et al. 2013).

Host range depends on the species of sea lice; most sea lice research has focused on two species: *L. salmonis* and *C. elongatus* (Boxaspen 2006). As a result, much more is known about these two species than others. *C. elongatus* is more of a generalist, having been recorded to parasitize over 80 species of both elasmobranch and teleost fish (Pike and Wadsworth 1999). On the other hand, *L. salmonis* is specialized in parasitizing salmonids. It has been observed to parasitize 13 species of salmonid (Johnson and Fast 2004, Pike and Wadsworth 1999). Under rare circumstances, it can also parasitize non-salmonid hosts (Johnson and Fast 2004, Pike and Wadsworth 1999).

During the feeding stages of its life cycle, sea lice can reduce a salmonid host's fitness. Sublethal effects that have been documented in salmon include reduced respiratory and osmoregulatory capacity; reduced growth and swimming capabilities; and compromised immune function (Fjelldal et al. 2019, Godwin et al. 2017, Johnson and Fast 2004, Tully and Nolan 2002, Wagner et al. 2004). These sublethal effects may indirectly lead to mortality, however it is less common that sea lice are directly responsible for the mortality of its salmonid host.

Sea lice infection intensity thresholds for sublethal and lethal effects are hard to define as they appear to be salmonid species and life-stage dependent. Generally, Atlantic salmon (*Salmo spp.*) appear to be more susceptible to these effects than Pacific salmon species (Costello 2006). In addition, earlier life stages are more susceptible than adults (Liu et al. 2011, Morton et al. 2016). A recent study using *L. salmonis* showed that after four weeks, Arctic Char (*Salvelinus alpinus*) smolts with a mean infection intensity of 0.33 (range 0.09-0.91) mobile lice per g of fish show significant declines in body growth (weight and length), and osmoregulatory abnormalities (Fjelldal et al. 2019). Furthermore, fish mortality showed an infection intensity-dependent trend, with 100% mortality at $\geq$ 0.7 mobile lice g^{-} fish (Fjelldal et al. 2019). In a different study, at 21 d post-infection, brown trout (*Salmo trutta*) smolts began to show physiological signs of infection at an *L. salmonis* load of 0.19-0.68 mobile lice per gram of fish, corresponding to approximately 13 sea lice per fish (Wells et al. 2006). These physiological endpoints included changes in plasma glucose, lactate, osmolarity, chloride, and cortisol (Wells et al. 2006). Deleterious effect thresholds of sea lice infection have not been thoroughly investigated for other species of salmonids. However, Costello (2006) suggests that if one accounts for laboratory, population, and on-farm

observations, a general guideline is that 5-10 sea lice per fish or 0.1 sea lice per gram of fish has the potential to be deleterious to all salmonid species of any life stage.

High host population densities on salmon farms are conducive of propagation of parasites including sea lice. The first recorded outbreak of sea lice on Atlantic salmon farms occurred in Norway in the 1960s (Pike and Wadsworth 1999). Similar findings occurred in the mid-1970s and late 1980s in Scotland and the Atlantic coast of North America, respectively (Pike and Wadsworth 1999). Of the species of sea lice that exist, there are five species that are reported most often in salmonid marine aquaculture globally: *Caligus clemensi* (Pacific Ocean) *Caligus elongatus* (Atlantic Ocean)*, Lepeophtheirus salmonis*, *Caligus teres*, and *Caligus rogercressyi* (Johnson et al. 2004). The latter two are found in the Southern Hemisphere, while the other three are found in the Northern Hemisphere (Johnson and Fast 2004). These five species are associated with the greatest economic and ecological burden.

Sea lice outbreaks on salmon farms have the potential to cause both economic and ecological impacts. Economically, the cost of management and the loss of product is responsible for a reduction in revenue for the aquaculture industry. Costello (2009a) estimated that globally, sea lice outbreaks cost salmonid aquaculture operations close to $500 million USD annually, which is 6% of the total production value. More recently, Abolofia et al. (2017) estimates that sea lice infestations on salmon farms result in a loss of revenue of between 2.27-13.10% depending on latitude and seasonality of salmon cohort introduction into net pens. Wild catch fisheries may also experience a loss in revenue due the spread of sea lice from farmed salmon to wild populations. Although the financial impact on wild fisheries have been more challenging to define. Furthermore, the impact on wild salmon populations also represents an environmental concern. Many studies have revealed that open net pens allow for transmission of sea lice to wild populations, increasing the sea lice infection load in these populations (Costello 2009b, Krkosek et al. 2005, Morton et al. 2004, Morton et al. 2008, Torrissen et al. 2013, Thorstad and Finstad 2018). In fact, the passive dispersal distance of the naupliar life-stage of sea lice can be greater than 100 km depending on ocean currents (Thorstad and Finstad 2018). Although sea lice are naturally occurring in wild populations, the added burden from this spill-over from salmon farms appears to be ecologically relevant. For example, two different salmon farms located in BC along wild salmon migration routes were modelled to increase sea lice infection pressure on juvenile wild pink (*Oncorhynchus gorbuscha*) and coho (*Oncorhynchus kisutch*) salmon by four to five orders of magnitude over natural infection rates (Krkosek et al. 2005). This was the case over a 75 km span along the migration route (Krkosek et al. 2005). Similar findings have been made in

BC, showing a significantly greater abundance of *L. salmonis* and *C. clemensi* on wild juvenile pink, chum, and sockeye (*Oncorhynchus nerka*) salmon inhabiting areas near salmon farms compared to populations not in the vicinity of farms (Morton et al. 2004, Morton et al. 2008, Price et al. 2011).

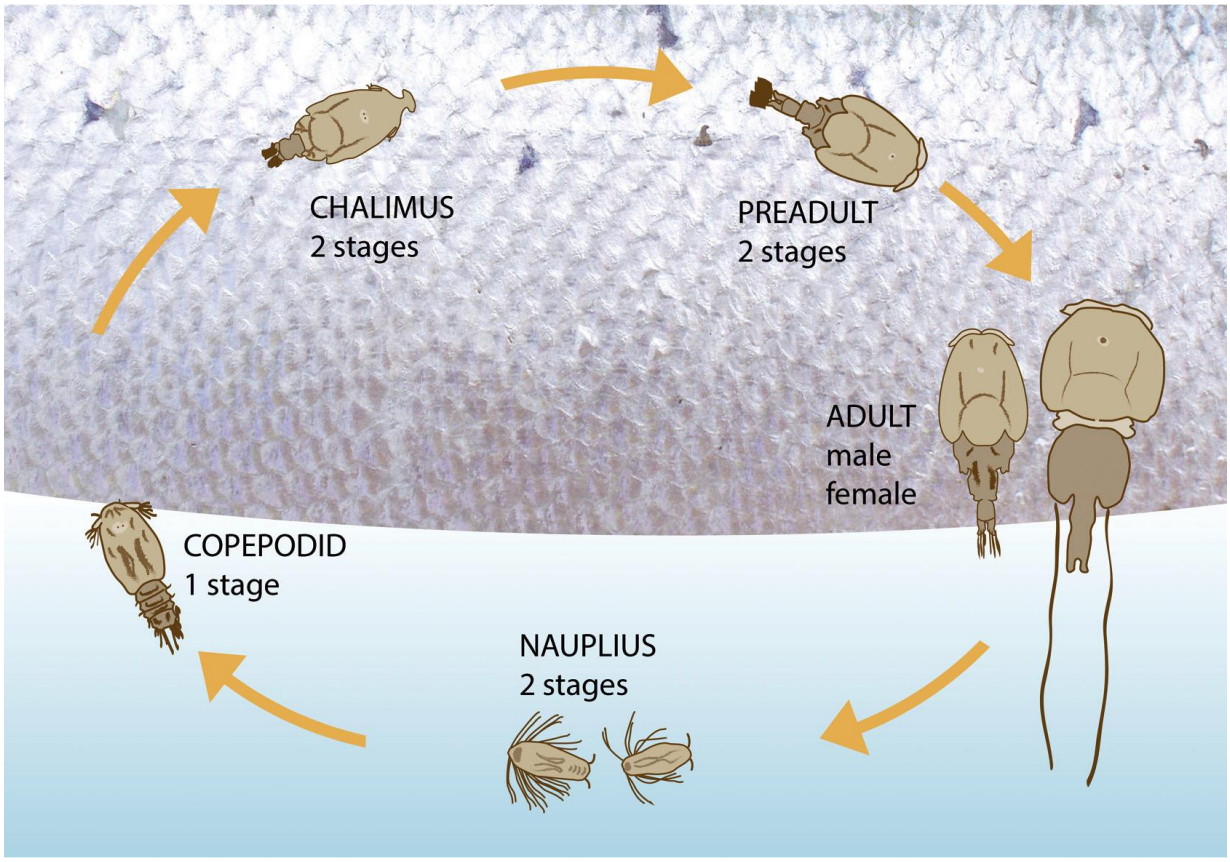


Figure 1: Depiction of salmon sea louse life cycle. The free-living planktonic stage is the nauplius (length: ~0.5-0.6 mm). This is followed by the juvenile life stages (copepodid and chalimus) when it begins feeding on the superficial tissue of the salmonid host. The preadult and adult phases can transfer from one host to another. Sizes of different life-stages are not shown to scale. The length of the copepodid and chalimus stages are 0.7 and 1.1-2.3 mm, respectively. The length of preadults is typically between 3.4-5.2 mm. The length of adults is 5-6 mm (males) and 8-12 mm (females). (Figure credit: Thorstad et al. 2015).

1.3. Sea lice management strategies

Methods for managing and preventing sea lice burdens can be broadly categorized into two different groups: (1) non-chemical and (2) chemical. Non-chemical means include the following: fallowing; biological controls; improved animal husbandry; mechanical and electrical barriers; traps; mechanical, thermal, and optical delousing; and selective breeding (DFO 2014, Overton et al. 2018, Sletmoen 2016). Chemical treatments combat sea lice with chemotherapeutants. Five groups of chemotherapeutic compounds have been, or are currently being used, worldwide in salmonid aquaculture: organophosphates (OPs), pyrethroids, hydrogen peroxide, avermectins, and benzoyl ureas (Aaen et al. 2015, Burridge et al. 2010, Haya et al. 2005, Roth 2000). The latter two groups are delivered as in-feed additives, while the former three are delivered through bath treatments (Haya et al. 2005). Although the use of chemotherapeutants is currently the most widely used approach to management, there is a large base of evidence for sea lice developing resistance to all groups of chemotherapeutants except for benzoyl ureas (Aaen et al. 2015). In fact, certain mechanisms behind resistance have even been elucidated. For example, a mutation in the gene that encodes acetylcholinesterase (AChE) is responsible for resistance to organophosphates (Aaet et al. 2015, Fallang et al. 2004). The trend towards increased resistance to chemotherapeutants demonstrates the importance of developing an integrated approach to management. Furthermore, use of chemotherapeutants can have potentially damaging ecological effects due to chemical pollution of the surrounding aquatic ecosystem. Therefore, the development and improvement of non-chemical treatments is an important future direction for an effective integrated pest management strategy.

1.3.1. Non-chemical treatments

Many non-chemical sea lice management and prevention efforts exploit the concept of reducing salmonid host population densities on farms and the density of farms within a certain geographical area (DFO 2014, Jansen et al. 2012, Kristoffersen et al. 2013). For example, fallowing is a management option which involves removing salmon hosts from within a net-pen for a certain duration to reduce host population density, and therefore also sea lice density (DFO 2014). An effective duration of fallowing depends on the life cycle length of sea lice, which is also affected by environmental conditions such as water temperature (DFO 2014). Another

consideration for effective fallowing is wild fish population densities near the salmon farm (DFO 2014). If nearby wild host fish population densities are high, fallowing may be less effective. As a preventative measure, some salmon farms may also permanently operate with reduced salmon net-pen densities (DFO 2014). Furthermore, with regards to both management by fallowing and prevention by reduced cage density, it is important to consider geographical salmon farm density. For example, in Chile and Norway, the density of farms in an area is positively correlated with sea lice abundance on farms in that area (Jansen et al. 2012, Kristoffersen et al. 2013). It is therefore recommended that farms not be situated too close to each other. Furthermore, it is recommended that all farms nearby each other make a concerted effort when adopting any management and prevention approach.

Biological controls can be divided into two methods: (1) Integrated Multitrophic Aquaculture (IMTA) using bivalves and (2) utilizing cleaner fish. Broadly speaking, IMTA looks to achieve a more ecologically sustainable approach by allowing nearby farmed seaweed or filter-feeding mollusks to recycle excess nutrients (for example from feed/waste) generated by aquaculture operations (DFO 2014). However, promoting the proliferation of filter feeding bivalves can also act as a method for biological control, since they can feed on the free-living zooplanktonic life-stage of sea lice (Alexander et al. 2016, DFO 2014). Although, the efficacy of this approach has yet to be investigated. The more well-established biocontrol method is the use of certain fish, termed ‘cleaner fish’, which graze directly on attached sea lice, reducing parasite load for hosts. The use of wrasse (*Labridae spp.*) as a cleaner fish began in 1989 in Norway and Scotland. Currently, five species of wrasse are used in those countries (DFO 2014). One major limitation to wrasse is that they are dormant during winter months and therefore unable to graze at that time (Powell et al. 2018). This prompted interest in the lumpfish (*Cyclopterus lumpus*), which can continue to feed on lice in winter months (Powell et al. 2018). The lumpfish has shown promise and is now the most used cleaner fish in Norwegian aquaculture (Barrett et al. 2020, Imsland et al. 2018, Powell et al. 2018). Although in Scotland and Norway cleaner fish are common practice on farms, in Canada this strategy has yet to be adopted. Promising species for use in eastern Canada include the cunner (*Tautogolabrus adspersus*) and the lumpfish which are endemic to this region (DFO 2014). There are no known candidates for the west coast of Canada, since no known cleaner fish candidates are endemic to this area (DFO 2014).

Selective breeding strategies for sea lice resistant strains of Atlantic salmon (Salmo salar) has the potential to become an important facet in sea lice management in aquaculture (Gharbi et al. 2015, Gjerde et al. 2011, Jones et al. 2002, Kolstad et al. 2005, Tsai et al. 2016). However, it

is still in need of development. Two major issues currently are that it lacks reliable protocols for identifying breeding value and its efficacy has not been sufficiently explored (Gharbi et al. 2015). It is also a work intensive and time-consuming process, which may not bear immediate results, and has no guarantee of working. Despite this, Atlantic salmon eggs for strains with increased sea lice resistance are now commercially available in Norway (DFO 2014). As it stands, selective breeding is an important future direction to consider in the development of non-chemical management strategies.

Delousing strategies can be categorized as follows: mechanical, temperature/salinity treatment, and optical. Mechanical delousing technologies involve removal of sea lice by flushing the salmon with seawater (Sletmoen 2016, Overton et al. 2018). Several patented systems exist, and some systems include a step where a brush is used to remove any sea lice that persist after the flushing stage (Sletmoen 2016, Overton et al. 2018). Depending on which system is used, it can remove up to 81-100% of attached lice on individual fish (Overton et al. 2018). Temperature treatment involves using thermal delousing technologies. These exploit sea lice's sensitivity to elevated temperatures. In this method, typically salmon are briefly flushed with hot water before entering a warm water bath (Sletmoen 2016). This method can remove between 75-100% of attached mobile sea lice (Overton et al. 2018). Salinity treatment involves utilizing an abrupt decrease in salinity to eliminate sea lice. For example, this may involve holding an affected fish in fresh water for between 2-3 h (Sletmoen 2016). This delousing strategy can eliminate up to 90% of sea lice (Sletmoen 2016). Finally, optical delousing uses a camera that can recognize sea lice attached to salmon, at which point it exterminates the sea lice by focusing a laser on the louse (Sletmoen 2016).

Mechanical and electrical barriers can prevent sea lice from entering salmon net pens. There are four main mechanical barrier technologies: snorkel (chimney) cages, nets, skirts, and aeration diffusers (BCFSA 2019, Bobadilla and Oidtmann 2017). Anti-sea lice nets are simply the addition of a plankton net (mesh size: 100 µm) around the net pen (Bobadilla and Oidtmann 2017). This is designed to prevent sea lice larvae from entering the net pen (Bobadilla and Oidtmann 2017). Doing this may reduce sea lice infection rates by up to 75% (Bobadilla and Oidtmann 2017). Anti-sea lice skirts involve a less permeable barrier by wrapping tarpaulin around the upper portion (up to depths of 10m) of a net pen (BCFSA 2019, Bobadilla and Oidtmann 2017). Since sea lice larvae tend to inhabit shallower water depths, closer to the surface, the skirt prevents entry at these depths (BCSFA 2019, Bobadilla and Oidtmann 2017). Snorkel cages have an angular net ceiling, which leads into a tarpaulin tube (Sletmoen 2016, Stien et al. 2016). This is

designed to encourage salmon to stay below the sea lice zone, while also allowing them to refill their swim bladders at the surface when necessary (Stien et al. 2016). Snorkel cages have been shown to reduce lice loads by between 24-84% (Geitung et al. 2019, Sletmoen 2016, Stien et al. 2016). Oppedal et al. (2017), demonstrated when using snorkel depths of 0, 4, 8, 12, and 16 m, there was exponential decline in sea lice infestation levels with increasing snorkel depth. Snorkel depths of 16 m resulted in reductions in sea lice infestation levels by 10 to 20-fold compared to those at 0 m (Oppedal et al. 2017). Aeration diffusers also known as 'bubble curtains', are installed around the circumference of a net pen at around a depth between 15-20 m (BCSFA 2019). These machines release air bubbles around the perimeter of the net pen, creating a barrier and preventing sea lice from entering net pens (BCSFA 2019). In addition to mechanical barriers, electrical barriers have also been used to deter sea lice infiltration into net pens. For example, a technology called the Seafarm Pulse Guard utilizes electronic netting which stuns free-living stage of sea lice (Sletmoen 2016).

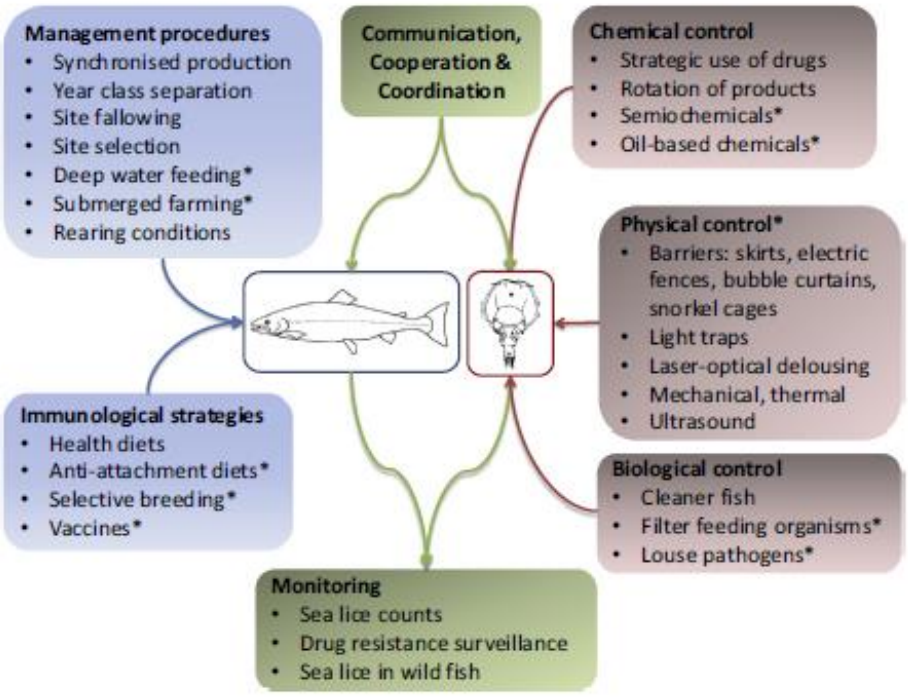
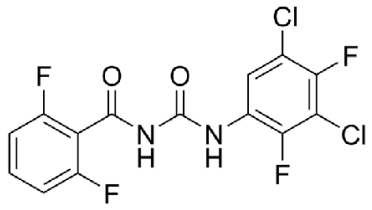
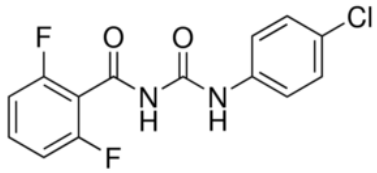


Figure 2: The integrated approach to pest management of sea lice on salmon farms. These methods can be broadly categorized into non-chemical and chemical management strategies. Management strategies can target either the host (e.g. improved husbandry, fallowing, selective breeding, etc.) or they can focus on the sea lice (e.g. biological control, barriers, delousing, chemical, etc.). (Figure credit: Bobadilla and Oidtmann 2017)

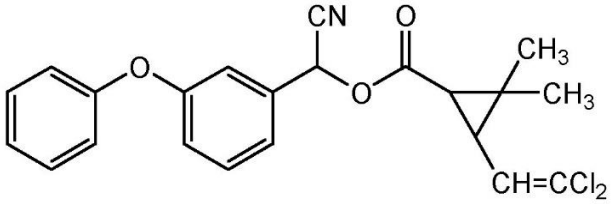
(a) Teflubenzuron



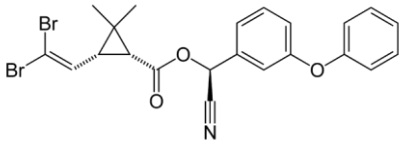
(b) Diflubenzuron



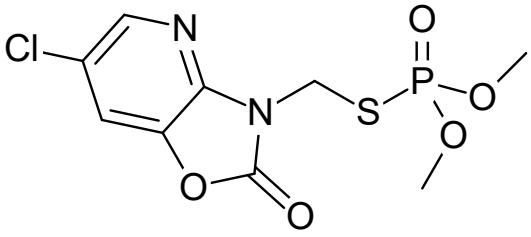
(d) Cypermethrin



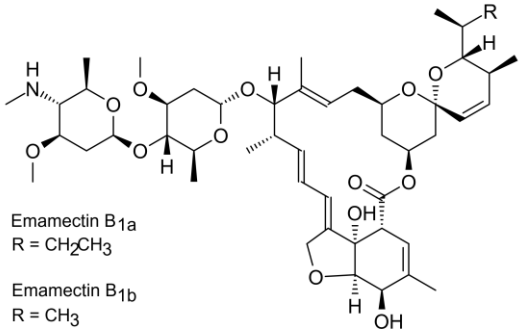
(c) Deltamethrin



(f) Azamethiphos



(e) Emamectin (B1a and B1b)



(g) Hydrogen Peroxide

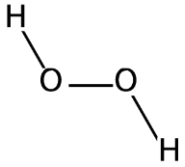


Figure 3: Chemical control of sea lice. Structures of 7 chemicals that are currently used worldwide as chemotherapeutants for sea lice (clinical registration and usage varies from country to country).

1.3.2. Sea lice chemotherapeutants: worldwide and in Canada

Although clinically registered treatments vary by country, seven compounds, found in a variety of sea lice formulations, are currently used in salmonid aquaculture globally: teflubenzuron and diflubenzuron (benzoyl ureas); cypermethrin and deltamethrin (pyrethroids); azamethiphos (OP); emamectin benzoate (avermectin); and hydrogen peroxide ([Figure 3] Aaen et al. 2015, Burridge et al. 2010, Torrissen et al. 2013). The use of chemotherapeutants to manage sea lice began in Norway in 1974 with the introduction of trichlorfon (metrifonate), an OP (Aaen et al. 2015, Torrisen et al. 2013). The use of another OP, dichlorvos, followed with its introduction in Scotland in 1979 (Aaen et al. 2015, Torrisen et al. 2013). Dichlorovos was subsequently used in Chile and Norway in 1985 and 1986, respectively (Aaen et al. 2015). Until the 1990s it became the treatment of choice for most salmonid aquaculture operations globally (Torrisen et al. 2013). Trichlorfon was phased out by the late 1990s in most countries (Aaen et al. 2015, Roth 2000). At the time, it was also becoming evident that resistance was starting to develop, therefore alternative chemotherapeutants were investigated for effective control of sea lice (Aaen et al. 2015).

In the early 1990s, several new chemotherapeutants became available to aquaculture operations globally. This began with the introduction of hydrogen peroxide in Scotland and Norway in 1993 (Aaen et al. 2015). Azimethiphos, an OP, was introduced shortly after in 1994 in those two countries (Aaen et al. 2015). Azimethiphos was an important break-through at the time as it was shown to be 10-fold more effective than dichlorvos, with a larger therapeutic margin in Atlantic salmon (*Salmo salar*) (Overton et al. 2018, Roth 2000). This discovery likely led to the discontinuation of dichlorovos, which was no longer used by the late 1990s in most countries (Aaen et al. 2015, Roth 2000). Around the same time, pyrethroids also began to be purposed for sea lice management, first having been used in Norway in 1994 (Aaen et al. 2015, Overton et al. 2018, Torrissen et al. 2013). Two pyrethroids, cypermethrin and deltamethrin, continue to be used for sea lice management today (Overton et al. 2018). In the late 1990s, two benzoyl ureas, teflubenzuron and diflubenzuron, began to be used in sea lice management (Haya et al. 2005). They were approved for use in Norway and Scotland in 1997 and 1999, respectively (Aaen et al. 2015, Haya et al. 2005).

The most recent addition to the chemical arsenal for managing sea lice is emamectin benzoate (EB). It became available in 1999 as a treatment option, and all salmonid-farming countries have adopted it since then (Aaen et al. 2015, Torrissen et al. 2013). It is part of a family

of drugs called avermectins, which are macrocyclic lactones synthetically derived from the bacterium *Streptomyces avermitilis* (Haya et al. 2005). In the past, two other avermectins, ivermectin (IVM) and doramectin, have been utilized less successfully as chemotherapeutants (Roth 2000, Horsberg 2012). Due to IVM's narrow therapeutic margin, it is an unsatisfactory candidate for sea lice control (Roth 2000, Torrissen et al. 2013). In fact, IVM has never been licensed for use in aquaculture (Horsberg 2012). However, it has been used 'off-label' in Canada, Chile, and Ireland at least until the year 2000 (Horsberg 2012). The chemical manufacturer Merck, Sharp, and Dohme (MSD) do not condone this use (Haya et al. 2005). The introduction of EB has presumably offset the off-label usage of IVM for control of sea lice (Haya et al. 2005). Usage of doramectin has only been previously documented in Chile (Roth 2000, Horsberg 2012). No studies have been published with regards to its efficacy as an anti-sea lice agent in salmonid fish, and it currently does not play a major role in sea lice management (Horsberg 2012).

In Canada, it was not until 1994 that chemotherapeutic means of sea lice control were explored and utilized in salmonid aquaculture (Burridge and Van Geest 2014). At that time, major sea lice outbreaks had occurred on Atlantic salmon farms in southwest New Brunswick. In response, the Pest Management Regulatory Agency (PMRA) of Health Canada granted emergency registration to a variety of formulations including the following: Salmosan® (active ingredient (AI): azamethiphos); Salartect® (AI: hydrogen peroxide); Excis® (AI: cypermethrin); a formulation containing ivermectin as the AI; and a formulation containing a mixture of naturally occurring pyrethroids (Burridge and Van Geest 2014). Between 1995 and 2000, Salmosan® and Salartect® were registered for use with the PMRA. Salmosan® was the more efficacious of the two. However, in 1999, the formulation SLICE® (AI: EB) became available under emergency registration. The use of SLICE® quickly overshadowed Salmosan® and Salartect®, which led to the manufacturers of the latter two formulations withdrawing their renewal application with the PMRA (Burridge and Van Geest 2014).

By 2009, SLICE® was fully registered with the PMRA (Burridge et al. 2010). The heavy reliance on SLICE® led to the development of resistance in eastern Canada in 2009, prompting another emergency registration of three formulations: Salmosan®, Paramove®50 (AI: hydrogen peroxide), and Alphamax® (AI: deltamethrin). Use of Alphamax® was discontinued the following year, but Salmosan® and Paramove®50 continue to be the preferred formulations for use in eastern Canadian salmonid aquaculture. On the other hand, SLICE® is the only formulation used in BC (Burridge and Van Geest 2014). Currently, there are three commercial formulations that are approved for use as sea lice chemotherapeutants in Canadian aquaculture: SLICE®,

Interox®Paramove® 50 (AI: hydrogen peroxide), and Calicide® (AI: teflubenzuron) (Yossa and Dumas 2016). The additional two formulations that are available only under emergency release are Salmosan® and Alphamax® (Yossa and Dumas 2016).

1.4. Emamectin Benzoate

The benzoate salt of emamectin (emamectin benzoate; EB) is found in a variety of pesticide formulations designed for both terrestrial agricultural and aquaculture. Slice®, containing 0.2% EB (active ingredient), is a formulation that has been designed for use as an anti-sea lice chemotherapeutant. It was developed by Schering-Plough Animal Health (Kenilworth, New Jersey). Emamectin benzoate is mixture of two avermectin homologues: 4'-epimethyamino-4'-deoxyavermectin B1a benzoate (MAB1a) (≥ 90%) and 4'-epimethyamino-4'-deoxyavermectin B1b benzoate (MAB1b) (≤ 10%) (Bright and Dionne 2005). These two molecules are relatively large (MAB1a: 1008.26 g/mol; MAB1b: 994.24 g/mol).

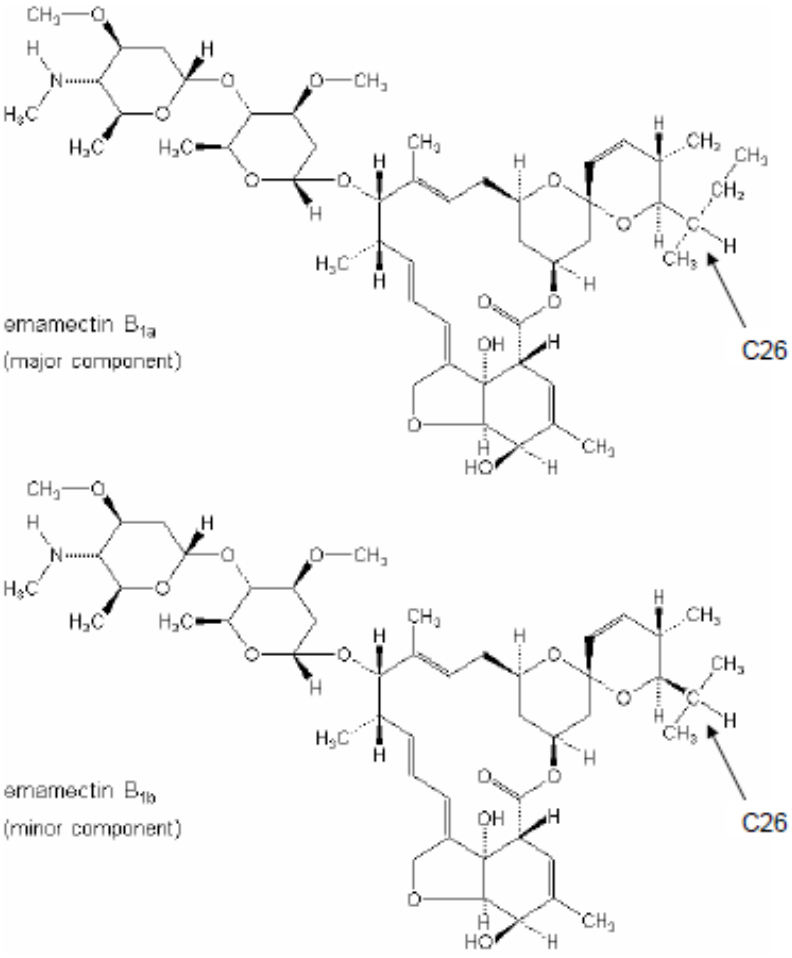


Figure 4: The two emamectin homologs which collectively are termed emamectin. 4'-epimethyamino-4'-deoxyavermectin B1a benzoate (MAB1a) (≥ 90%) and 4'-epimethyamino-4'-deoxyavermectin B1b benzoate (MAB1b) (≤ 10%). (Figure credit: Bright and Dionne 2005).

1.4.1 Environmental fate

EB is relatively lipophilic (log K_{ow} value = 5 at pH 7.0) and has a low solubility in water (24 - 320 mg/L in freshwater and 5.5 mg/L in seawater) (Bright and Dionne 2005). It has negligible volatility with a vapor pressure below 1 mm Hg (Bright and Dionne 2005). It degrades at a variety of rates depending on the environmental medium and conditions. If suspended in aqueous solution, it rapidly decays due to photolysis, with a degradation half-life (DT_{50}) of 1.4 to 22.4 d (Bright and Dionne 2005). Between a pH of 5.2 and 8, it is stable to hydrolysis, but at pH 9 at 25 ^{o}C, the DT_{50} due to hydrolysis is 136.5 d (Bright and Dionne 2005). The DT_{50} in aerobic and

anaerobic soils are reported to be 193.4 and 427 d, respectively (Bright and Dionne 2005). In marine sediment collected from underneath an active aquaculture operation, EB (in the form of Slice®) had a DT_{50} of 404 d (Benskin et al. 2016). While, Bright and Dionne (2005) report a DT_{50} of 164-175 d in marine sediment. Its environmental persistence in sediment has been a cause for concern for aquatic ecosystems subject to chemical run-off from salmon farms.

On salmon farms, EB and its metabolites can accumulate in the aquatic environment via uneaten, medicated food pellets treated with Slice® or contaminated excrement from medicated fish (Bright and Dionne 2005). Due to EB's high log K_{ow} value and low water solubility, it has a strong tendency towards binding to particulate matter when in an aqueous environment (Bright and Dionne 2005). Once bound, it will sink to the benthic zone of an aquatic environment. This suggests that it will build-up in the sediment underneath and adjacent to salmon farms. While tightly bound to sediment particulates, it may also gradually desorb into the aqueous interstitial spaces in sediment, transferring it into the water column (Bright and Dionne 2005). A small fraction of it will reside in the water column; laboratory studies indicate that typically 2 to 3% of it will reside in seawater (Bright and Dionne 2005). This fraction will rapidly decay by photolysis if it is exposed to appreciable levels of light from the water surface.

Detectable levels of EB and its main desmethyl metabolite (4'-deoxy-4'-epi-amino avermectin B1a; AB1) can be found in sediment and water near aquaculture operations. In the Broughton Archipelago, BC, Canada, measurable concentrations of EB in surface sediment and sub-surface seawater after a 7-d treatment regimen with Slice® at two separate active marine salmonid aquaculture operations were found (DFO 2011b). Measurements were taken over the course of 4 months and at varying distances (up to 150m) from the edge of the salmon pen. On one of the sites, the surface sediment concentration of EB peaked at 35 µg/kg (i.e. parts per billion), measured at the edge of the salmon pen (0 m distance interval), 2 to 3 weeks following the first day of Slice® application (DFO 2011b). The other site had maximal levels of only 0.33 µg/kg at the same distance and time interval. The two orders of magnitude difference between the maximal concentration found on these two sites was attributed to the difference between the hydrogeology of the two sites (DFO 2011b). Over the course of 4 months, this maximal detected concentration decreased only slightly (DFO 2011b). Furthermore, EB concentrations decreased as a result of increased distance from the salmon pen, where it began to be close to, or below, the limit of detection of 0.12 µg/kg at distance intervals of 100 and 150 m (east or west) from the edge of the net pens. In addition, detectable levels of EB (approximately 3 µg/kg) persisted when measured 1.5 years after Slice® treatment (DFO 2011b). The measured concentration of AB1

were approximately less than 30% those of EB in surface sediment. Previous studies have had comparable results where EB and AB1 in surface sediment was measured at concentrations of 2.2 and 0.6 µg/kg, respectively, one week after Slice® treatment at 10 m from a salmon pen (Bright and Dionne 2005).

EB levels in the water column were much lower and shorter-lived than those in surface sediments (DFO 2011b). The highest detectable levels were measured in water at 0.6 ng/L (i.e. parts per trillion) within 50 m of both salmon farms one day after the first Slice® application (DFO 2011b). These levels dissipated quickly to below the detectable limit (0.006 ng/L) after 4 to 5 weeks after Slice® treatment (DFO 2011b). The concentration of AB1 was approximately 30% those of EB, which reflects similar results to those in surface sediment (DFO 2011b).

1.4.2. Toxicity to non-target organisms

Aquatic toxicity data from EB exposures with aquatic organisms was compiled and is presented in Table 1 and 2 for water and sediment exposures, respectively. A large portion of the resources and data were found in Lumaret et al. (2012). These values were verified with the original source wherever possible. However, some data reported in this review article were extracted from classified reports, so this was not always possible. Additional data was also extracted from Park (2013), which had carried out a similar compilation at that time. A thorough literature review was also conducted to address any data gaps in Lumaret et al. (2012) and Park (2013), and to find data from more recent years.

Water exposures primarily involved administration through dissolving EB into test water; a small proportion of studies involved administration by incorporating EB into feed. Five out of the six species of aquatic vertebrates had toxicity values available from toxicity testing which involved dissolved administration. The 96 h LC50 of these five species ranged from 18 to 1340 µg/L. The least sensitive vertebrate species was *Cyprinus carpio* (common carp) with a 96 h LC50 of 1340 µg/L. It is challenging to say which is the most sensitive species since life-stage is not reported in some cases. However, the early life-stages of *Pimephales promelas* (fathead minnow) had the lowest 96 h LC50 value at 18 µg/L. The two studies which involved in-feed administration found that juvenile *O. mykiss* and *S. salar* had similar sublethal 96 h NOAELs of 218 and 173 µg/kg, respectively. In comparison to vertebrates, toxicity data was available for a greater number of species of aquatic invertebrate species. The 96 h LC50 of the least and most sensitive

invertebrate species differed by more than four orders of magnitude. The most sensitive invertebrate species was *Mysidopsis bahia* with a 96 h LC50 of 0.04 µg/L. The least sensitive invertebrate species was *Nephrops norvegicus* with a 96 h LC50 of 983 µg/L.

Sediment exposures assessed toxicity over a longer duration of exposure than most water exposures. Marine amphipods had 10 d LC50s ranging from 153-890 µg/kg (either dry or wet weight of sediment), for the species *Corophium volutator, Eohaustorius estuarius,* and *Monocorophium insidiosum*. Another species of crustacean, *H. americanus*, had a rather comparable 10 d LC50 of 250.23 µg/kg (wet weight) to these three marine amphipod species. Two species of annelids, *Hediste diversicolor* and *Arenicola marina* had 10 d LC50s that differed by an order of magnitude from each other at 1368 and 111 µg/kg (wet weight), respectively. A third species, *Neanthes virens*, was also assessed over a much longer duration of 30 d and found to have a NOEC of 171 µg/kg (wet weight). Only one species of mollusk, *Cerastoderma edule*, had been used in sediment toxicity tests, and the resulting 28 d LC50 could not be calculated but was greater than 1000 µg/kg (dry weight).

WATER EXPOSURES									
Test organism	**Life-stage**	**Measured or nominal**	**Formulation or active ingredient**	**Effect measurement**	**Dose descriptor**	**Notes**	**Exposure duration**	**Concentration or dose**	**Reference**
VERTEBRA TES									
Oncorhynchus mykiss (Rainbow trout)				Mortality	LC50 / NOEC	Fresh water	96 h	174 / 48.7 µg L-1	McHenery & Mackie (1999), Schering-Plough Anim. Health (2002) – in Lumaret et al. (2012)
	Juvenile (166-387 g)	M	AI	Lethargy, Appetite, Coloration, and Histology	NOAEL / LOAEL*	Sea water; Feed	96 h	218 / 413 µg kg-1	Roy et al. 2000
Lepomis macrochirus (Bluegill sunfish)	Juvenile (0.42 g)		AI	Mortality	LC50 / NOEC	Fresh water	96 h	180 / 87 µg L-1	OPP 2000
Salmo salar (Atlantic Salmon)	Juvenile (289-484 g)	M	AI	Lethargy, Appetite, Coloration, and Histology	NOAEL / LOAEL*	Sea water; Feed	96hr	173 / 356 µg kg-1	Roy et al. 2000
	Smolts (41-89 g)	M	F	Behavior, weight, histology	NOAEL*	Sea water; Feed	7 d	54 µg kg-1	Stone et al. (2002)
Pimephales promelas (Fathead minnow)	Adult			Mortality	LC50 / NOEC		96 h	194 / 156 µg L-1	McHenery & Mackie (1999), Schering-Plough Anim. Health (2002) – in Lumaret et al. (2012)
	Early Life Stages			Mortality	LC50 / NOEC	Fresh water	96 h	18 /12 µg L-1	
				Mortality	LOEC		96 h	28 µg L-1	

Cyprinodon variegatus (Sheepshead minnow)	Mortality	LC50 / NOEC	Fresh water	96 h	1,340 / 860 µg L-1	McHenery & Mackie (1999);, Schering-Plough Anim. Health (2002) – in Lumaret et al. (2012)
Cyprinus carpio (Common carp)	Mortality	LC50	Fresh water	96 h	260 – 444 µg L-1	Wallace (2001b) in Park (2013)
INVERTEBRATES						
Nephrops norvegicus (Dublin Bay prawn)	Mortality	LC50 / NOEC	Sea water	96 h	983 / 814 µg L-1	McHenery & Mackie (1999), Schering-Plough Anim. Health (2002) – in Lumaret et al. (2012)
	Mortality	LC50 / NOEC	Sea water	192 h	572 / 440 µg L-1	
	Mortality	LC50 / NOEC	Feed	96 h	> 68.2 / 68.2 mg L-1	
	Mortality	LC50 / NOEC	Feed	192 h	> 68.2 / 68.2 mg L-1	
Crangon crangon (Bay shrimp)	Mortality	LC50 / NOEC	Sea water	96 h	242 / 161 µg L-1	McHenery & Mackie (1999), Schering-Plough Anim. Health (2002) – in Lumaret et al. (2012)
	Mortality	LC50 / NOEC	Sea water	192 h	161 / < 161 µg L-1	
Artemia salina (Brine shrimp)	Immobilization	IC50	Sea water	6 h	1.73 µg L-1	McHenery & Mackie (1999), Schering-Plough Anim. Health (2002) – in Lumaret et al. (2012)
Mysidopsis bahia (Mysid shrimp)	Mortality	LC50 / NOEC	Sea water	96 h	0.04 / 0.02 µg L-1	McHenery & Mackie (1999), Schering-Plough Anim. Health (2002)– in

									Lumaret et al. (2012)
Americamysis bahia (Mysid shrimp)				Growth	NOEC	Sea water	28 d	0.0087 µg L-1	Blankinship et al. 2002b – in Park (2013)
Pseudocalanus elongatus (Copepod)	Nauplii (N6)	N	AI	Immobilization	EC50		48 h	0.12 µg L-1	Willis & Ling (2003)
	Copepodites (C1)	N	AI	Immobilization	EC50	Sea water	48 / 96 h	0.14 / 0.17 µg L-1	Willis & Ling (2003)
	Copepodites (C6)	N	AI	Immobilization	EC50		48 / 96 h	0.45 / 10.9 µg L-1	Willis & Ling (2003)
Temora longicornis (Copepod)	Nauplii (N6)	N	AI	Immobilization	EC50		48 h	0.23 µg L-1	Willis & Ling (2003)
	Copepodites (C1)	N	AI	Immobilization	EC50	Sea water	48 h	0.41 µg L-1	Willis & Ling (2003)
	Copepodites (C6)	N	AI	Immobilization	EC50		48 h	2.8 µg L-1	Willis & Ling (2003)
Oithona similis (Copepod)	Nauplii (N6)	N	AI	Immobilization	EC50		48 / 96 h	>15.8 µg L-1	Willis & Ling (2003)
	Copepodites (C1)	N	AI	Immobilization	EC50	Sea water	48 / 96 h	15.86 / 14.75 µg L-1	Willis & Ling (2003)
	Copepodites (C6)	N	AI	Immobilization	EC50		48 / 96 h	232 / 113 µg L-1	Willis & Ling (2003)
Acartia clausi (Copepod)	Nauplii (N6)	N	AI	Immobilization	EC50		48 / 96 h	0.57 / 0.48 µg L-1	Willis & Ling (2003)
	Copepodites (C1)	N	AI	Immobilization	EC50	Sea water	48 / 96 h	0.28 / 0.13 µg L-1	Willis & Ling (2003)
		N	AI						Willis & Ling (2003)
	Copepodites (C6)	N	AI	Immobilization	EC50		48 / 96 h	0.29 / 5.27 µg L-1	Willis & Ling (2003)
	Adult	N	AI	Egg Production	LOEC/NOEC		7 d	0.158 / 0.05 µg L-1	Willis & Ling (2003) Willis & Ling (2003)

Corophium volutator (mud shrimp)				Mortality	LC50 / NOEC	Sea water	10 d	6.32 / 3.2 µg L-1	McHenery & Mackie (1999), Schering-Plough Anim. Health (2002) – in Lumaret et al. (2012)
Daphnia magna				Mortality	LC50 / NOEC	Sea water	48 h	1.0 / 0.3 µg L-1	McHenery & Mackie (1999), Schering-Plough Anim. Health (2002) – in Lumaret et al. (2012)
				Reproduction	EC50 / LOEC	Sea water	21 d	0.16 / 0.09 µg L-1	
				Mortality	LC50	Sea water; Feed	21 d	0.13 µg kg-1	
Daphnia spp.	24 hr old	N	AI	Mortality	LC50	Sea water; Feed	48hr	0.24 µg L-1	Raja et al. 2020
	24 hr old	N	AI	Immobilization	EC50	Sea water; Feed	48hr	0.16 µg L-1	Raja et al. 2020
Mytilus galloprovincialus (Mediterranean mussel)				Development	EC50	Sea water	48 h	314 µg L-1	Aufderheide (2002) – in Park (2013)
				Mortality	LC50	Sea water	48 h	> 713 µg L-1	
Crassostrea virginica (Eastern oyster)				Shell deposition	EC50 / NOEC	Sea water	96 h	530 / 260 µg L-1	Zelinka et al. (1994a) – in Park (2013)
				Mortality	LC50 / NOEC	Sea water	96 h	665 / 260 µg L-1	
Capitella capitata (Polychaete)				Mortality	LC50 / NOEC	Sea water	21 d	1.04 / 0.46 µg L-1	McHenery & Mackie (1999), Schering-Plough Anim. Health (2002) – in Lumaret et al. (2012)

*interpreted from data

Table 1: Summary of toxicity values found for emamectin benzoate for water exposures (i.e. water is the only environmental medium present, but routes of administration may vary – e.g. dissolved, injected, or feed). *interpreted from data

SEDIMENT EXPOSURES								
Test organism	**Life-stage**	**Measured or nominal**	**Formulation or active ingredient**	**Effect measurement**	**Dose descriptor**	**Exposure duration**	**Concentration**	**Reference**
INVERTEBRATES								
Corophium volutator (mud shrimp)				Mortality	LC50 / NOEC	10 d	193 / 115 µg kg-1	McHenery & Mackie (1999), Schering-Plough Anim. Health (2002) – in Lumaret et al. (2012)
		N	F	Mortality	LC50	10 d	153 µg kg-1 w.w.	Mayor et al. (2008)
		N	AI	Mortality	LC50/NOEC	28 d	316 / 100 µg kg-1 d.w.	Cheng et al. (2020)
		N	AI	Growth	NOEC	28 d	30 µg kg-1 d.w.	Cheng et al. (2020)
Eohaustorius estuarius (amphipod)		M	F	Mortality	LC50	10 d	185 µg kg-1 w.w.	Kuo et al. (2010)
Monocorophium insidiosum (amphipod)		N	AI	Mortality	LC50	10 d	890 µg kg-1 d.w.	Tucca et al. (2014)
				Biochemical response (GST act./TBARS)	LOEC*	10 d	100 / 50 µg kg-1 d.w.	
Hediste diversicolor (Rag worm)		N	F	Mortality	LC50	10 d	1368 µg kg-1 w.w.	Mayor et al. (2008)
Arenicola marina (Lungworm)				Mortality	LC50 / NOEC	10 d	111 / 56.0 µg kg-1 w.w.	McHenery & Mackie (1999), Schering-Plough Anim. Health (2002)

								– in Lumaret et al. (2012)
		N	AI	Mortality	LC50/NOEC	28 d	> 1000 / ≥1000 µg kg-1 d.w.	Cheng et al. (2020)
Cerastoderma edule (common cockle)		N	AI	Mortality	LC50/NOEC	28 d	> 1000 / ≥1000 µg kg -1 d.w.	Cheng et al. (2020)
Homarus ameri (American lobster)	Juvenile (Stage IV)	M	F	Mortality	LC50	5 d	607.05 µg kg -1 w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Mortality	LC50	10 d	250.23 µg kg -1 w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Mortality	LC50	15 d	68.82 µg kg -1w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Mortality	LC50	20 d	31.66 µg kg -1 w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Mortality	LC50	25 d	20.42 µg kg -1w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Mortality	LC50	30 d	17.87 µg kg -1 w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Body positioning	EC50	10 d	636.62 µg kg -1w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Body positioning	EC50	15 d	96.16 µg kg -1 w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Body positioning	EC50	20 d	53.14 µg kg -1w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Body positioning	EC50	25 d	85.08 µg kg -1 w.w.	Daoud et al. (2018)
Homarus ameri (American lobster)	Juvenile (Stage IV)	M	F	Moulting	EC50	15 d	32.72 µg kg -1 w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Moulting	EC50	20 d	41.13 µg kg -1 w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Moulting	EC50	25 d	44.51 µg kg -1 w.w.	Daoud et al. (2018)
	Juvenile (Stage IV)	M	F	Moulting	EC50	40 d	10.49 µg kg -1 w.w.	Daoud et al. (2018)

*interpreted from data

Table 2: Summary of toxicity values for emamectin benzoate in a variety of taxa of aquatic organisms for sediment exposures (i.e. concentration is based wet weight (w.w.) or dry weight (d.w.) of sediment). *interpreted from data

1.5. Ivermectin

Ivermectin (IVM) is a mixture of ≥ 80% 22,23-dihydroavermectin-B_{1a} and ≤20% 22,23-dihydroavermectin-B_{1b} (Halley et al. 1989). It is not commonly used for sea lice management in aquaculture; it has never been approved for the treatment of sea lice in any of the major salmon-producing countries (Canada, Chile, Norway, Scotland, and Ireland) (Horsberg 2012). However, it has been used off-label on salmon farms in Canada, Chile, and Ireland (Haya et al. 2005, Horsberg 2012, Torrissen et al. 2013). The following three formulations containing IVM have been recorded as being used off-label in aquaculture: a 1% injectable solution (Ivomec®); a 1% oral drench (Eqvalan®); and a 0.6% medicated premix (Ivomec® Premix for Swine 0.6%) (Davies and Rodger 2000, Horsberg 2012, Johnson and Margolis 1993, Palmer et al. 1987). All of these have been used as an in-feed application – including the injectable solution, which was just homogenized into feed instead of being injected.

1.5.1 Environmental fate

As IVM is part of the same chemical group as EB, namely avermectins, they share many chemical properties. IVM is lipophilic (log K_{ow} value = 3.2), has low water solubility (4 mg/L), and is not particularly volatile (vapor pressure = < 1.5 x 10^{-9} mm Hg) (Halley et al. 1989, Lumaret et al. 2012). It also has a relatively high absorption coefficient for soil (K_d = 227-333) (Lumaret et al. 2012). As a result, IVM has a similar environmental fate to EB in the aquatic environment. Like EB, it enters the aquatic environment via uneaten medicated pellets or excrement (both its metabolites and parent compound) from organisms which have ingested it. It has a strong tendency to bind to particulates while in the water column, and otherwise undergoes rapid photolysis when in the aqueous phase (DT_{50}= < 0.5 d) (Lumaret et al. 2012). As a result, if used for treatment of sea lice, most of it will accumulate in the benthic zone, having adsorbed to sediment particulates where it will degrade much more slowly. In studies conducted with soil, it has been shown to have a DT_{50} between 7 to 240 d, heavily depending on light and temperature (Lumaret et al. 2012). In a laboratory study with IVM residing in high organic matter soil at 22°C in the dark, it had a DT_{50} ranging between 93-240 d (Halley et al. 1989).

1.5.2. Toxicity to non-target organisms

Toxicity reference values for IVM-exposed aquatic vertebrates, invertebrates, and algae were mostly found in Lumaret et al. (2012) and Davies and Rodger (2000). These values were verified with the original source wherever possible. However, several articles were not accessible. A thorough literature review was also conducted for all IVM toxicity data involving aquatic organisms to identify and fill any data gaps in these review articles. Table 3 and 4 summarize the aquatic toxicity values found in literature for water and sediment exposures, respectively.

Water exposures involved administration through dissolving, injecting, or incorporating IVM into feed. Some studies used formulations and others used the purified active ingredient. In vertebrates, *A. anguilla* was the most sensitive species, with a 24 h LC50 value of 0.2 µg/L. The least sensitive species *was D. rerio*, which surprisingly showed the greatest resistance to toxicity at the embryonic life-stage compared to the juvenile and adult life-stages. The 144 h LC50 of *D. rerio* embryos was 518.5 µg/L as compared to the 96 h juvenile and adult LC50 of 17.21 and 74.88 µg/L, respectively. The two most sensitive species of invertebrates were *D. magna* and *N. integer*, which had almost identical 48 h LC50s of 0.025 and 0.026 µg/L. The least sensitive invertebrate species was the gastropod *Hydrobia ulvae*, which had a 96 h LC50 value of >10000 µg/L. The two species of algae which data were available for, namely *Chlorella pyrenoidosa* and *Pseudokirchneriella subcapitata*, also were resistant to toxic effects of IVM. These two species had a 14 d NOEC (biomass) and a 72 h EC50 (biomass) of 5100 and > 4000 µg/L, respectively.

Sediment exposures only involved invertebrate species. The most sensitive species was *D. magna* with a 48 h LC50 of 6.5 µg/kg (sediment dry weight). The two least sensitive species, *A. rubens* and *L. variegatus*, had respective LC50 values of 23600 µg/kg d.w. (10 d exposure) and 6440 µg/kg d.w. (28 d exposure). Most other species for which data existed, had toxicity values in the approximate range of 10 to 200 µg/kg (wet or dry weight) for exposure durations ranging between 96 h to 30 d, with assessment of either lethal or sublethal endpoints.

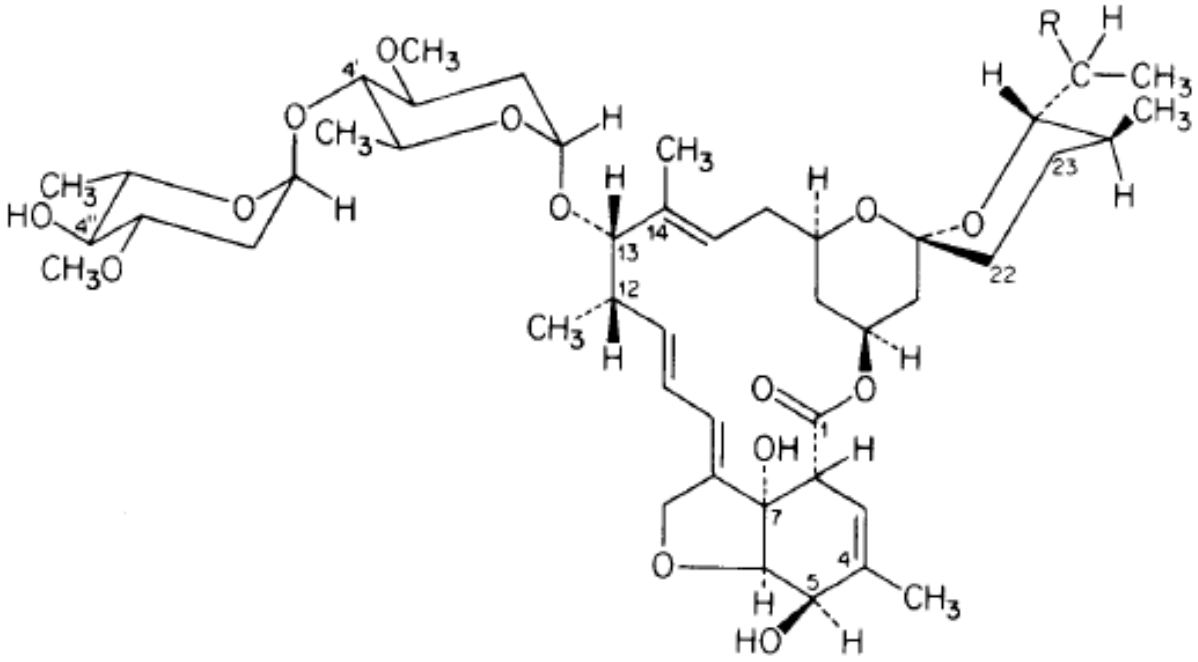


Figure 5: Chemical structure of Ivermectin (≥ 80% 22,23-dihydroavermectin B1a: R=C2H5; ≤ 20% 22,23-dihydroavermectin B1b: R=CH3). (Figure credit: Halley et al. 1989).

WATER EXPOSURES

Test organism	Life-stage	Measured or nominal	Formulation or active ingredient	Effect measurement	Dose descriptor	Notes	Exposure duration	Concentration or dose	Reference
VERTEBRATES									
Anguilla anguilla (eel)			F	Mortality	LC50	dissolved; freshwater	24 hr	0.2 µg L-1	Geets et al. 1992
Lepomis macrochirus (Bluegill sunfish)			AI	Mortality	LC50	dissolved; freshwater	96 hr	4.8 µg L-1	Halley et al. 1989
Salmo gardneri (Rainbow trout)			AI	Mortality	LC50 / NOEC	dissolved; freshwater	96 hr	3.0 / 0.9 µg L-1	Halley et al. 1989
Salmo gardneri (Rainbow trout)				Mortality	LC50	dissolved state	96 hr	3 µg kg-1	Kilmartin et al. 1997 in Lumaret et al. 2012
Salmo salar (Atlantic salmon)				Mortality	LC50	dissolved	96 hr	17 µg L-1	Kilmartin et al. 1996 in Davies and Rodger 2000
Salmo salar (Atlantic salmon)				Mortality	LC50	injection	96 hr	500 µg kg-1	Kilmartin et al. 1997 in Lumaret et al. 2012
Salmo trutta (Brown trout)				Mortality	LC50	injection	96 hr	300 µg kg-1	Wislocki et al. 1989 in Lumaret et al. 2012
Sparus aurata (Sea bream)	40 g weight	N	F	Mortality	0% mortality	single peritoneal injection	35 d	doses between 100-800 µg kg-1	Katharios et al. 2001
Sparus aurata (Sea bream)	40 g weight	N	F	Hematocrit	LOEC	single peritoneal injection	35 d	800 µg kg-1	Katharios et al. 2001
Danio rerio (Zebrafish)	embryo	N	AI	Mortality	LC50	dissolved; freshwater	144 hr	518.5 ug L-1	Oliveira et al. 2016

Danio rerio (Zebrafish)	juvenile	N	AI	Mortality	LC50	dissolved; freshwater	96 hr	17.21 ug L-1	Oliveira et al. 2016
Danio rerio (Zebrafish)	adult	N	AI	Mortality	LC50	dissolved; freshwater	96 hr	74.88 ug L-1	Oliveira et al. 2016
Danio rerio (Zebrafish)	embryo	N	AI	Lethargy	EC50	dissolved; freshwater	72 hr	93.16 ug L-1	Oliveira et al. 2016
Danio rerio (Zebrafish)	juvenile	N	AI	Lethargy	EC50	dissolved; freshwater	72 hr	19.50 ug L-1	Oliveira et al. 2016
Danio rerio (Zebrafish)	adult	N	AI	Lethargy	EC50	dissolved; freshwater	72 hr	229.91 ug L-1	Oliveira et al. 2016
Danio rerio (Zebrafish)	embryo/larva e	N	AI	Development (posture and spine)	EC50	dissolved; freshwater	96 hr	379.55 ug L-1	Oliveira et al. 2016
Danio rerio (Zebrafish)	embryos	N	AI	Biochemical (ChE and CAT activity)	NOEC / LOEC	dissolved; freshwater	96 hr	40 / 80 ug L-1	Oliveira et al. 2016
Danio rerio (Zebrafish)	adult	N	AI	Biochemical (GST activity)	NOEC / LOEC	dissolved; freshwater	96 hr	20 / 40 ug L-1	Oliveira et al. 2016
Danio rerio (Zebrafish)	adult	N	AI	Mortality	LC50	dissolved; freshwater	96 hr	73.3 ug L-1	Domingues et al. 2016
Danio rerio (Zebrafish)	adult	N	AI	Feeding	EC50	dissolved; freshwater	21 d	5.1 ug L-1	Domingues et al. 2016
Clarias gariepinus	juvenile		AI	Mortality	LC50	dissolved; freshwater	96 hr	15 ug L-1	Ogueji et al. 2019
INVERTEBRATES									
Daphnia magna		N	AI	Mortality	LC50 / NOEC	dissolved; freshwater	48 hr	0.025 / 0.01 µg L-1	Halley et al. 1989
Daphnia magna		N	AI	Immobilisation	EC50	static; freshwater	48 hr	0.0057 µg L-1	Garric et al. 2007
Daphnia magna		N	AI	Growth	NOEC / LOEC	semi-static; freshwater	21 d	0.0003 / 0.001 ng L-1	Garric et al. 2007
Daphnia magna		N	AI	Reproduction	NOEC / LOEC	semi-static; freshwater	21 d	0.0003 / 0.001 ng L-1	Garric et al. 2007
Daphnia magna		N	AI	Sex ratio	NOEC / LOEC	semi-static; freshwater	21 d	0.0003 / 0.001 ng L-1	Garric et al. 2007

Daphnia magna	N	AI	Immobilisation	EC50	dissolved; freshwater	48 hr	0.59 µg L-1	Bundschuh et al. 2016
Artemia salina (Anostraca)		AI	Mortality	LC50	dissolved; seawater (35 ppt)	24 hr	> 300 µg L-1	Grant and Briggs 1998
Sphaeroma rugicauda (Isopoda)		AI	Mortality	LC50	dissolved; brackish water (3.5 ppt)	96 hr	348 µg L-1	Grant and Briggs 1998
Crangon septemspinosa (Decapoda)	N	F	Mortality	LC50	feed	96 hr	8.5 mg kg-1 (feed)	Burridge and Haya 1993
Crangon septemspinosa (Decapoda)	N	F	Mortality	LC50	dissolved	96 hr	> 21.5 µg L-1	Burridge and Haya 1993
Palaemonetes varians (Decapoda)		AI	Mortality	LC50	dissolved; brackish water (3.5 ppt)	96 hr	54 µg L-1	Grant and Briggs 1998
Gammarus duebeni (Amphipoda)		AI	Mortality	LC50	dissolved; brackish water (3.5 ppt)	96 hr	0.33 µg L-1	Grant and Briggs 1998
Gammarus zaddachi (Amphipoda)		AI	Mortality	LC50	dissolved; brackish water (3.5 ppt)	96 hr	0.33 µg L-1	Grant and Briggs 1998
Carcinus maenas (Decapoda)		AI	Mortality	LC 50	dissolved; brackish water (17.5 ppt)	96 hr	957 µg L-1	Grant and Briggs 1998
Neomysis integer (Mysidacea)		AI	Mortality	LC50	dissolved	48 hr	0.026 µg L-1	Grant and Briggs 1998
Neomysis integer (Mysidacea)	M	AI	Mortality	LC50	dissolved	96 hr	0.07 µg L-1	Davies et al. 1997
Nereis diversicolor (Polychaeta)		AI	Mortality	LC50	dissolved; brackish	96 hr	7.5 µg L-1	Grant and Briggs 1998

						water (17.5 ppt)			
Lumbriculus variegates		N	F	Mortality	LC50	dissolved; freshwater	72 hr	490 µg L-1	Ding et al. 2001
Lumbriculus variegates		N	F	Swimming	EC50	dissolved; freshwater	3 hr	0.96 µg L-1	Ding et al. 2001
Lumbriculus variegates		N	F	Reversal	EC50	dissolved; freshwater	3 hr	14.0 µg L-1	Ding et al. 2001
Lumbriculus variegates		N	F	Crawling Frequency	EC50	dissolved; freshwater	3 hr	79.6 µg L-1	Ding et al. 2001
Lumbriculus variegates		N	F	Crawling Speed	EC50	dissolved; freshwater	3 hr	44.6 µg L-1	Ding et al. 2001
Caenorhabditis elegans				Reproduction	NOEC	Followed ISO/CD 10872 test protocol; water only exposure	96 hr	≤ 1.0 µg L-1	Liebig et al. 2010
Crassostrea gigas (Bivalvia)	Larvae			Mortality	LC50	dissolved	96 hr	80-100 µg L-1	Kilmartin et al. 1997 in Davies et al. 1997
Crassostrea gigas (Bivalvia)	Spat			Mortality	LC50	dissolved	96 hr	460 µg L-1	Kilmartin et al. 1997 in Davies et al. 1997
Mytilus edulis (Bivalvia)				Mortality	LC50	dissolved	96 hr	400 µg L-1	Kilmartin et al. 1997 in Davies et al. 1997
Pecten maximus (Bivalvia)				Mortality	LC50	dissolved	96 hr	300 µg L-1	Kilmartin et al. 1997 in Davies et al. 1997
Tapes semidecussata (Bivalvia)	Larvae			Mortality	LC50	dissolved	96 hr	380 µg L-1	Kilmartin et al. 1997 in Davies et al. 1997

Tapes semidecussata (Bivalvia)	Spat			Mortality	LC50	dissolved	96 hr	600 µg L-1	Kilmartin et al. 1997 in Davies et al. 1997
Monodonta lineata (Gasteropoda)				Mortality	LC50	dissolved	96 hr	780 µg L-1	Davies and Rodger 2000
Biomphlaria glabrata (Gasteropoda)		N	AI	Mortality	LC50	dissolved	24 hr	30 µg L-1	Matha and Weiser 1988 in Lumaret et al. 2012
Hydrobia ulvae (Gasteropoda)			AI	Mortality	LC50	dissolved; brackish water (17.5 ppt)	96 hr	> 10000 µg L-1	Grant and Briggs 1998
Potamopyrgus jenkinsii (Gasteropoda)			AI	Mortality	LC50	dissolved; brackish water (3.5 ppt)	96 hr	< 9000 µg L-1	Grant and Briggs 1998
Littorina littorea (Gasteropoda)			AI	Mortality	LC50	dissolved; brackish water (17.5 ppt)	96 hr	> 1000 µg L-1	Grant and Briggs 1998
Littorina littorea (Gasteropoda)				Mortality	LC50	dissolved	96 hr	580 µg L-1	Kilmartin et al. 1997 in Davies et al. 1997
Nucella lapillus (Gasteropoda)				Mortality	LC50	dissolved	96 hr	390 µg L-1	Kilmartin et al. 1997 in Davies et al. 1997
Patella vulgata (Gasteropoda)				Mortality	LC50	dissolved	96 hr	600 µg L-1	Kilmartin et al. 1997 in Davies et al. 1997
Dugesia gonocephala (Platyhelminthes)		N	AI	Immobilisation	EC50	dissolved; freshwater	96 hr	675.2 µg L-1	Bundschuh et al. 2016

Caenorhabditis elegans (Nematoda)	N	AI	Immobilisation	EC50	dissolved; freshwater	24 hr	17.5 µg L-1	Bundschuh et al. 2016
Brachionus calyciflorus (Rotifera)	N	AI	Immobilisation	EC50	dissolved; freshwater	24 hr	1961 µg L-1	Bundschuh et al. 2016
Tubifex tubifex (Oligochaeta)	N	AI	Immobilisation	EC50	dissolved; freshwater	96 hr	1866 µg L-1	Bundschuh et al. 2016
Radix ovata (Gastropoda)	N	AI	Immobilisation	EC50	dissolved; freshwater	96 hr	17 µg L-1	Bundschuh et al. 2016
G. pulex (Amphipoda)	N	AI	Immobilisation	EC50	dissolved; freshwater	96 hr	1.4 µg L-1	Bundschuh et al. 2016
A. aquaticus (Isopoda)	N	AI	Immobilisation	EC50	dissolved; freshwater	96 hr	390.3 µg L-1	Bundschuh et al. 2016
A. sulciollis (Plecoptera)	N	AI	Immobilisation	EC50	dissolved; freshwater	96 hr	14.3 µg L-1	Bundschuh et al. 2016
ALGAE								
Chlorella pyrenoidosa	N	AI	Biomass	NOEC / LOEC*	dissolved	14 d	5100 / 9100 µg L-1	Halley et al. 1989
Pseudokirchneriella subcapitata	N	AI	Biomass	EC50 / NOEC	Using OECD 201 Guideline (OECD 2002)	72 hr	> 4000 / 391 µg L-1	Garric et al. 2007

Table 3: Summary of toxicity values for aquatic organisms exposed to IVM in water exposures. *interpreted from data

SEDIMENT EXPOSURES									
Test organism	**Life-stage**	**Measured or nominal**	**Formulation or active ingredient**	**Effect measurement**	**Dose descriptor**	**Notes**	**Exposure duration**	**Concentration**	**Reference**

INVERTEBRATES									
Chironomus riparius (Diptera)	larvae	N	AI	Mortality	LC50 / NOEC	Followed OECD Guideline 218 (OECD 2004)	10 d	64 / 25 µg kg -1 d.w.	Egeler et al. 2010
Chironomus riparius (Diptera)	larvae	N	AI	Length	NOEC / LOEC	Followed OECD Guideline 218 (OECD 2004)	10 d	12.5 / 25 µg kg -1 d.w.	Egeler et al. 2010
Chironomus riparius (Diptera)	larvae	N	AI	Biomass	NOEC / LOEC	Followed OECD Guideline 218 (OECD 2004)	10 d	3.1 / 6.3 µg kg -1 d.w.	Egeler et al. 2010
Chironomus riparius (Diptera)	larvae	N	AI	Emergence (male)	EC50 / NOEC	Followed OECD Guideline 218 (OECD 2004)	28 d	19.5 / 12.5 µg kg -1 d.w.	Egeler et al. 2010
Chironomus riparius (Diptera)	adult	N	AI	Emergence (females)	EC50 / NOEC	Followed OECD Guideline 218 (OECD 2004)	28 d	9.0 / 6.3 µg kg -1 d.w.	Egeler et al. 2010
Chironomus riparius (Diptera)	adult	N	AI	Development (males)	NOEC / LOEC	Followed OECD Guideline 218 (OECD 2004)	28 d	25 / >25 µg kg -1 d.w.	Egeler et al. 2010
Chironomus riparius (Diptera)	adult	N	AI	Development (females)	NOEC / LOEC	Followed OECD Guideline 218 (OECD 2004)	28 d	6.3 / 12.5 µg kg -1 d.w.	Egeler et al. 2010
Lumbriculus variegatus (Oligochaeta)		N	AI	Survival/Reproduction	EC50 / NOEC	Followed OECD Guideline 225 (OECD 2007)	28d	6440 / 160 µg kg -1 d.w.	Egeler et al. 2010
Lumbriculus variegatus (Oligochaeta)		N	AI	Biomass	EC50 / NOEC	Followed OECD Guideline 225 (OECD 2007)	28d	2980 / 160 µg kg -1 d.w.	Egeler et al. 2010

Daphnia magna		N	AI	Abundance/Biomass	NOEC	IVM-spiked cattle dung added to water-sediment test system	10 d	53 µg kg -1 dung d.w.	Schweitzer et al. 2010
Corophium volutator (Amphipoda)		N	F	Mortality	LC50		10 d	180 µg kg -1 d.w.	Davies et al. 1998
Asterias rubens (Asteroida)		N	F	Mortality	LC50		10 d	23600 µg kg-1 d.w.	Davies et al. 1998
Arenicola marina (Polychaeta)			AI	Mortality	LC50		10 d	23 µg kg-1 d.w.	Grant and Briggs 1998
Arenicola marina (Polychaeta)		N	F	Mortality	LC50 / NOEC		10 d	18 / 12 µg kg-1 w.w.	Thain et al. 1997
Arenicola marina (Polychaeta)		N	F	Feeding	NOEC		10 d	< 5 µg kg-1 w.w.	Thain et al. 1997
Homarus americanus (Decapoda)	Juvenile (Stage IV)	M	F	Mortality	LC50		10 d	212.1 µg kg-1 w.w.	Daoud et al. 2018
Homarus americanus (Decapoda)	Juvenile (Stage IV)	M	F	Mortality	LC50		30 d	11.6 µg kg-1 w.w.	Daoud et al. 2018
Homarus americanus (Decapoda)	Juvenile (Stage IV)	M	F	Body positioning	EC50		10d	42.1 µg kg-1 w.w.	Daoud et al. 2018
Homarus americanus (Decapoda)	Juvenile (Stage IV)	M	F	Body positioning	EC50		25 d	11.7 µg kg-1 w.w.	Daoud et al. 2018
Homarus americanus (Decapoda)	Juvenile (Stage IV)	M	F	Moulting	EC50		15 d	14.0 µg kg-1 w.w.	Daoud et al. 2018
Homarus americanus (Decapoda)	Juvenile (Stage IV)	M	F	Moulting	EC50		25 d	7.3 µg kg-1 w.w.	Daoud et al. 2018

Caenorhabditis elegans	Reproduction	NOEC	Followed ISO/CD 10872 test protocol; sediment exposure	96 hr	100 ug kg-1 d.w.	Liebig et al. 2010

Table 4: Summary of toxicity values for aquatic organisms exposed to IVM in sediment exposures.

1.6. Toxic mechanism of action of avermectins

Avermectins target ligand-gated chloride channels in the nervous system of invertebrates. They act as positive modulators of two types of these channels: glutamate-gated chloride channels (GluClRs) and ionotropic subtypes of GABA receptors (GABARs) (Song et al. 2016, Wolstenholm and Rodgers 2005, Wolstenholme 2010). Binding of avermectins to these receptors results in an influx of chloride ions, hyperpolarizing the nerve or muscle cell membrane, inhibiting action potential propagation at the post-synaptic membrane (Wolstenholme 2010). GluClRs are only found in invertebrates, at the neuromusculuar junction (Lankas et al. 1997, Wolstenholme 2010). This is thought to be the main reason why avermectins cause flaccid paralysis and subsequent death in invertebrates (Lankas et al. 1997). However, some have also attributed these adverse effects to interaction with GABARs, which are also involved in locomotion in invertebrates (Lumaret et al. 2012, Lunt 1991, Song et al. 2016).

The mechanism of toxicity in the vertebrate nervous system is less well understood (Chen et al. 2014, Wolstenholme 2010). However, similarly to invertebrates, avermectins have been shown to interact with ligand-gated chloride channels, such as glycine receptors and GABARs (Lumaret et al. 2012, Wolstenholm 2010). These receptors are only found in the central nervous system in vertebrates. In addition, it has been recognized that vertebrates are potentially less prone than invertebrates to the toxic effects of avermectins. There are three possible reasons for this: (1) GluClRs are not present in vertebrates (2) avermectins have a higher affinity (approximately 100 times higher) for invertebrate isoforms of GABARs and (3) avermectins do not readily cross the blood-brain barrier of vertebrates which is necessary to access their target receptors (Fisher and Mrozik 1992, Kohler 2001, Lumaret et al. 2012, Reddy 2013).

1.7. Toxicity modifying factors

Many environmental factors can alter a xenobiotic substance's toxicity in both the aquatic and terrestrial environment. These can be divided into three categories: (1) chemical, (2) physical, and (3) biological. Chemical factors describe the chemical properties of an environmental medium (e.g. pH, cationic/anionic profile, organic matter content, etc.). Physical factors describe the physical properties of a given environmental medium (e.g. sediment grain size; water/sediment turbidity; light intensity; temperature, etc.). Biological factors are those that pertain to biota present

in an environmental medium (e.g. microbial activity). Collectively, all these environmental factors which alter exposure or bioavailability, and hence internal dose and toxicity can be termed exposure and toxicity modifying factors (ETMFs) (CCME 2007). ETMFs in an aquatic environment can further be divided into two matrix-dependent categories: (1) water characteristics and (2) sediment and porewater characteristics.

1.7.1 Water characteristics

In the water column, recognized ETMFs include the following: suspended solids, turbidity, light intensity, temperature, resident microbial activity, pH, hardness, alkalinity, dissolved oxygen (DO), dissolved organic carbon (DOC), particulate organic carbon (POC), and cationic/anionic profile (e.g. Mg^{2+}, Ca^{2+}, K^{+}, Na^{+}, Cl^{-}, CO_3^{-}) (CCME 2007, Di Toro et al. 2001, Loverage 2016, Santore et al. 2001, Smith and Lizotte 2007, Wang 1987). These are all factors to consider when deriving a water quality guideline for any environmental contaminant (CCME 2007). Many of these factors have been shown to alter the toxicity of organic compounds (Akkanen and Kukkonen 2001, Bostrom and Berglund 2015, Tsui and Chu 2003, Smith and Lizotte 2007). For example, the toxicity of Roundup® (AI: N-phosphonomethylglycine) was shown to significantly increase with increasing pH (ranging between 6 to 9) (Tsui and Chu 2003). The 48 h LC50 of *Ceriodaphnia dubia* in the most alkaline treatment (pH = 9) was over 4-fold lower than the most acidic treatment (pH = 6). Furthermore, Bostrom and Berglund (2015) showed that the toxicity of six pharmaceuticals depended on pH, which determines whether these compounds are ionized or not. For all the compounds tested, the 48 h EC50 (immobilization) of *D. magna* increases at the pH with the highest fraction of ionized compound. This demonstrates that the neutral form of a compound is typically more toxic to the exposed organism. In another study, Smith and Lizotte (2007) show that water hardness, turbidity, suspended solids, and phytoplankton density all had a significant negative linear correlation with the toxicity of two pyrethroids, ʎ-cyhalothrin and ɣ-cyhalothrin, in *Hyalella azteca*. The 48 h EC50 (immobilization) values ranged more than 10-fold between 1.4 to 15.7 ng/L and 0.6 to 13.4 ng/L for ʎ-cyhalothrin and ɣ-cyhalothrin, respectively, depending on the level of these ETMFs.

1.7.2. Sediment and porewater characteristics

Characteristics of sediment and its porewater which can alter toxicity of contaminants during sediment exposures can be divided into three main categories: (1) persistent physical characteristics of the medium, (2) persistent chemical characteristics of the medium, and (3) non- or less-persistent chemical characteristics of the medium. Persistent physical characteristics of sediment include the following: grain size, grain angularity, sediment consolidation and water-retention capacity (Bentivegna et al. 2004, Lapota et al. 2000, Simpson and Kumar 2016, Word et al. 2005). Persistent chemical characteristics include the following: sediment mineral constituents (oxides of Fe, Al, Mn), total organic carbon (TOC), and total organic nitrogen (TON) (Bentivegna et al. 2004, Besser et al. 2004, Lapota et al. 2000, Simpson and Kumar 2016). Non- or less-persistent chemical characteristics are those that predominantly describe sediment porewater characteristics, which are heavily influenced by the surrounding sediment, including the following: ammonia, pH, DO, salinity, and sulfide content (Lapota et al. 2000, Word et al. 2005); these characteristics have been observed to be notably different when compared to overlying water (Lapota et al. 2000). Porewater DOC and POC are not included in this category, because these together with sediment-adsorbed OC, make-up sediment TOC, which is categorized as a persistent chemical characteristic.

1.8. Organic carbon as a toxicity modifying factor

Organic matter (OM) is the term for the heterogenous mixture of organic compounds derived from biomass at various stages of decomposition (Swift 1996, Thurman 1985). It is typically measured as organic carbon (OC), due to the ease with which OC can be accurately measured in comparison to OM (Thurman 1985). OC includes just the carbon skeleton of OM molecules, which may otherwise contain other elements such as oxygen and hydrogen (Thurman 1985). Generally, by weight, OM content is approximately twice that of OC, however this depends on which compounds make up the OM (Thurman 1985).

In an aqueous environment, OC can broadly be categorized into two components: (1) dissolved organic carbon (DOC) and (2) particulate organic carbon (POC). All organic carbon that is greater than 0.45 µm is considered POC, and anything less than that threshold is DOC. In most aquatic environments, POC makes up only a very small portion of OC (Thurman et al. 1985).

DOC is primarily comprised of humic substances (approximately 50-75%) which are composed of humins, humic acids, and fulvic acids (Steinberg 2003). The distinction between these three categories of humic substances is based on traditional fractionation methods of soil humic substances (Steinberg 2003). Adding alkali to humic substances dissolves the humic and fulvic acids, leaving behind the undissolved humins (Steinberg 2003). Then, if one acidifies this alkaline extract, the humic acids precipitate separating them from the fulvic acids which remain dissolved (Steinberg 2003). Humic substances have anionic chemical moieties arising predominantly from carboxylic functional groups, and to a lesser extent hydroxyl and phenolic groups. Hypothetical molecular structures of humic and fulvic acids are represented in Figure 7. The remainder of DOC is made-up of biological macromolecules such as carbohydrates (e.g. lignin from plants), amino acids, fatty acids, and hydrocarbons (Thurman 1985). While POC on the other hand is made up of all detrital material exceeding 0.45 µm (Thurman 1985). Total organic carbon (TOC) is the sum of DOC and POC. In sediment, this refers to the sum of the DOC and POC in porewater, in addition to OC adsorbed to sediment. The mechanism by which OM can alter toxicity involves complexation with environmental contaminants resulting in reduced bioavailability and altered environmental fate of the contaminant. In this way, humic substances can reduce toxicity of xenobiotic compounds and metals by lowering the internal dose received by the organism (Gonzalez-Guadarrama et al. 2018, Steinberg 2003). Xenobiotic compounds chemically interact with humic substances in a variety of ways. This can involve adsorption or covalent bonding (Bollag and Myers 1992, Steinberg 2003). Adsorption can be the result of the following types of chemical bonds: Van der Waals forces, hydrophobic interactions, ionic bonding (i.e. electrostatic attraction), charge-transfer (π- π) bonds, and hydrogen bonding (Bollag and Myers 1992, Laird and Koskinen 2008, Steinberg 2003). Hydrophilic xenobiotic compounds tend to predominantly interact with humic substances via ionic and hydrogen bonding (Steinberg 2003). However, other mechanisms of molecular attraction, including hydrophobic interactions, Van der Waals forces, and π- π bonds play a lesser role in adsorption for some hydrophilic compounds (Laird and Koskinen 2008). The theoretical interaction between the hydrophilic compound keto-s-triazine, a triazine herbicide, and a humic substance molecule is depicted in Figure 8. This figure shows the variety of different chemical bonds that can occur simultaneously between these two molecules including hydrogen bonding, ionic bonding, and hydrophobic interactions.

Hydrophobic organic contaminants (HOCs) complex with humic substances predominantly by hydrophobic interactions and Van der Waals forces (Steinberg 2003). These xenobiotics have a high affinity for non-polar regions on humic substance molecules, especially the aromatic rings (Moeckel et al. 2013, Steinberg 2003). This affinity is typically expressed as a

binding constant, K_{DOC}, which is the proportion of humic-substance-bound HOC relative to unbound HOC in an aqueous environment (Li et al. 2015). The method used to determine this is called the complexation flocculation (CF) method (Laor and Rebhun 1994). Using this method, Li et al. (2015) determined the log K_{DOC} values or thirty-three HOCs belonging to four chemical classes of contaminants: polyaromatic hydrocarbons (PAHs), phthalic acid esters (PAEs), polybrominated diphenyl ethers (PBDEs), and organic chlorine pesticides (OCPs). The log K_{DOC} for these contaminants ranged between 4.16-7.14. This aptly demonstrates the binding affinity HOCs have for DOC in an aqueous environment; where the HOCs with the highest affinity have a DOC-bound fraction that is 6 or 7 orders of magnitude greater than the unbound fraction. Furthermore, the hydrophobicity of a compound (expressed as log K_{ow}) is positively correlated with its log K_{DOC} (Li et al. 2015). This makes sense, given that complexation between HOCs and humic substances is dependent on hydrophobic interactions between the molecules. The log K_{ow} values of the compounds studied by Li et al. (2015) ranged from 3.27-8.55. Given that the log K_{ow} values of EB and IVM are 5 and 3.2, respectively, it makes sense that their bioavailability and resulting toxicity could be modified by humic substances. In fact, EB and IVMs log K_{OC} values are 3.54-4.38 and 4.10-4.20, respectively, suggesting they have a high affinity for OC (Lumaret et al. 2012).

Organic carbon is a well-established ETMF. In most scenarios, it will ameliorate toxicity, however at high concentrations it may exacerbate toxicity additively (Cloran et al. 2010, Nadella et al. 2009). Most studies investigating the effects OC has on toxicity of environmental contaminants have focused on the effect DOC in the water column has on toxicity of metals, especially copper (Table 1). Fewer studies have explored the relationship between either water DOC or sediment TOC and toxicity of non-metals (Table 1 and 2). Table 1 and 2 summarize the toxicity modifying effect that OC has in water and sediment exposures, respectively, for metal and non-metal contaminants in a variety of taxa. In most of the sediment and water exposures involving metals and non-metals, OC significantly mitigated toxicity.

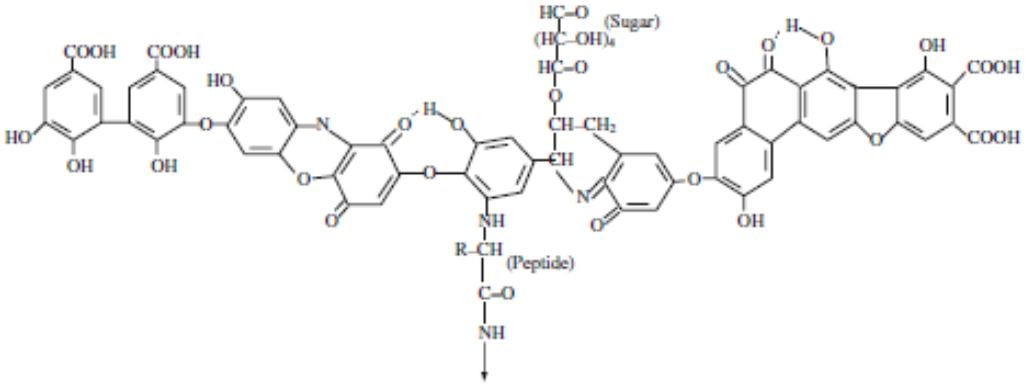
There is substantial evidence for organic carbon reducing toxicity of metals. Studies which have documented this have shown that toxicity of the following metals are modified by the presence of DOC: copper (Cu), chromium (Cr), cadmium (Cd), zinc (Zn), nickel (Ni), silver (Ag), arsenic (As), and uranium (U) (Table 1). This protective effect had a broad range, increasing the LCx or ECx by approximately 2- to 50-fold in the presence of the highest DOC treatments tested in these studies. Most studies did not exceed a maximum concentration of DOC of 30 mg C/L. Within this range, most studies clearly demonstrated that there was a DOC-concentration-

dependent trend, with a negative correlation between DOC-concentration and toxicity. This correlation was typically linear, however there were a few exceptions (Cloran et al. 2010, Nadella et al. 2009, Wang et al. 2011). In rare instances, toxicity was not significant altered by OC. For example, in Koukal et al. (2003) Cd and Zn did not have a significant effect on photosynthetic activity of *Pseudokirchneriella subcapitata*, when fulvic acids were added from the Suwannee River. On other hand, in that same study, Cd and Zn did have a significant effect on photosynthetic activity when the source of the DOC was in the form of peat and soil humic acids (Koukal et al. 2003). This may suggest that the source of the humic substances plays a role in their potential to reduce toxicity. Two studies also found that OC exacerbated toxicity of metals (Cloran et al. 2010, Nadella et al. 2009). Cloran et al. (2010) attributed this to the fact that the highest two concentrations of DOC (48 and 80 mg C/L) when tested in the absence of Ni, significantly reduced survival; DOC had intrinsic toxicity at these higher concentrations. It appeared in that study that DOC and Ni were acting additively. Nadella et al. (2009) showed an 11-fold decrease in the EC50 when exposed to copper in the presence of 20 mg C/L. These findings were peculiar, since at that DOC concentration range, all other studies observed a protective against toxicity. The authors were not sure how to explain this result, but the source of the water they used seemed to make a significant difference. Also, intermediate OC levels did show a protective effect, so the highest concentration may be explained by intrinsic DOC toxicity as in Cloran et al. (2010).

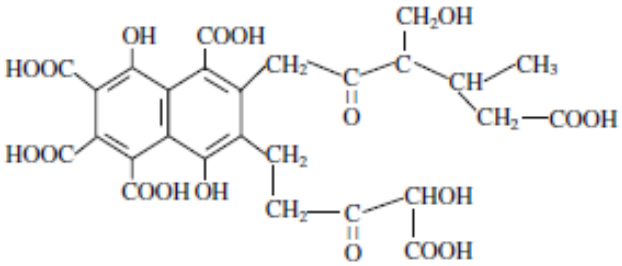
The effect of OC on toxicity in water exposures involving non-metals were less consistent. Toxicity of the following substances was reduced in the presence of DOC: triclosan, chlorphyrifos, DDT, formaldehyde, and γ-hexachlorocyclohexane. However, of those that did show significant effects on toxicity, the magnitude of this effect ranged between a 1.4- to a 21.6-fold increase in LC50 or EC50 values. No studies showed any evidence for DOC exacerbating toxicity. This may be attributed to the fact that the highest concentration of DOC evaluated in these studies was 21.7 mg C/L. This is below the higher concentrations of DOC which showed intrinsic toxicity in the studies that investigated metal exposures. In sediment exposures for non-metals, the following four compounds were significantly affected by sediment TOC: tributyltin, bifenthrin, glyphosate, and alkylbenzene sulfonate. The reduction in toxicity ranged between a 1.6- to a 5.7-fold increase in LC50 or EC50 values. The greatest effect on toxicity could be seen at a sediment TOC level of 4.43%, but levels as low as 0.59% had a significant effect on toxicity.

Accumulation of sediment OC may be especially relevant to aquaculture open net pen operations. The high fish population densities on farms result in above normal discharge of organic waste which can accumulate in sediment directly below or adjacent to a net pen (Brown

et al. 1987, Carroll et al. 2003, Chou et al. 2002, Findlay et al. 1997, Sutherland et al. 2000, Tsutsumi et al. 2006). In Japan, the flux of OC to the sea floor has been shown to be 2.5-fold higher (2.11 gC/m^2/d) below red seabream fish farms (*Pagrus major*) relative to nearby reference sites (Tsutsumi et al. 2006). Remarkably similar results have been found salmonid net-pens in BC (Sutherland et al. 2000). Beside the bottom of the net-pen, carbon flux was approximately 200 mg/m^2/h which was 2-fold higher compared to the control site with a carbon flux of approximately 100 mg/m^2/h (Sutherland et al. 2000). It appears that this carbon flux only significantly increases TOC % of sediment near the salmon farm. For example, Brown et al. (1987) showed that at 3 m from salmon pens, the sediment TOC was 1.6-fold higher compared to 11 m from salmon pens. They also found that all sediment 15 m and further (in intervals up to 1400 m) from the salmon pen was highly similar, and approximately 2.3 to 2.4-fold lower than at 3 m (Brown et al. 1987). Given these results, it seems the radius of effect for the increased sediment OC is relatively small, within the range of approximately 10 m. Thus, the major environmental implications of organic matter pollution, such as hypoxia in the water column, which have been discussed in other literature (Tsutsumi et al. 2006) may only be relevant to benthic organisms within this radius of effect. On the contrary, these increased OC levels may be beneficial by acting as a toxicity modifying factor underneath active salmon farms, mitigating the toxic effects of sea lice chemotherapeutants.



(a) **Humic Acid** (Stevenson 1982)



(b) **Fulvic Acid** (Buffle 1977)

Figure 6: Hypothetical chemical structures of humic and fulvic acids. There are many variations to the structures humic and fulvic acids can assume in the environment (Figure credit: Wang and Mulligan 2006)

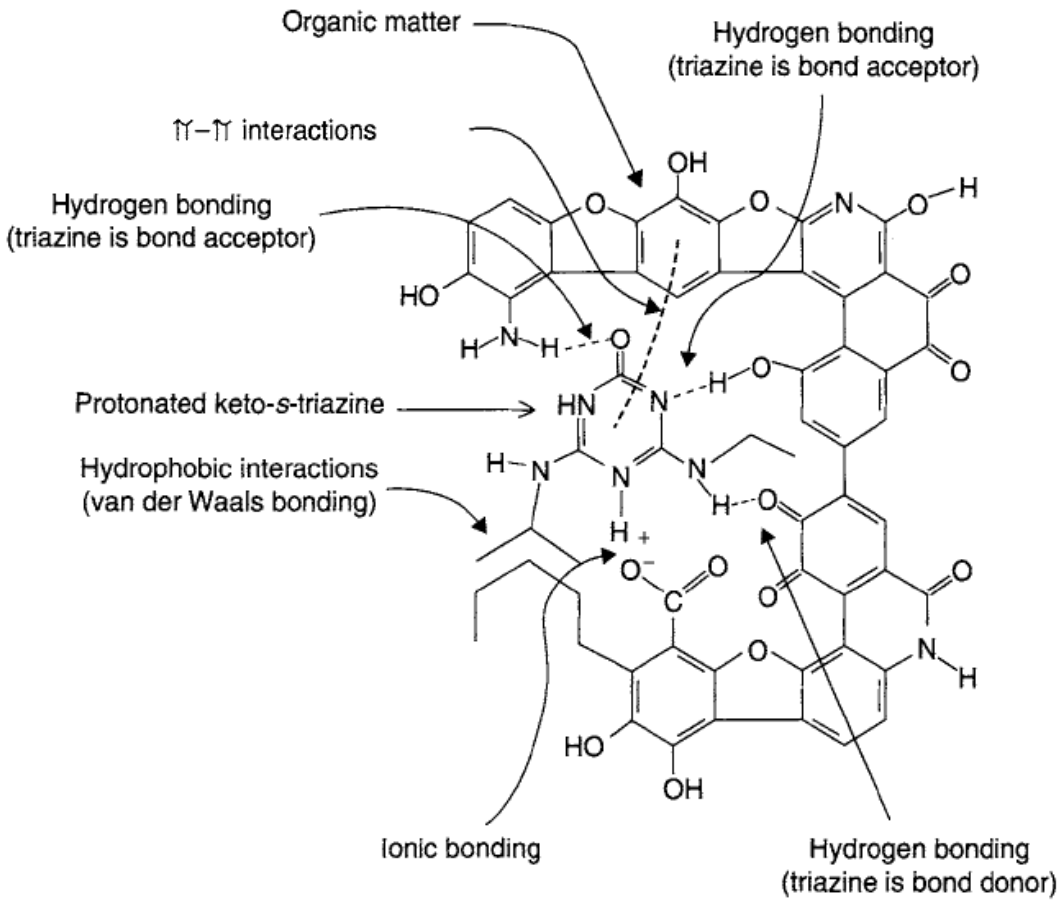


Figure 7: An example of chemical interactions between organic matter and organic contaminants, showing the proposed chemical interactions between keto-s-triazine (hydrophilic environmental contaminant) and organic matter (humic or fulvic acid molecule). (Figure credit: Laird and Koskinen 2008).

WATER EXPOSURES

Substance	Duration	Endpoint	Species	OC Measure	Lowest OC tested	Highest OC tested	Toxicity Modifying Effect	Notes	pH	Hardness (mg/L CaCO3)	Reference
METALS											
Cadmium (II) (CdCl2)	1 hr	Photosynthetic activity	*Pseudokirchneriella subcapitata*	DOM	0 mg/l	5 mg/l	5-fold and 2-fold decrease (significant) in photosynthetic inhibition (peat and soil humic acids, respectively)	Bioavailability: metal complexed with colloids at higher DOC	8.5 ± 0.1	N/R	Koukal et al. 2003
Cadmium (II) (CdCl2)	1 hr	Photosynthetic activity	*Pseudokirchneriella subcapitata*	DOM	0 mg/l	5 mg/l	FAs had no significant effect on toxicity	Source of FAs: Suwannee River (GA, USA)	8.5 ± 0.1	N/R	Koukal et al. 2003
Chromium (III) (chrome lignosulfonate)	96 hr	Mortality	*Daphnia pulex*	DOC	0 mg/L	50 mg/L	1.2-fold increase in LC50 (significant)	Bioavailability shown to decrease (decrease in free chromium ion)	8.0 ± 0.1	92 ± 8	Stackhouse and Benson 1989
Chromium (III) (CrCl3)	96 hr	Mortality	*Daphnia pulex*	DOC	0 mg/L	50 mg/L	~50-fold increase in LC50*	Bioavailability shown to decrease (decrease in free chromium ion)	8.0 ± 0.1	92 ± 8	Stackhouse and Benson 1989
Chromium (VI) (K2Cr2O7)	48hr	Immobilization	*Daphnia magna*	DOC	0 mg/l	80 mg/l	1.5-fold increase in EC50	DOC did not have a significant effect on toxicity	7	250	Park et al. 2009
Copper (CuCl2)	3hr	Mechanosensory (hair cell) cell death	*Danio rerio*	DOC	0.1 mg/L	4.3 mg/l	4.4-fold increase in EC50	EC50 and DOC highly correlated (r^2 = 0.975)	6.97 - 7.46	45	Linbo et al. 2009

Copper (II) (CuCl2)	48hr	Embryo development	*Mytilus trossolus*	DOC	0 mg/L	20 mg/L	4-fold increase to 11-fold decrease in EC50 values*****		7.88	N/R	Nadella et al. 2009
Copper (II) (CuSO4)	48hr	Mortality	*Diaptomus clavipes*	TOC	0 mg/l	26.2-30.1 mg/l	3- to 10-fold increase in LC50*	Change in LC50 depended significantly on temperature (10, 20, 30 degrees celsius)	7.2-7.6	10-50	Boeckman and Bidwell 2006
Copper (II) (CuSO4)	48hr	Mortality	*Daphnia pulex*	TOC	0 mg/l	14.0-16.9 mg/l	~20- to 25-fold increase in LC50* (depending on temperature treatment)	LC50 and TOC highly correlated (p<0.0001; r^2 = 0.91)	7.2-7.6	32-52	Boeckman and Bidwell 2006
Copper (II) (CuSO4)	4 d	Mortality	*Villosa iris*	DOC	0.5 mg/L	10 mg/L	5-fold increase in LC50 (significant)	LC50 and DOC highly correlated (r^2=0.90)	8.3-8.4	98-169	Wang et al. 2011
Copper (II) (CuSO4)	2 d	Mortality	*Ceriodaphnia dubia*	DOC	0.5 mg/L	10 mg/L	11-fold increase in LC50 (significant)	LC50 and DOC highly correlated (r^2=0.98)	8.3-8.4	102-174	Wang et al. 2011
Copper (II) (CuSO4)	28 d	Mortality	*Villosa iris*	DOC	0.5 mg/L	10 mg/L	5-fold increase in LC20 (significant)	EC20 and DOC highly correlated (r^2=0.99)	8.3-8.4	98-169	Wang et al. 2011
Copper (II) (CuSO4)	7 d	Mortality	*Ceriodaphnia dubia*	DOC	0.5 mg/L	10 mg/L	17-fold increase in LC20 (significant)	EC20 and DOC highly correlated (r^2=0.94)	8.3-8.4	102-174	Wang et al. 2011
Copper (II) (CuSO4)	28d	Biomass	*Villosa iris*	DOC	0.5 mg/L	10 mg/L	5-fold increase in EC20 (significant)	EC20 and DOC highly correlated	8.3-8.4	98-169	Wang et al. 2011

								(r^2=0.99); exponential fit			
Copper (CuSO4)	7d	Reproduction	*Ceriodaphnia dubia*	DOC	0.5 mg/L	10 mg/L	4-fold increase in EC20 (significant)	EC20 and DOC correlated (r^2=0.79); logistic fit	8.3-8.4	102-174	Wang et al. 2011
Copper (II) (CuCl2)	48hr	Immobilization	*Daphnia magna*	DOC	0 mg/l	80 mg/l	20-fold increase in EC50	EC50 and DOC highly correlated (p<0.0001; r^2=0.904)	7	250	Park et al. 2009
Copper (II) (CuSO4)	48hr	Lethality	*Daphnia magna*	DOC	0 mg/l	3.75 mg/l	~10-fold increase in LC50*	Bioavailability: DOC significantly reduced the amount of free ionic copper in solution	7	N/R	Meador 1991
Nickel	48 hr	Mortality	*Daphnia magna*	DOC	0 mg/l	80 mg/l	Survival lower than 25% at a DOC-free LC75 of Ni****		7.89 ± 0.04	100 ± 2	Cloran et al. 2010
Silver (I) (AgNO3)	96 hr	Mortality	*Oncorhynchus mykiss* (rainbow trout)	DOC	0.3 mg/l	5.8 mg/l	4.1-fold increase in LC50 (significant)	Bioavailability: no significant decrease in Ag+ ions	6.76 ± 0.07		Bury et al. 1999
Silver (I) (AgNO3)	96 hr	Mortality	*Pimephales promelas*	DOC	0.3 mg/l	5.8 mg/l	2.7-fold increase in LC50 (significant)	Bioavailability reduced; shown by significant decrease in free Ag+ ions			Bury et al. 1999
Uranium (II) (UO2SO4)	72 hr	Growth Rate	*Chlorella sp.*	DOC	0 mg/l	20 mg/l	19.6-fold increase in IC50 (significant***)	Water source: Synthetic Magela Creek water	6.2 (6.0-6.4)	3.6 (3-3.9)	Trenfield et al. 2011
Uranium (II) (UO2SO4)	96 hr	Population Growth Rate	*Hydra viridissima*	DOC	0 mg/l	20 mg/l	7.5-fold increase in	Water source: Synthetic	6.1 (6.0-6.2)	3.6 (3-3.9)	Trenfield et al. 2011

							IC50 (significant***)	Magela Creek water			
Uranium (II) (UO2SO4)	96 hr	Survival	*Mogurnda mogurnda*	DOC	0 mg/l	20 mg/l	4.7-fold increase in LC50 (significant***)	Water source: Synthetic Magela Creek water	6.2 (6.0-6.4)	3.6 (3-3.9)	Trenfield et al. 2011
Uranium (II) (UO2SO4)	72 hr	Growth Rate	*Chlorella sp.*	DOC	0 mg/l	10 mg/l	11.5-fold increase in LC50 (significant***)	Water source: Sandy Billabong water	6.0 (5.9-6.3)	4.6 (3.5-6)	Trenfield et al. 2011
Uranium (II) (UO2SO4)	96 hr	Population Growth Rate	*Hydra viridissima*	DOC	0 mg/l	10 mg/l	2.3-fold increase in IC50 (significant***)	Water source: Sandy Billabong water	6.1 (5.8-6.4)	4.6 (3.5-6)	Trenfield et al. 2011
Uranium (II) (UO2SO4)	96 hr	Survival	*Mogurnda mogurnda*	DOC	0 mg/l	10 mg/l	1.8-fold increase in LC50 (significant***)	Water source: Sandy Billabong water	6.2 (5.9-6.4)	4.6 (3.5-6)	Trenfield et al. 2011
Zinc (II) (ZnSO4)	1 hr	Photosynthetic activity	*Pseudokirchneriella subcapitata*	DOM	0 mg/l	5 mg/l	2- and 10-fold decrease (significant) in photosynthetic inhibition (peat and soil humic acids, respectively)	Bioavailability: metal complexed with colloids at higher DOC	8.5 ± 0.1	N/R	Koukal et al. 2003
Zinc (II) (ZnSO4)	1 hr	Photosynthetic activity	*Pseudokirchneriella subcapitata*	DOM	0 mg/l	5 mg/l	FAs had no significant effect on toxicity	Source of FAs: Suwannee River	8.5 ± 0.1	N/R	Koukal et al. 2003
						NON-METALS					
Chlorpyrifos	24 hr	Mortality	*Ceriodaphnia dubia*	DOC	0.6 mg/l	21.7 mg/l	Significant 4.8-fold increase of survival % (from 19.1% to 91.6%)	Exposure to 82 ng/L Chlorphyrifos	N/R	53.2	Mezin and Hale 2004
Chlorpyrifos	24 hr	Mortality	*Americamysis bahia*	DOC	2.2 mg/l	14.8 mg/l	1.2-fold decrease in	Exposure to 322 ng/L Chlorphyrifos	N/R	N/R	Mezin and Hale 2004

							survival (not significant)					
DDT	24 hr	Mortality	*Ceriodaphnia dubia*	DOC	0.6 mg/l	21.7 mg/l	Significant 2-fold increase in survival (50.6% to ~100%)	Exposure to 1100 ng/l of DDT; protective effect maxes out at 30 mg DOM/l	N/R		53.2	Mezin and Hale 2004
DDT	24 hr	Mortality	*Americamysis bahia*	DOC	2.2 mg/l	14.8 mg/l	1.2-fold decrease in survival (not significant)*	Exposure to 1100 ng/l of DDT	N/R	N/R		Mezin and Hale 2004
Formaldehyde	144 hr	Embryo mortality**	*Danio rerio*	DOC	0 mg/l	5 mg/l	21.6-fold increase in LC50 (significant)	Using hard water	7.71 - 7.77		125.6	Meinelt et al. 2005
Formaldehyde	144 hr	Embryo mortality**	*Danio rerio*	DOC	0 mg/l	5 mg/l	1.4-fold increase in LC50 (significant)	Using soft water	7.54 - 7.86		53.6	Meinelt et al. 2005
gamma-Hexachlorocycl-ohexane	144 hr	Bioaccumulation	*Marsilea minuta* (aquatic fern)	DOM	0%	1%	47-78% reduction in tissue concentration	Bioaccumulation reduction depends on segment of plant tissue sampled and light intensity	N/R	N/R		Misra et al. 2000
Triclosan	48 hr	Immobilization	*Gammarus pulex*	DOC	Not reported	11 mg/l (added)	1.4-fold increase in EC50 (significant)	Water source: Synthetic Fresh Water (SFW)	8.35 - 8.39			Rowett et al. 2016
Triclosan	48 hr	Immobilization	*Gammarus pulex*	DOC	Not reported	11 mg/l (added)	1.29-fold increase in EC50 (not significant)	Water source: Synthetic Fresh Water (SFW)	7.25 - 7.27			Rowett et al. 2016

*approximation based on visual inspection of graph

**unhatched embryos considered dead

***not reported, but inferred based on non-overlapping 95% confidence intervals

****Protective at 18 mg/l DOC: ~1.5 fold greater survival than expected at LC75 of Ni; exposure to 48 and 80 mg/L DOC alone resulted in significant mortality (15 and 55%)

*****Results depended on water source; intermediate OC levels (<10 mg C/L) still showed protective effect: appeared to have a inverted U shape in terms of OC versus EC50 for 2 out of 3 of the water sources tested

Table 5: The toxicity modifying effect of organic carbon on metals and non-metals in water exposures with aquatic organisms. Most quantifications of toxicity modifying are expressed as the change in LC/ECx value that results at the highest organic carbon (OC) or organic matter (OM) treatment compared to the lowest OC or OM treatment. Dissolved and total OC are abbreviated as DOC and TOC, respectively; dissolved OM is abbreviated as DOM. Where possible, the valency (roman numerals) and salt of the metal used in the toxicity study was included (e.g. copper in $CuCl_2$ with a valency of 2).

SEDIMENT EXPOSURES											
Substance	**Duration**	**Endpoint**	**Species**	**OC Measure**	**Lowest OC tested**	**Highest OC tested**	**Toxicity Modifying Effect**	**Notes**	**Particle Size**	**pH**	**Reference**
NON-METALS											
Alkylbenzene sulfonate	48 hr	Mortality	*Hyalella azteca*	TOC	0.35%	4.64%	4.6-fold increase in LC50 (significant)	Sediment OC series (0.35, 0.65, 0.84, 1.47, and 4.64); dry weight	N/R	N/R	Cano et al. 1996
Bifenthrin (pyrethroid)	10 d	Mortality	*Hyalella azteca*	TOC	0.56%	4.43%	5.7-fold increase in LC50 (significant)	dry weight of sediment	Low OC: 14 : 62 : 24 (Sand : Silt : Clay); High OC: (46 : 47: 7)	6.76 ± 0.13 (overlying water)	Hardwood et al. 2012
Bifenthrin (pyrethroid)	10 d	Immobilization	*Hyalella azteca*	TOC	0.56%	4.43%	4.9-fold increase in EC50 (significant)	dry weight of sediment	Low OC: 14 : 62 : 24 (Sand : Silt : Clay); High OC: (46 : 47: 7)	6.76 ± 0.13 (overlying water)	Hardwood et al. 2012

Bifenthrin (pyrethroid)	10 d	Mortality	*Chironomus dilutus*	TOC	0.56%	4.43%	2.2-fold increase in LC50 (significant)	dry weight of sediment	Low OC: 14 : 62 : 24 (Sand : Silt : Clay); High OC: (46 : 47: 7)	6.76 ± 0.13 (overlying water)	Hardwood et al. 2012
Bifenthrin (pyrethroid)	10 d	Immobilization	*Chironomus dilutus*	TOC	0.56%	4.43%	1.6-fold increase in EC50 (significant)	dry weight of sediment	Low OC: 14 : 62 : 24 (Sand : Silt : Clay); High OC: (46 : 47: 7)	6.76 ± 0.13 (overlying water)	Hardwood et al. 2012
Glyphosate	48 hr	Mortality	*Ceriodaphnia dubia*	TOC	0.0%	2.10%	3.1-fold increase in LC50 (significant)	Formulation: 'Roundup'	N/R	8.0	Tsui and Chu 2004
Glyphosate	48 hr	Mortality	*Ceriodaphnia dubia*	TOC	0.0%	2.10%	1.3-fold decrease (not significant)	Formulation: 'Roundup Biactive' **	N/R	8.0	Tsui and Chu 2004
Tributyltin	10 d	Mortality	*Rhepoxynius abronius* (Amphipoda)	TOC	0.12%	0.59%	Concentration resulting in 20% mortality at highest OC is ~ 2-fold higher* than LC50 at lowest OC***		97% and 90% sand for high and low OC sediment, respectively	7.8 ± 0.06 (porewater)	Meador et al. 1997
Tributyltin	10 d	Mortality	*Armandia brevis* (Polychaeta)	TOC	0.30%	0.87%	Concentration resulting in 33% mortality at highest OC is ~3-fold higher* than LC50 at lowest OC****		84% sand for both high and low OC sediment	7.8 ± 0.1 (porewater)	Meador et al. 1997

*Approximation based on visual inspection of graph

**The only difference compared to regular 'Roundup' formulation is the surfactant found in this formulation

***Mortality at highest TBT concentration in highest OC concentration treatment resulted in 20% mortality (preventing LC50 calculation)

****Mortality at highest TBT concentration in highest OC concentration treatment resulted in 33% mortality (preventing LC50 calculation)

Table 6: The toxicity modifying effect of organic carbon (OC) on non-metals in sediment (with overlying water) exposures with aquatic organisms. Most quantifications of toxicity modifying are expressed as the change in LC/ECx value that results at the highest OC treatment compared to the lowest OC treatment. Total OC content of sediment is abbreviated as TOC.

1.9. Chemical residence time as a toxicity modifying factor

Besides sediment and porewater characteristics, the duration of contact between a chemical and a certain environmental medium (i.e. chemical residence time) may also play a role in modifying a substance's toxicity. There are two proposed mechanisms that underly this and can be categorized as follows: (1) bond strength and (2) sequestration. When a substance complexes with humic substances, the strength of the bond between them can increase over time (Clark and Choppin 1990). With relevance only to sediment/soil matrices, a substance may also gradually sequester in pockets of the soil matrix which are inaccessible to even the smallest of microorganisms (Alexander 2000). Both mechanisms may result in a decrease in bioavailability of a substance over time.

Clark and Choppin (1990) conducted an experiment that adds validity to the mechanism involving modification of bond strength. After 15 min of contact, it was found that 100% of trivalent europium (Eu) was bound to humic acids, but only 4% of the bonding was characterized as bonds with high affinity. However, after 2 d of contact, 38% of the bonds were high affinity bonds (Clark and Chopping 1990). Therefore, it appears that bond strength for contaminant-DOC complexes can increase over time, reducing the bioavailability of an environmental contaminant, and thus reducing its toxicity. However, few published toxicological studies to date have confirmed this. On the contrary, another experiment showed that copper (Cu) and cadmium (Cd) accumulation on the gills of juvenile *Oncorhynchus mykiss* were unaffected by the age of the metal-DOC complex, when these complexes were aged for a period of three weeks (Hollis et al. 1996). The ability of DOC to keep these metals from accumulating on the gill surface is the main mechanism for its protective effect (Hollis et al. 1996). Therefore, these findings do not support the idea that chemical residence time affects toxicity in the case of these two metals. However, it is possible that the three weeks of aging in the study was not sufficient to observe a significant influence on toxicity. No other literature could be found regarding the age of DOC-contaminant complexes, and its effect on toxicity.

Some literature exists regarding a chemical's residence time on the bioaccessibility of various compounds in soil. Alexander (2000) summarizes results from a variety of studies which found that all of the following 7 compounds are less accessible to microbes for mineralization after aging in soil: naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, atrazine, and 4-nitrophenol. For example, Chung and Alexander (1998) measured microbial mineralization of phenanthrene and atrazine at various time-points over the course of 200 d, in 16 different soil

types. After 200 d of aging in soil, both atrazine and phenanthrene were significantly less mineralizable by microbes in 15 out of 16 soil types tested, when compared to initial un-aged conditions (Chung and Alexander 1998). In many cases, this reflected an approximately two-fold decrease in percentage of microbial mineralization of these two compounds (Chung and Alexander 1998).

1.10. Study goal and objectives

The objective of this study was to assess the effect of chemical residence time and sediment OC content on the toxicity of ivermectin (pure chemical) and the formulation Slice® (a.i. EB) to benthic invertebrates. Toxicity was assessed for both lethal and sublethal endpoints using two species of benthic invertebrates: *Eohaustorius estuarius* (Amphipoda) and *Neanthes virens* (Polychaeta). Three levels of sediment OC (low, medium, and high) and chemical residence time (0-, 2-, and 4-months) were included, for a total of 9 combinations of the two factors. This design also allowed for the assessment of any interaction between these factors. The lowest organic carbon treatment (0-OC) had a sediment TOC content below the limit of detection (<0.05%) and the highest (1-OC) was 0.42%, with the intermediate OC treatment (0.5-OC) being a 50/50 mixture of the highest and lowest OC sediments. Exposure durations for *E. estuarius* and *N. virens* were 48 h and 10 d, respectively. Sublethal endpoints involved behavioral assays which included light-avoidance and burrowing behavior for *E estuarius* and *N. virens*, respectively. Combined exposures to both avermectins were also included to investigate the toxic effects that may result upon simultaneous exposure to both avermectins.

Chapter 2. The effects of sediment organic carbon and chemical residence time on lethal and sublethal avermectin toxicity to benthic invertebrates

2.1 Introduction

Sea lice are an ectoparasitic copepods which can be found in marine or brackish waters (Boxaspen 2006). Following attachment sea lice feed on the surface tissues of the host. In salmonid hosts, the resulting sublethal effects can include reduced respiratory and osmoregulatory capacity; reduced growth and swimming capabilities; and compromised immune function (Fjelldal et al. 2019, Godwin et al. 2017, Johnson and Fast 2004, Tully and Nolan 2002, Wagner et al. 2004). These sublethal effects may indirectly lead to mortality, however it is less common that sea lice are directly responsible for the mortality of its salmonid host (Costello 2006). Farmed salmon host fitness decreases can become an economic burden to the salmonid aquaculture industry; it has been estimated that sea lice are responsible for annual losses of $500 million USD, accounting for 6% of global salmonid aquaculture production (Costello 2009a). Although there are over 500 species of sea lice known (Ahyong et al. 2011), only 5 of them have been observed in salmonid aquaculture operations in both Europe and the Americas: *Caligus clemensi* (Pacific Ocean) *Caligus elongatus* (Atlantic Ocean)*, Lepeophtheirus salmonis*, *Caligus teres*, and *Caligus rogercressyi* (Johnson and Fast 2004). The latter two are found in the Southern Hemisphere, while the other three are found in the Northern Hemisphere (Johnson and Fast 2004). Besides the economic cost associated with sea lice, they are also a concern for wild salmon populations; densely populated open net pens allow for the cultivation of sea lice, which can then spread to wild populations (Krokosek 2004, Morton et al. 2008, Thorstad and Finstad 2018, Torrisen et al. 2013). The economic and ecological toll has prompted management efforts worldwide to contain this costly marine pest.

Chemical management strategies have been used since the 1970s in Norway, starting with the use of trichlorfon (i.e. metrifonate) (Aaen et al. 2015, Torrisen et al. 2013). Currently 7 compounds, found in a variety of sea lice formulations, are used in salmonid aquaculture globally: teflubenzuron and diflubenzuron (benzoyl ureas); cypermethrin and deltamethrin (pyrethroids); azamethiphos (organophosphates); emamectin benzoate (avermectin); and hydrogen peroxide

(Aaen et al. 2015, Burridge et al. 2010, Torrissen et al. 2013). Clinically registered treatments vary by country. In Canada there are currently 5 commercial formulations that can be used in aquaculture: Slice® (active ingredient (AI): emamectin benzoate), Interox®Paramove®50 (AI: hydrogen peroxide), Calicide® (AI: teflubenzuron), Salmosan® (AI: azamethiphos), and Alphamax® (AI: deltamethrin) (Yossa and Dumas 2016). The latter two are only available if emergency registration is granted by Health Canada's Pest Management Regulatory Agency (PMRA) (Yossa and Dumas 2016). In British Columbia, Slice® is the only formulation used (Burridge and Van Geest 2014).

The in-feed formulation Slice® contains 0.2% emamectin benzoate (EB) which is part of a class of compounds called avermectins (Lumaret et al. 2012). Avermectins are macrocyclic lactones which are derived from the bacteria *Streptomyces avermitilis* (Reddy 2013). EB is a mixture of two homologues of avermectins: 4'-epimethyamino-4'-deoxyavermectin B_{1a} benzoate (MAB_{1a}) and 4'-epimethyamino-4'-deoxyavermectin B_{1b} benzoate (MAB_{1b}) (Bright and Dionne 2005). The compounds are relatively large with a molecular weight of 1008.3 and 994.24 g/mol, respectively (Bright and Dionne 2005). They are hydrophobic ($\log K_{ow}$=5 at pH=7), therefore they tend to partition to sediment, allowing EB to build-up in sediment underneath salmon pens, with minimal levels residing in the water column (Bright and Dionne 2005). In an aquatic environment, the dispersal radius for EB is not particularly large; EB has been shown to be almost undetectable (limit of detection: 0.06 µg/kg) in sediment beyond 100 to 150 m from the edge of an open net pen after a typical 7-d treatment regimen of Slice® (DFO 2011b). However, it does have relatively high environmental persistence in sediment, with a degradation half-life (DT_{50}) ranging between 164 to 404 d in marine sediment (Benskin et al. 2006, Bright and Dionne 2005). Conversely, if suspended in the water column, it rapidly decays due to photolysis, with a DT_{50} between 1.4 and 22.4 d (Bright and Dionne 2005).

Ivermectin (IVM) is also an avermectin compound and has been previously used as an off label chemotherapeutant in aquaculture (Haya et al. 2005). It has never been licensed for use in aquaculture, however countries including Ireland, Canada, and Chile had used it until the year 2000 (Horsberg et al. 2012). It is not clear how frequently it is used currently, but it is not a popular method for sea lice control. Like EB, it is administered by incorporation into fish feed. Three formulations have been used historically in aquaculture: a 1% injectable solution (Ivomec®); a 1% oral drench (Eqvalan®); and a 0.6% swine premix (Ivomec® Premix for Swine) (Horsberg et al. 2012). It then disperses into the marine environment by leaching from uneaten food pellets, or from the excrement of treated fish (Bright and Dionne). It also has similar chemical characteristics

to EB, being hydrophobic (log K_{ow} value = 3.21), with a tendency to accumulate in sediments under aquaculture operations and is found only minimally in the water column. Its DT_{50} in sediment is comparable to that of EB. When residing in a soil-feces mixture at 22°C in a laboratory setting, the DT_{50} ranges between 93 to 240 d (Halley et al. 1989). The tendency of IVM and EB to build-up in the sediment and persist for long periods is a concern for non-target sediment-dwelling organisms.

The toxic mode of action of avermectins is linked to their affinity for ligand-gated chloride channels in both invertebrates and vertebrates (Lumaret et al. 2012, Song et al. 2016, Wolstenholme 2010). However, they are generally regarded as being more toxic to invertebrates due to a high selectivity for glutamate-gated chloride channels, which are only found in invertebrates (Fisher and Mrozik 1992, Lumaret et al. 2012, Reddy 2013). Many aquatic toxicity studies have looked at mortality as a toxicological endpoint for both EB and IVM in non-target species. In aquatic vertebrates, the 96-h LC50 of EB for water-only exposures ranges between 18 and 1340 µg/L, when considering the following five species of fish: *Oncorhynchus mykiss* (juvenile), *Lepomis macrochirus* (juvenile), *Pimephales promelas* (adult and early life stages), *Cyprinodon variegatus*, *Cyprinus carpio* (Lumaret et al. 2012, OPP 2000, Wallace 2001b in Park 2013). In aquatic invertebrates, the 96-h LC50 of EB for water-only exposures ranges between 0.04 and 983 µg/L, when considering 4 species of invertebrates: *Nephrops norvegicus*, *Crangon crangon*, *Mysidopsis bahia*, *Crassostrea virginica* (Lumaret et al. 2012, Zelinka et al. 1994 in Park 2013). In comparison, the 96-h LC50 values in water-only exposures to IVM in vertebrates ranges between 0.2 and 74.88 µg/L when considering the following 6 species: *Clarias gariepinus*, *Danio rerio*, *Anguilla Anguilla*, *Lepomis macrochirus*, *Salmo gardneri*, *Salmo salar* (Davies and Rodger 2000, Domingues et al. 2016, Geets et al. 1992, Halley et al. 1989, Oliveira et al. 2016). For invertebrates, the 96-h LC50 values in water-only exposures to IVM ranges between 0.07 and > 10,000 µg/L when considering the following 19 species: *Sphaeroma rugicauda* (Isopoda), *Crangon septemspinosa* (Decapoda) *Palaemonetes varians* (Decapoda) *Gammarus duebeni* (Amphipoda) *Gammarus zaddachi* (Amphipoda) *Carcinus maenas* (Decapoda) *Neomysis integer* (Mysidacea) *Nereis diversicolor* (Polychaeta) *Crassostrea gigas* (Bivalvia) *Mytilus edulis* (Bivalvia) *Pecten maximus* (Bivalvia) *Tapes semidecussata* (Bivalvia) *Monodonta lineata* (Gasteropoda) *Biomphlaria glabrata* (Gasteropoda) *Hydrobia ulvae* (Gasteropoda) *Potamopyrgus jenkinsii* (Gasteropoda) *Littorina littorea* (Gasteropoda) *Nucella lapillus* (Gasteropoda) *Patella vulgata* (Gasteropoda) (Grant and Briggs 1998 Burridge and Haya 1993 Davies et al. 1997 Davies and Rodger 2000 Matha and Weiser 1988).

EB- and IVM-induced mortality has also been investigated in non-target species of invertebrates in exposures involving sediment and overlying water. The 10-d LC50 of EB ranges between 111 and 1368 μg per kg (dry/wet weight) of sediment when considering the following 6 species of benthic invertebrates: *Corophium volutator* (Amphipoda), *Eohaustorius estuarius* (Amphipoda), *Monocorophium insidiosum* (Amphipoda), *Homarus americanus* (Decapoda), *Arenicola marina* (Polychaeta), *Hediste diversicolor* (Polychaeta) (Daoud et al. 2018, Kuo et al. 2010, Lumaret et al. 2012, Mayor et al. 2008, Tucca et al. 2014). The latter two species correspond to the lowest and highest LC50 values, respectively. The 10-d LC50 of IVM ranges between 18 and 212 μg per kg (dry/wet weight) of sediment when considering the following 4 benthic invertebrate species: *Chironomus riparius* (Diptera), *Corophium volutator* (Amphipoda), *Arenicola marina* (lugworm, Polychaeta), and *Homarus americanus* (American lobster) (Daoud et al. 2018, Davies et al. 1998, Egeler et al. 2010, Grant and Briggs 1998, Thain et al. 1997). The latter 2 species have the lowest and highest LC50 values, respectively. However, in *Asterias rubens* (Asteroida) the 10-d LC50 for IVM has been reported to be 23,600 μg/kg (dry weight of sediment) (Davies et al. 1998).

Besides mortality, both EB and IVM exposure can result in a suite of sublethal toxic effects in both aquatic invertebrates and vertebrates. Sublethal effects previously observed in aquatic invertebrates exposed to either EB or IVM include the following: loss of locomotory control, paralysis, developmental effects (e.g. reduced growth and failure to molt), and reduction in reproductive capacity (Bundschuh et al. 2016, Cheng et al. 2020, Daoud et al. 2018, Ding et al. 2001, Egeler et al. 2010, Liebig et al. 2010, Song et al. 2016, Willis & Ling 2003). In aquatic vertebrates, the following sublethal effects have been observed upon exposure to EB: reduced appetite; lethargy; loss of motor coordination; changes in coloration (Roy et al. 2000, Stone et al. 2002). The following sublethal effects in IVM-exposed aquatic vertebrates have been observed: reduction in hematocrit and appetite; changes in cholinesterase, catalase, and glutathione S-transferases activity; lethargy; postural abnormalities and spine deformities (Katharios et al. 2001, Oliveira et al. 2016).

Many environmental factors can alter a substance's toxicity in both the aquatic and terrestrial environment. These can be divided into three categories: (1) chemical, (2) physical, and (3) biological. Chemical factors describe chemical properties of the environmental medium such as pH, cationic/anionic solute profile, organic matter content, dissolved oxygen. Physical factors describe physical properties of an environmental medium (e.g. water/sediment turbidity, sediment grain size, light intensity, temperature, etc.). Biological factors are those that pertain to

biota present in an environmental medium such as microbial activity. Collectively, all these environmental factors which alter exposure or bioavailability, and hence internal dose and toxicity can be termed exposure and toxicity modifying factors (ETMFs) (CCME 2007). In the aquatic environment, this can be divided into two matrix dependent categories: (1) water column characteristics and (2) sediment/porewater characteristics.

In the water column, recognized ETMFs include the following: suspended solids turbidity, light intensity, temperature, resident microbial activity, pH, hardness, alkalinity, dissolved oxygen (DO), dissolved organic carbon (DOC), particulate organic carbon (POC), levels of major cations/anions (e.g. Mg^{2+}, Ca^{2+}, K^{+}, Na^{+}, Cl^{-}, CO_3^{-}) (CCME 2007, Di Toro et al. 2001, Loverage 2016, Santore et al. 2001, Smith and Lizotte 2007, Wang 1987). Many of these factors have been shown to alter the toxicity of organic compounds (Akkanen and Kukkonen 2001, Bostrom and Berglund 2015, Tsui and Chu 2003, Smith and Lizotte 2007). In sediment, recognized ETMFs include the following: grain size and angularity, sediment consolidation, water-retention capacity, mineral constituents (e.g. oxides of Fe, Al, and Mn), total organic carbon (TOC), and total organic nitrogen (TON) (Bentivegna et al. 2004, Lapota et al. 2000, Simpson and Kumar 2016, Word et al. 2005). Sediment porewater characteristics, which are heavily influence by surrounding sediment, that are considered ETMFs include the following: ammonia, pH, DO, and acid-volatile sulfides (AVS) (Lapota et al. 2000, Word et al. 2005).

Organic matter (OM), predominantly made up of humic substances, is a well-recognized toxicity modifying factor (Ferraz et al. 2020, Steinberg 2003, Thurman 1985). Organic carbon (OC) includes only the carbon constituent of OM molecules and is the typical method of quantifying OM (Thurman 1985). The bioavailability of non-metal contaminants can be reduced by a variety of chemical interactions with humic substances including ionic and covalent bonding, hydrophobic interactions, Van der Waals forces, π-π stacking, and hydrogen bonding (Bollag et al. 1992, Bollag and Meyers 1992, Steinberg 2003). Several studies have shown the protective effect DOC has on the bioavailability and toxicity of non-metals in experiments involving exposure in the water column (Mezin and Hale 2004, Meinelt et al. 2005, Misra et al. 2000, Rowett et al. 2016). This effect can be dramatic, but also depends on water chemistry parameters such as hardness and pH. For example, Meinelt et al. (2005) show 5 mg/L of DOC in hard water (125.6 mg $CaCO_3$/L) corresponded to a significant 21.6-fold increase in the 144-h LC50 of embryonic stage *D. rerio* exposed to formaldehyde compared to the control group (0 mg/L of DOC). In contrast, they found that when using soft water (53.6 mg $CaCO_3$/L) instead, the increase in the LC50 was only 1.4-fold relative to the control group. Although this still resulted in a significant

increase in the LC50 relative to the control group, the toxicity mitigating effect of DOC is much less pronounced when soft water was used instead of hard water. Rowett et al. (2016) found that 11 mg/L of DOC in slightly alkaline water (pH = 8.35 to 8.39) corresponded to a significant 1.4-fold increase in the 48-h EC50 (immobilization) relative to the control group (0 mg/L of DOC) of *Gammarus pulex* exposed to triclosan. However, when using more neutral water (pH = 7.25-7.27), they found that the increase in the EC50 was only 1.29-fold, and not statistically significant. These two examples illustrate that water chemistry parameters, such as hardness and pH, can influence the protective effect of DOC. Other studies which did not involve a point-estimate (i.e. LC/ECx) metric for toxicity have also found toxicity-ameliorating effects of DOC. For example, Mezin and Hale (2004) found that 21.7 mg/L of DOC significantly reduced mortality of *Ceriodaphnia dubia* exposed to chlorpyrifos and dichlorodiphenyltrichloroethane (DDT) relative to a low-DOC treatment group at 0.6 mg/L of DOC. However, in this same study they found that 14.8 mg/L of DOC had no effect on mortality-induced by these two compounds in *Americamysis bahia*; although in this case it was relative to a low-DOC treatment group of 2.2 mg/L. Overall, DOC has been shown to reduce the toxicity of non-metals in the water column, albeit inconsistently. This reduction in toxicity generally appears to be less than two-fold, as represented by changes in LC/ECx's, but can also be as high as approximately 20-fold.

Some studies have also focused on the effects OC has on the toxicity of non-metals in sediment exposures (Cano et al. 1996, Harwood et al. 2013, Meador et al. 1997, Tsui and Chu 2004). For example, Harwood et al. (2013) showed that a TOC in sediment of 4.43% significantly increased the 10-d LC50 and EC50 values by 5.7- and 4.9-fold, respectively, in *Hyalella azteca* exposed to a pyrethroid pesticide, bifenthrin. This increase was shown relative to a control group of 0.56% sediment TOC. Cano et al. (1996) made a comparable finding where 4.64% sediment TOC corresponded to a significant 4.6-fold increase in the 48 h LC50 of *H. azteca* exposed to alkylbenzene sulfonate when compared to a low-TOC treatment of 0.35%. Other studies have shown that a much lower sediment TOC content can also result in a reduction in toxicity. For example, Meador et al. (1997) found that a sediment TOC of 0.59% resulted in a significant reduction in mortality of *Rhepoxynius abronius* (Amphipoda) after 10 d exposures to tributyltin, relative to a control sediment with 0.12% TOC. In this case, an LC50 for the 0.59% TOC treatment could not be calculated due to insufficient mortality at the highest concentration of tributyltin tested. However, the concentration resulting in 20% mortality in this treatment was two-fold higher than the LC50 of the 0.12% TOC treatment. Meador et al. (1997) conducted a similar experiment with Armandia brevis (Polychaeta), finding that the concentration resulting in 33% mortality with a 0.87% TOC treatment was 3-fold higher than the LC50 at the lower TOC treatment of 0.30%.

Toxicity of glyphosate has also been shown to be reduced by sediment TOC in exposures with *Ceriodaphnia dubia* (Tsui and Chu 2004). A sediment TOC of 2.10% resulted in a statistically significant 3.1-fold higher 48-h LC50 value relative to a 0.0% TOC control group with exposures using the glyphosate-containing formulation Roundup®. However, when the glyphosate-containing formulation Roundup Biactive® was used, the 48 h LC50 was 1.3-fold lower than the control group. The only difference between these 2 formulations is the surfactant used. No studies have addressed the modifying effects on the toxicity of OM on EB or IVM, or any avermectins. Since the environmental fate of EB and IVM dictates that the environmental medium they will predominantly reside in is sediment, rather than water, it is imperative to understand the effects varying levels of sediment TOC will have on toxicity of these compounds.

Increasing chemical residence time (the length of time that a substance spends in contact with sediment) has been shown to reduce bioavailability (the portion of a compound which can be absorbed by an organism, through any route of exposure, from both the physically available and unavailable pools of that compound) of persistent organic pollutants (Alexander 2000, Chung and Alexander 1998, Conrad et al. 2002, Kukkonen and Landrum 1998, Landrum et al. 1992, Taylor et al. 2019). One recognized mechanism for this decrease in bioavailability with increasing chemical residence time is that contaminants gradually sequester into pockets of the sediment matrix which are inaccessible to even the smallest of organism, such as soil microbes (Alexander 2000). This represents a reduction in a contaminant's physical availability: the portion of a compound that can come in direct contact with the exterior or interior surfaces of an organism. For example, Chung and Alexander (1998) demonstrate that soil microbes were significantly less able to mineralize – and therefore, presumably, were less in contact with – phenanthrene and atrazine after 200 d of being aged with sediment. However, this may not accurately reflect the exposure scenario in multicellular organisms which have organs with extractive functions, like the gastrointestinal tract, enabling more effective extraction of contaminants from sediment particles than single-celled microbes. Nevertheless, a reduction in bioavailability of contaminants with increasing chemical residence time has been previously observed in multicellular organisms including *Lumbriculus variegatus* (aquatic oligochaete), *Diporeia spp.* (benthic amphipod), and *Eisenia foetia* (earthworm) exposed to persistent organic pollutants (Conrad et al. 2002, Kukkonen and Landrum 1998, White et al. 1999, You et al. 2009). Furthermore, it was found that physical occlusion of the contaminant was responsible for 21% of the overall reduction in pyrene bioavailability in *L. variegatus*, after 220 d of chemical residence time (Conrad et al. 2002). This indicates that physical occlusion of contaminants, with increasing chemical residence time, plays

a role in bioavailability reductions of these contaminants in more complex organisms than microbes.

The decrease in physical availability of a contaminant is not solely responsible for a decrease in its bioavailability. For example, the bioavailability, normalized to physical availability of pyrene (i.e. only when considering the physically available pool of pyrene in sediment) in *L. variegatus* decreased by about 58% over the course of 220 d of chemical residence time (Conrad et al. 2002). This represents a decrease in bioavailability that is attributed to reasons other than a contaminant's physical availability. It is worth noting that a large amount (approximately 60%) of the decrease in bioavailability of pyrene can be accounted for after the first d of sediment aging. In fact, Conrad et al. (2002) found that the reduction in bioavailability over the course of 220 d followed 3-stage progression. The first stage: a rapid reduction in bioavailability by approximately 30% over the course of the first day. The second stage: a plateau in bioavailability between day 14 to 70. The third stage: between day 70 to 220, a further 28% decrease in bioavailability. This demonstrates that the reduction in bioavailability might be a rapid process initially. Besides bioavailability, the chemical extractability, representing the physically available fraction, of pyrene decreased by 50% after 220 d of chemical residence time (Conrad et al. 2002). Therefore, the recorded 70% decrease in body burden of pyrene in the test species used in that study over the course of 220 d of sediment aging, represents a decrease in the overall bioavailability of pyrene. Furthermore, to address the confounding-effect of chemical degradation of pyrene, the authors did confirm that pyrene did not decay during the entire 220 d of the experiment.

Besides pyrene, the bioavailability of other persistent organic pollutants has been shown to decrease with increasing chemical residence time. For example, the bioavailability of benzo(a)pyrene to the benthic amphipod *Diporeia spp*. decreased by 42% after a period of 13 months of chemical residence time (Kukkonen and Landrum 1998). A vast majority (approximately 90%) of this decrease in bioavailability occurred after the first week of sediment aging. Other studies have also shown that dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), and other polycyclic aromatic hydrocarbons (PAHs) also experience a reduction in bioavailability with increasing chemical residence time (Landrum et al. 1992, Menchai et al. 2008, Taylor et al. 2019). The role that chemical residence time plays in the bioavailability of EB and IVM has not previously been investigated.

The aim of this study was to investigate the effects of sediment OC content and chemical residence time on the toxicity of two compounds in sea lice chemotherapeutants: EB and IVM. Both single-chemical and combination exposures to EB and IVM were conducted. Combination

exposures allowed for insight into the consequences of using these two compounds concurrently on the same salmon farm. The test organisms used were two benthic marine invertebrates: the polychaete *Neathes virens* and the amphipod *Eohaustorius estuarius*. These sediment-dwelling organisms potentially represent particularly sensitive and highly exposed ecological entities.

2.2 Materials and Methods

2.2.1. Organisms and Holding Conditions

E. estuarius were provided by Nautlius Environmental (Burnaby, BC). *N. virens* were provided by Aquatic Research Organisms (Hampton, NH). Before being used in experiments, both species were acclimatized at 11ºC, with a 12 : 12 light : dark schedule, for a minimum of 1 week. Both species were provided with sediment and seawater in their holding vessels. Sea water was aerated during holding. Between 30 to 50 *N virens* were placed in plastic totes (41.9 cm x 26.7 cm x 16.5 cm) filled 33% (v/v) with uncontaminated sediment collected from Centennial Beach (Tsawwassen, BC, Canada; coordinates: 49.017095, -123.040231) (not from the intertidal zone, unlike the low OC sediment [described in Section 2.2.3.]). Plastic totes were submerged in 170 L plexiglass tanks (185 cm x 36.8 cm x 25.4 cm) almost entirely full of seawater. Four totes were placed in each 170 L plexiglass tank. *E. estuarius* were held in plastic containers (10.2 cm x 10.2 cm x 6.4 cm) filled 50% with sediment provided with the plastic containers by Nautilus Environmental (Burnaby, BC). Each container held 110 *E. estuarius*. The containers were submerged in a large plastic tote (55.9 cm x 36.8 cm x 15.2 cm), filled 75% full of seawater. For *E. estuarius*, seawater was not filtered or changed during holding. For *N. virens*, in addition to continuous filtering of seawater with canister filters, all seawater was changed daily during holding to prevent the build-up of waste products. *E. estuarius* were not fed while in holding. *N. virens* were fed Phytogold-S® (Brightwell Aquatics; Fort Payne, AL); a few drops of Phytogold® were delivered into their holding tanks once per week. However, it is likely that these organisms were feeding on particulate matter that existed in the sediment they were provided with. Seawater was pH 8.1, salinity 28ppt.

2.2.2. Chemicals and Formulations

SLICE® 0.2% Premix (Merck Animal Health, Intervet Canada Corp., Kirkland, QC), which contains 0.2% EMB w/w, was obtained from Fisheries and Oceans Canada (DFO). IVM (CAS Number 70299-86-7), which is a solid white powder, was obtained from Sigma-Aldrich (Oakville, ON). IVM was stored in the dark at 4°C, while Slice® was stored in the dark at room temperature.

2.2.3. Sediment

Marine sediment was collected from the intertidal zone during low tide at Centennial Beach, Tsawwassen, BC, at the following coordinates: 49.014819, -123.038693. The sediment was wet sieved with a 2 mm sieve, stored at room temperature, and allowed to dry for approximately 2 months before use. Allowing the sediment to dry facilitated OC degradation. Sediment with a total organic carbon (TOC) percent that was below the limit of detection (<0.05%) was used as the low OC (0-OC) sediment treatment. The sediment used for the high OC (1-OC) sediment treatment was collected from the Tofino Mudflats (Tofino, BC), at the following coordinates: 49.125609, -125.883488. This sediment had a TOC content 0.42% (4200 mg C/kg). After collection, this sediment was kept hydrated with a top layer of seawater to prevent degradation of OC. This sediment was then sieved to a particle size of 6.35 mm to remove rocks and marine debris. It was then stored at 4 °C, to minimize degradation of OC. After processing the 0-OC and 1-OC sediment, it was sent to Maxxam Analytics (Burnaby, BC) for analysis of the following characteristics: pH, total organic matter (TOM), TOC, and particle-size distribution. For a summary of these sediment characteristics, refer to *Appendix A* (Figure A.1, Figure A.2, & Table A.*1*). The sediment for the intermediate OC (0.5-OC) level was a 50:50 mixture, by mass, of the 0-OC and 1-OC sediments.

2.2.4. Sediment Spiking and Incubation

For *N. virens* exposures, 250 g of sediment was added to 1 L glass mason jars to a depth of 2.5 cm. For *E. estaurius* exposures, 100 g of sediment was added to a 500 mL glass mason jars and prepared in a similar manner. A shallow layer of seawater was then added to a level of

approximately 2-4 cm above the sediment. Sediment was then spiked with either Slice®, IVM, or a combination, using seawater as a solvent for delivery. Spiked sediment was stirred vigorously with a glass stirring rod. Spiked and control sediment jars were incubated for three different lengths of time: overnight (0 months), 60 d (2 months), and 120 d (4 months). The 0-month treatment was incubated overnight in the dark at room temperature, while the 2- and 4-month groups were incubated in the dark at 4 °C. After incubation, seawater was added to jars so that an approximate seawater volume to sediment volume ratio of 4 : 1 was attained as recommended for sediment-water toxicity tests with aquatic oligochaetes (OECD 2007). A similar ratio was used as recommended for use with marine amphipods, including *E. estuarius* (USEPA 2016). These jars were then used for exposures.

2.2.5. Exposures

At the beginning and end of exposures, the following water quality parameters were measured: pH, salinity, dissolved oxygen (DO), temperature. The duration of exposure was different for the two species: for *N. virens* it was 10 d, while for *E. estuarius* it was 48 h. Exposures were conducted in a temperature-controlled room at 11 °C. The light-dark cycle in this room followed a 12 : 12 light : dark rotation. Seawater in all jars was lightly aerated. All exposures were static (no water changes took place for the duration of the exposure).

For single-chemical exposures, *N. virens* were exposed to the following 8 nominal concentrations of EB for all 3 organic carbon levels: 10, 20, 40, 80, 800, 2400, 4800, 9600 µg/kg. The following 8 nominal concentrations were used for IVM: 4, 8, 20, 40, 80, 400, 1200, 4800 µg/kg. All concentrations are expressed as µg of chemical per kg of sediment wet weight (w.w.). For combination exposures, the following concentrations (EB/IVM) were used: 10/4, 10/8, 20/4, 80/20, 80/40, 800/20, 800/40, 2400/400 µg/kg. For example, the ‘10/4’ combination treatment corresponds to 10 µg/kg of EB and 4 µg/kg of IVM. Eight replicate jars were included for each of the 5 highest EB concentrations. Four replicate jars were included for each of the 3 lowest EB concentrations. Eight replicate jars were included for each of the following IVM concentrations: 20, 40, 400, 1200, and 4800 µg/kg, while only 4 replicate jars were included for each of the following IVM concentrations: 4, 8, and 80 µg/kg. The imbalance in replicates was because exposures were split into 2 groups based on endpoint measurements: lethal and sublethal. The lethal group included higher concentrations and had 8 replicate jars for each concentration. The

sublethal (where lethality was also measured) group included lower concentrations and had 4 replicates jars for each concentration. This was done for each of the 9 chemical residence time and OC co-treatments. Mortality was measured for all replicate jars used in this study (i.e. either 4 or 8 replicates depending on concentration); meanwhile sublethal endpoint measurements were only carried out for 4 replicates at each of the 6 lowest concentrations for EB, IVM, and combination exposures. In cases where 8 replicate jars existed at a given concentration, 4 jars were randomly selected for sublethal measurements. Concentrations for combination exposure groups had the same number of replicate jars as their corresponding single-chemical concentration had. Each replicate jar contained 1 organism. For mortality, 12 replicate solvent (seawater) control jars were included for each OC and chemical residence time combination. This was because 4 and 8 control jar replicates were included for the sublethal and lethal concentration series groups, respectively. For sublethal endpoints, 8 control jars were assessed for sublethal endpoints for each OC and chemical residence time combination: 4 randomly selected from the lethal concentration series group, and 4 included for the sublethal concentration series group.

E. estuarius were exposed to a different series of concentrations for each of the 3 OC levels, since preliminary testing suggested that a different concentration series would be necessary for reliable point-estimate methods of statistical analysis. Refer to Table 7 for the list of nominal concentrations for EB, IVM, and combination exposures. Three replicate jars were included for each concentration, except for the 2000 and 1500 µg/kg concentrations of EB for the 0.5-OC and 0-OC groups, respectively. For these concentrations 3 additional replicates were included. Each replicate jar contained ten organisms. Six replicate solvent (seawater) control jars were included for each combination of chemical residence time and OC: the reason 6 replicates were included was because concentration series were split into two groups: a lower (sublethal) and higher (lethal) concentration series, like with *N. virens*. Three control replicates were included in each of these 2 concentration series groups.

	***E. estuarius* Concentration Series**								
	0-OC			**0.5-OC**			**1-OC**		
Concentration	[EB] (µg/kg)	[IV] (µg/kg)	[EB/IVM] (µg/kg)	[EB] (µg/kg)	[IVM] (µg/kg)	[EB/IVM] (µg/kg)	[EB] (µg/kg)	[IVM] (µg/kg)	[EB/IVM] (µg/kg)
C1	10	2.5	10/2.5 (C1/C1)	50	5	50/5 (C1/C1)	50	5	50/5 (C1/C1)

C2	50	5	50/5 (C2/C2)	100	10	100/10 (C2/C2)	150	10	150/10 (C2/C2)
C3	100	10	100/10 (C3/C3)	200	50	200/50 (C3/C3)	500	25	500/50 (C3/C4)
C4	500	25	100/50 (C3/C4)	500	25	500/25 (C4/C4)	1000	50	500/100 (C3/C6)
C5	1000	50	500/10 (C4/C3)	1000	75	500/75 (C4/C5)	1500	75	1000/25 (C4/C3)
C6	1500*	80	1000/25 (C5/C4)	2000*	135	1000/25 (C5/C4)	2000	100	1500/50 (C5/C4)
C7	2500	120	500/50 (C4/C5)	3500	100	1000/75 (C5/C5)	3000	150	1500/100 (C5/C6)
C8	5000	150	1500/120 (C6/C7)	6000	200	2000/135 (C6/C6)	5000	250	3000/150 (C7/C7)
C9		300			500		8500	600	

Table 7: Summary of concentrations (C1-C9; in µg/kg) of emamectin benzoate ([EB]), ivermectin ([IVM]), and combinations of the two ([EB]/[IVM]) that E. estuarius were exposed to during toxicity tests. OC levels are unitless (relative scale) and each level has a unique concentrations series. Three replicate jars were used for all concentrations, except for those indicated with the asterisk (*), where 6 replicates were used. Shaded boxes indicate concentrations at which sublethal testing was conducted.

2.2.6. Toxicological endpoints

Mortality

At the end of the exposure period, mortality was assessed and recorded; for both species, if an organism was not moving spontaneously, it was gently prodded 3 times and observed for any movement. For *N. virens*, tissues of the body and tail segments may move with necrotic and decaying head tissue; in these cases, the organism was considered dead.

Behavioural assays

A light-avoidance assay was used to test for the sublethal effects of toxicity on *E. estuarius.* After 48 h exposures, *E. estuarius* were placed in a small, hollowed-out block (15.9 cm x 2.8 cm x 2.8 cm) divided evenly into 2 sides: one side was transparent, allowing light to enter; the other side was covered and dark (referred to as light-dark blocks). These light-dark blocks were arranged in 2 groups of 6 on a level surface. Each group of 6 blocks was comprised of three blocks adjacent lengthwise, with the light side of these blocks touching the light side of another three blocks adjacent lengthwise. The assay chamber was surrounded by black curtain to prevent

changes in light in the surrounding environment from affecting behavior. Three lamps illuminated the curtain-enclosed assay chamber, 2 of which were each placed over a different group of 6 light-dark blocks. The third lamp was placed roughly in the middle of the 2 groups of light-dark blocks. All light-dark blocks were filled half full of sea water prior to introducing *E. estuarius*. Two live amphipods randomly selected from the same replicate jar were introduced into the light side of each of these light-dark blocks. A video camera was mounted on a tripod and placed approximately 25 cm above each of the 2 groups of 6 light-dark blocks, for a total of 12 light-dark blocks being recorded simultaneously. Refer to Figure A.3 (*Appendix A*) for an example of 1 group of 6 light-dark blocks being filmed by a single camera. Upon video review, time spent per individual in the light v. dark was calculated over the course of 12 min. This was then expressed as percentage of total time spent in the dark v. total time of the assay.

A burrowing behavioral assay was conducted with *N. virens* at the termination of exposures. This assay was conducted in plastic totes (41.9 cm x 26.7 cm x 16.5 cm) divided by plexiglass sheets (18 cm x 20.3 cm) into 4 equal-sized quadrants. These totes were filled with 4 kg (dry weight) of uncontaminated sediment, to a depth of 4.5 cm. A shallow top-layer of seawater was added over the sediment to a depth of approximately 2-4 cm. Uncontaminated sediment used for this assay was the same that was used for *N. virens* holding tanks, as described in Section 2.2.1. This sediment was characterized by a coarser grain size than either the 0-OC or 1-OC sediment (qualitative observation). Video cameras were placed at a height of approximately 1 m above the water. Prior to introducing *N. virens* into plastic totes, video recording began. One *N. virens* was placed on sediment in each quadrant of the tote. As soon as the organism touched the sediment, they were filmed to determine whether they were completely burrowed after 15 min had elapsed. Organisms were only considered completely burrowed if no portion of their body was visible on inspection of video recordings.

2.2.7. Statistical Analysis

Single Chemical Exposures

For all single-chemical exposures, EB and IVM concentrations were treated as numeric (continuous) variables. For concentration-response analysis of *E. estuarius* mortality data, the *drc* package (Version 3.0-1) for RStudio Statistical Software Version 3.5.3 (RStudio PBC; Boston, MA) was used to generate sigmoidal concentration-response curves using a log-logistic 3-parameter model with an upper limit of 1 (Ritz et al. 2015, Ritz and Strebig 2016). Concentration-

response curves were generated for each combination of chemical residence time and OC treatments for single chemical exposures. This was done for EB and IVM separately. To determine whether there was a significant concentration-dependent increase in mortality, it was tested whether the slope parameter (proportional to the slope at the inflection point of the curve) (Ritz 2010) of the log-logistic model was significantly different from 0 with a t-test. The concentration-response curves were used to calculate LC50 values and their associated 95% confidence intervals for each combination of chemical residence time and OC. To determine the effects of OC and chemical residence time on toxicity of EB and IVM, multiple pairwise comparisons were made between LC50 values, using the *EDcomp* function within the *drc* package. This method uses a t-test to evaluate whether LCx values are statistically different from each other. To determine the effects of OC on mortality, pairwise comparisons were made between LC50 values across OC levels and within chemical residence time treatments. To determine the effects of chemical residence time on mortality, pairwise comparisons were made between LC50 values across chemical residence times and within OC levels. To address the inflation of type 1 statistical errors caused by multiple comparisons, a Bonferroni correction was applied: the threshold p-value for statistical significance (α) was divided by the number of pairwise comparisons (29); thus, the Bonferroni corrected α value was 0.00172.

JMP Statistical Software (version 15.0) and RStudio Statistical Software Version 3.5.3 (RStudio PBC; Boston, MA) were used to analyze *E estuarius* light-avoidance bioassay data. Unlike lethality data, point estimates (i.e. ECx values) were not determined due to the poor linear correlation between concentration and response. Instead, analysis focused on determining whether a linear correlation existed between concentration and response for each combination of chemical residence time and OC. Light avoidance was expressed as percentage of time spent in the dark during the 12-min period of observation. For each chemical residence time and OC treatment combination, percentage of time in the dark (response variable) and chemical concentration were incorporated into a linear regression model to determine whether a concentration-response relationship existed in each group. This was done separately for EB and IVM single-chemical exposures. To determine whether concentration had a significant effect on percentage of time spent in the dark, a one-way ANOVA was conducted to test whether the slope of the curves was significantly different from 0; this was done using JMP Statistical Software (version 15.0). To investigate the effect OC had on concentration-response relationships, an ANCOVA was carried out with an interaction term to evaluate whether a significant difference existed between slopes of the concentration-response curves of the 3 OC levels (0-, 0.5-, and 1-OC) at each chemical residence time (0-, 2-, and 4-months), separately. To determine the effect

chemical residence time had on concentration-response relationships, an ANCOVA was carried out with an interaction term to evaluate whether a significant difference existed between slopes of the 3 chemical residence time treatments for each OC level separately. All analyses were done separately for EB and IVM single-chemical exposures. Follow-up pairwise comparisons between slopes were made using a posthoc test with a Tukey adjustment. All ANCOVA analyses were conducted using RStudio Statistical Software Version 3.5.3 (RStudio PBC; Boston, MA); the *emmeans* package (Version 1.5.3) was used for pairwise statistical comparisons between slopes of concentration-response curves.

N. virens mortality and re-burrowing data was analyzed differently than *E. estuarius* data because it was binary (nominal) data for both lethality and burrowing. In addition, the same concentration series was used for each OC level, which allowed for a direct comparison between the three OC levels. For mortality data, either an organism was dead (1) or alive (0). For the re-burrowing assay, either an organism was unburrowed (1) or burrowed (0). For binary mortality and re-burrowing data, concentration-response analysis and LC50 and EC50 estimates were generated using JMP Statistical Software (version 15.0). To determine whether concentration-response relationships were significant for each chemical residence time and OC combination, binomial logistic regressions (logit) were conducted with concentration as the only factor in the model for each of these time-OC treatment combinations. This was done for EB and IVM separately. To determine whether concentration had a significant effect on response (either mortality or burrowing), a maximum likelihood ratio test was performed, using a chi-squared test statistic. The logistic regression models that used mortality data were used to calculate LC50 values and their associated 95% confidence intervals for each time-OC combination, for EB and IVM separately. EC50 values for burrowing were not calculated due to an inability to establish a consistent baseline response for each chemical residence time and OC treatments.

To determine whether chemical residence time and OC influenced lethal and sublethal toxicity, a 3-factor binomial nominal logistic regression model was run with the following three factors: concentration, chemical residence time, and OC. All 2-way interactions between all 3 factors were included as terms in the model. A maximum likelihood ratio test, using a chi-squared test statistic, was carried out to determine statistical significance of main effects and statistical interactions. To reduce the complexity of models, increasing statistical power, all non-significant terms in the model were dropped from the model. With these simplified models, maximum likelihood ratio tests were conducted to determine significant terms in the model. If no interaction existed between chemical residence time and OC, then follow-up paired comparisons between

treatment groups could be made for each of these 2 factors independently, without consideration of the level of the other factor: for example, paired comparisons between the 0-, 0.5-, and 1-OC groups would be made, pooling data across all chemical residence times. However, if an interaction was detected, then paired comparisons would need to be made amongst all nine chemical residence time and OC groups. This was done across OC levels, and within chemical residence time treatments, to determine the effects of OC. Conversely, this was done across chemical residence times, and within OC levels, to determine the effect of chemical residence time. Post-hoc tests with a Tukey-Kramer adjustment were used to locate differences in paired comparisons using the *emmeans* package in RStudio Statistical Software Version 3.5.3 (RStudio PBC; Boston, MA).

Combination Exposures

For all combination exposures, concentration was treated as a categorical (nominal) variable. JMP Statistical Software (version 15.0) was used to conduct all statistical analyses on *E. estuarius* combination exposure data. To investigate the effects of chemical residence time and concentration on mortality and light-avoidance in *E. estuarius*, a 2-way ANOVA model with an interaction term was fitted to the data for combination exposures. The 2 factors were chemical residence time and concentration. These 2 factors and their interaction were treated as fixed-effect factors in the model. Models were generated for each OC level separately because the concentration series for each OC-level differed. For mortality data, since a statistically significant interaction was found between chemical residence time and concentration for all 3 OC levels, the main effects of these factors on their own could not be used to draw conclusions. Instead, to determine whether chemical residence time influenced mortality, a one-way ANOVA was conducted to test for significant differences in mean mortality between the 3 chemical residence time treatments at each of the nine concentration levels (including the control group). To determine whether concentration influenced mortality, a one-way ANOVA was conducted to test for a statistical difference in mean mortality between concentration treatments for each chemical residence time. Subsequently, a Tukey HSD post-hoc test was used to statistically compare mean mortality at the 8 EB/IVM combination concentrations to the control group within the same chemical residence time treatment; this was done to determine whether there were concentration-dependent changes in mortality for each chemical residence time treatment. For light-avoidance data, significant interactions between concentration and chemical residence time were only found at the 0- and 0.5-OC levels. Therefore, the same methods as mentioned for mortality data were used to assess whether concentration and chemical residence time treatments significantly

influenced light avoidance. In the case of the 1-OC level, where an interaction did not exist, the main effects could be analyzed for each of the two factors on their own. In this case, a post-hoc Tukey HSD test was conducted to locate statistical differences between the 3 different chemical residence times and the 6 different concentrations, separately.

To investigate the effects of OC on mortality and light-avoidance in *E. estuarius*, a subset of 3 combination concentrations which overlapped between all 3 OC levels (refer to Table 7) were used to observe differences in response between OC levels. These 3 concentrations were 0 (control), 50/5, 1000/25 (EB/IVM; in μg/kg). A 2-way ANOVA model was run with the following 2 factors (independent variables): concentration and OC. A 2-way interaction term was included in this model. For mortality data, a statistically significant interaction between OC and chemical residence time was found in a preliminary 3-way ANOVA model, therefore a separate 2-way ANOVA model was run for each chemical residence time. Since a significant statistical interaction between OC and concentration was detected, OC's effect on mortality was evaluated at all 3 concentrations separately. To determine whether there were concentration-dependent changes in mortality, a post-hoc Tukey HSD test was carried out to detect pairwise statistical differences between concentration treatments and the control group within the same OC treatment. For light-avoidance data, there was no statistically significant interaction between OC and chemical residence time, therefore light-avoidance data was pooled across chemical residence times for all 3 OC groups and all 3 concentrations. The 2-way ANOVA model found no interaction between concentration and OC, therefore the main effects of these two factors were evaluated on their own.

For *N. virens*, to determine the effect of OC and chemical residence time on each endpoint, 3-factor binomial logistic regression models (logit) were run for each in RStudio Statistical Software Version 3.5.3 (RStudio PBC; Boston, MA). These models included the following 3 factors and all possible 2-way interactions between these factors: chemical residence time, OC, and concentration. These models were used to conduct a maximum likelihood ratio test (chi-squared test statistic) to determine whether each of the 3 factors had a significant effect on response and whether any significant interactions existed between factors. To reduce the complexity of models, increasing statistical power, all non-significant terms in the model were dropped from the model. With these simplified models, maximum likelihood ratio tests were conducted. This was followed by post-hoc tests using a Tukey-Kramer adjustment to locate statistically significant differences in pairwise comparisons using the *emmeans* package in RStudio Statistical Software Version 3.5.3 (RStudio PBC; Boston, MA). To determine

concentration's effect on each endpoint, a Pearson's chi-squared test was conducted for each. Pairwise comparisons were made using a posthoc test with a False Discovery Rate adjustment (Benjamini-Hochberg procedure) for multiple comparisons, with the *chisq.bintest* function in the *RVAideMemoir* (Version 0.9-78) package in RStudio Statistical Software Version 3.5.3 (RStudio PBC; Boston, MA). For mortality data, due to a significant interaction detected between concentration and chemical residence time, the Pearson's chi-squared test and subsequent posthoc test were done for each chemical residence time separately. While for burrowing data, this was done pooling data across all chemical residence time and OC cotreatments.

2.3. Results

2.3.1. Water quality parameters

A summary of the water quality parameters measured in both *E. estuarius* and *N. virens* exposures can be found in Table A.2 and Table A.3 (*Appendix A*), respectively. The mean temperature for each chemical residence time and OC treatment group ranged from 9.48 ± 0.13 to 13.44 ± 0.18 ^{o}C, and 8.65 ± 0.16 to 11.43 ± 0.15 ^{o}C, for *E. estuarius* and *N. virens* exposures, respectively. Seawater pH generally ranged between 7 to 8 in most exposure groups. Dissolved oxygen was > 80% in all groups except for the 0-0.5 and 0-1 group for *E. estuarius*, where it was 78 and 73%, respectively. Salinity was the most consistent water quality parameter typically ranging between 29 to 30 ‰.

2.3.2. Control mortality

Refer to Table A.4 in *Appendix A* for a summary of control survival for all sediment OC and chemical residence time treatment combinations. For *E. estuarius*, the control mortality did not exceed 10% for any sediment OC content or for any length of chemical residence time, ranging between 0 and 8.3%. For *N. virens*, the mean control mortality did not exceed 20% in any group, ranging between 0 to 16.7%.

2.3.3. Lethal toxicity: concentration-response analysis

In single chemical exposures, IVM caused a significant concentration-dependent increase in mortality in *E. estuarius* for all chemical residence time and OC treatment combinations (Figure 8). The estimated 48-h LC50 value for IVM in *E. estuarius* ranged between 32.38 to 468.2 µg/kg, depending on the chemical residence time and OC treatment group (Table 8). For EB exposures, there was a significant concentration-dependent increase in mortality for 8 out of 9 combinations of chemical residence time and OC: at 4 months of chemical residence, the 0-OC treatment did not show a concentration-dependent increase in mortality (p=0.12; Figure 8). Concentration-response curves are shown in Figure 8. The estimated 48-h LC50 value for EB-exposed *E. estuarius* ranged between 1875.45 µg/kg to > 6532.97 µg/kg, depending on the combination of chemical residence time and OC (Table 8). The effect of OC and chemical residence time on *E. estuarius* 48-h LC50 values for EB and IVM single-chemical exposures is discussed in *Section 2.3.4* and *2.3.5* (for OC and chemical residence time, respectively).

	E. estuarius	
	48 h LC50 values	
Time-OC	**EB**	**IVM**
0-0	2009 (1571-2447)	32.4 (11.9-52.8)
0-0.5	2413 (2029-2797)	121.0 (99.2-142.8)
0-1	2093 (1597-2589)	107.3 (82.1-132.5)
2-0	1876 (1497-2254)	39.6 (25.0-54.2)
2-0.5	2931 (2601-3260)	91.9 (78.9-105.0)
2-1	2482 (2158-2806)	154.9 (114.8-194.9)
4-0	>5000	200.3 (163.1-237.4)
4-0.5	>6000	276.7 (225.3-328.2)
4-1	6533 (5826-7240)	468.2 (373.0-563.4)

Table 8: Summary of emamectin benzoate (EB) and ivermectin (IVM) 48-h LC50 values for *E. estuarius* at each combination of chemical residence time (0-, 2-, and 4-months) and organic carbon (0-, 0.5-, and 1-OC; relative scale) [Time-OC]. The 0-, 0.5-, and 1-OC organic carbon (OC) treatments used sediments with total OC contents of < 0.05, ~ 0.2, and 0.42%, respectively. All LC50 values are in µg / kg of sediment (wet weight) and were calculated using nominal concentrations for EB and IVM. 95% upper and lower confidence limits are in brackets.

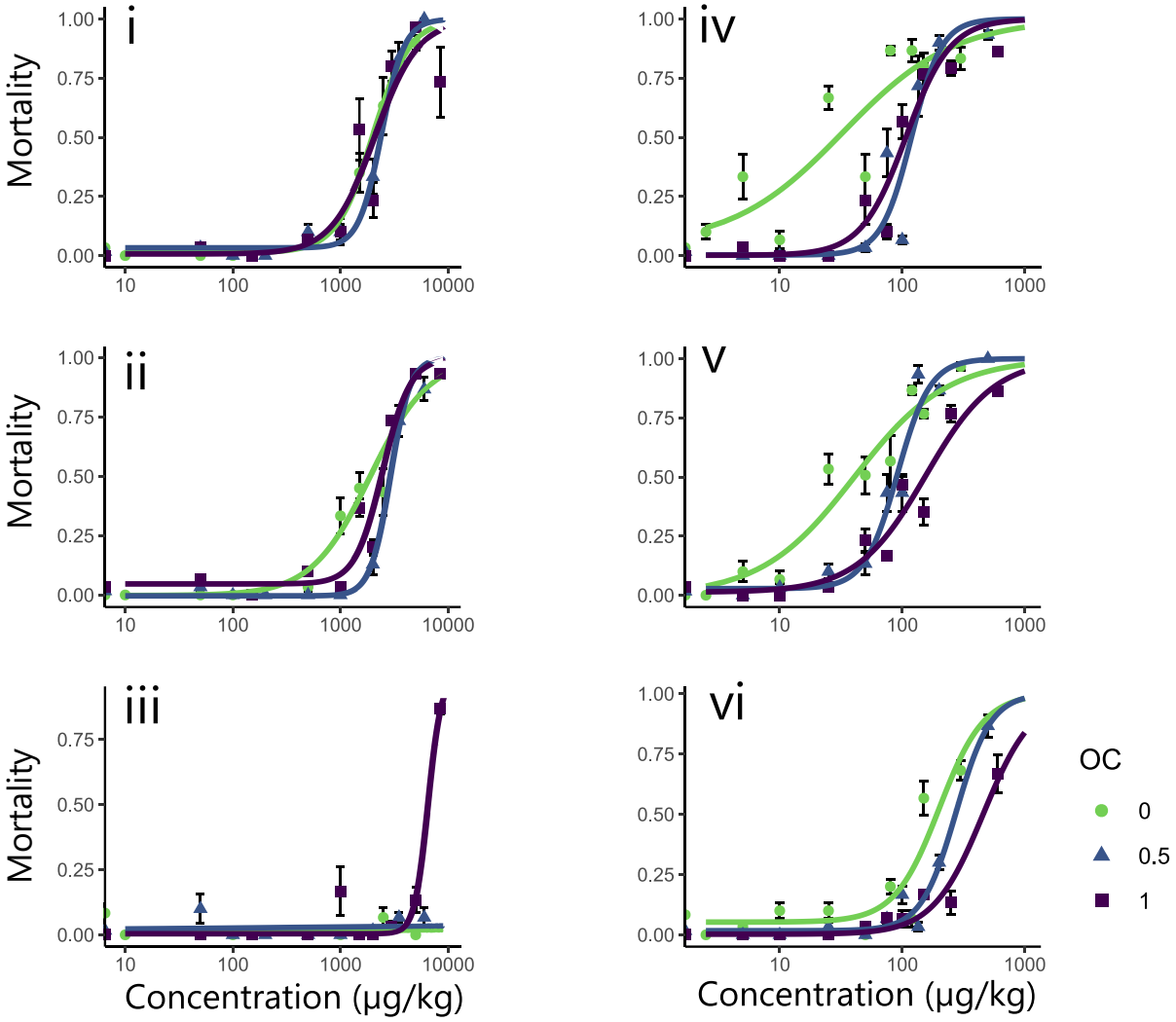


Figure 8: *E. estuarius* emamectin benzoate (EB) concentration-response curves for the 3 organic carbon (OC) levels (refer to legend) at (i) 0-, (ii) 2- , and (iii) 4-months of chemical residence, and ivermectin (IVM) concentration-response curves at (iv) 0- , (v) 2-, and (vi) 4-months of chemical residence time. The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Concentration is in µg/kg (w.w. sediment). Each data point represents the mean mortality proportion (± 95% confidence intervals). Lines represent 3-parameter log-logistic models fitted to data, which were used to calculate LC50 values.

For *N. virens* IVM exposures, 6 out of 9 combinations of chemical residence time and OC showed a significant concentration-dependent increase in mortality (Figure 9). At 0 months of chemical residence time, the 0- and 0.5-OC groups showed a significant concentration-dependent increase in mortality ($p<0.0001$ and $p=0.0350$, respectively), while the 1-OC group did not ($p=0.1033$). At 2 months of chemical residence time, the 0-, 0.5-, and 1-OC groups all showed a highly significant concentration-dependent increase in mortality ($p<0.0001$ for all 3). At 4 months

of chemical residence time, only the 0-OC group showed a significant concentration-dependent increase in mortality (p=0.0311), while the 0.5- and 1-OC groups did not (p=0.0994 and 0.7905, respectively). The 10-d LC50 for IVM-exposed *N. viren*s ranged from 537.63 to > 4800 µg/kg, depending on chemical residence time and OC treatment (Table 9). The effect of OC and chemical residence time on lethal toxicity will be discussed in *Section 2.3.4 and 2.3.5.*, respectively. Unlike for *E. estuarius*, a statistical comparison between LC50 values of the various chemical residence time and OC treatments was not conducted for *N. virens*. Instead, direct comparison of mortality data, pooling across all concentrations, were made between the various OC and chemical residence time treatment groups with binomial logistic regression models (described in *Section 2.2.7.*). This is because for *N. virens,* the concentration series used for the various chemical residence time and OC treatments were identical, unlike for *E. estuarius* where the concentration series differed between OC treatments.

For *N. virens* EB exposures, 8 out of 9 combinations of chemical residence time and OC showed significant concentration-dependent increase in mortality (Figure 9). At 0 months of chemical residence time, the 0-, 0.5-, and 1-OC groups all showed a significant concentration-dependent increase in mortality at 0 months of chemical residence time (p=0.0002, 0.0077, 0.0387, respectively). At 2 months of chemical residence time, the 0.5- and 1-OC groups showed a significant concentration-dependent increase in mortality (p=0.0037 and 0.0053, respectively), while 0-OC did not (p=0.2655). At 4 months of chemical residence time, the 0-, 0.5-, and 1-OC groups all showed a highly significant concentration-dependent increase in mortality (p<0.0001 for all 3). The 10-d LC50 for EB-exposed *N. virens* ranged between 4414.87 to > 9600 µg/kg (Table 9).

	N. virens	
	10-d LC50 values	
Time-OC	**EB**	**IVM**
0-0	7107 (4855-13042)	2694 (1646-5637)
0-0.5	7539 (4559-23804)	4753 (2590-54238)
0-1	>9600	>4800
2-0	>9600	538 (43.2-6275)
2-0.5	8216 (5290-20720)	689 (424-1380)
2-1	9155 (5963-24524)	2206 (1262-5165)
4-0	4415 (3073-7119)	3546 (1779-45455)
4-0.5	7534 (5331-12862)	>4800
4-1	9413 (7341-14249)	>4800

Table 9: Summary of emamectin benzoate (EB) and ivermectin (IVM) 10-d LC50 values for *N. virens* at each combination of chemical residence time (0-, 2-, and 4-months) and organic carbon (0-, 0.5-, and 1-OC; relative scale) [Time-OC]. The 0-, 0.5-, and 1-OC organic carbon (OC)

treatments used sediments with total OC contents of < 0.05, ~ 0.2, and 0.42%, respectively. All LC50 values are in µg / kg of sediment (wet weight) and were calculated using nominal concentrations for EB and IVM. 95% upper and lower confidence limits are in brackets.

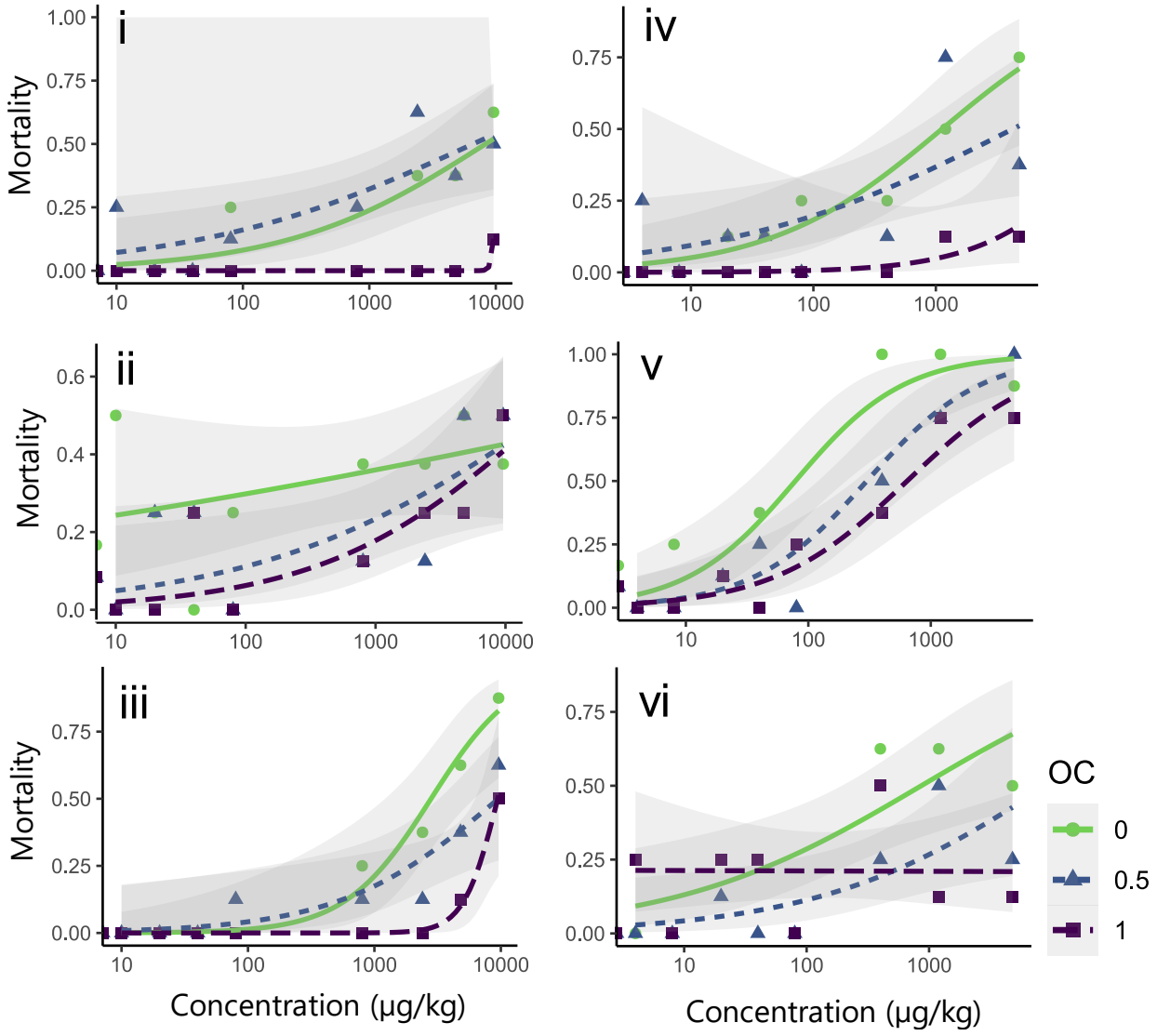


Figure 9: *N. virens* emamectin benzoate concentration-response curves for all 3 organic carbon (OC) levels (refer to legend) at (i) 0-, (ii) 2-, and (iii) 4-months of months of chemical residence; ivermectin concentration-response curves at (iv) 0-, (v) 2-, and (vi) 4-months of chemical residence time. The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Concentration is in µg/kg (w.w. sediment). Each data point represents the observed mortality proportion. Lines represent binomial logistic regression models fitted to data (used to calculate LC50 values). Shaded grey regions represent 95% confidence bands of logistic regression curves.

Combination (EB/IVM) exposures with *E. estuarius* showed a concentration-dependent trend of increasing mortality, as with the single chemical exposures. This trend depended on the chemical residence time and OC treatment combination. Concentration significantly influenced mortality in 8 out of 9 combinations of chemical residence time and OC. There was a statistically significant interaction between concentration and chemical residence time for each OC treatment ($p<0.0001$ for all 3 OC levels; Figure 19). Therefore, the effect of concentration on mortality was assessed for each chemical residence time separately, instead of pooling data across chemical residence times; this was done for each OC level. For the 0- and 0.5-OC group, concentration had a significant effect on mortality for all 3 chemical residence time treatments ($p<0.0001$ for all 3 chemical residence times for both OC levels; Figure 19 i and ii). For the 1-OC group, concentration had a highly significant effect on mortality only for the 0 and 2 months of chemical residence time treatments ($p<0.0001$ for both; Figure 19 iii); at 4 months of chemical residence time, concentration did not have a significant effect on mortality ($p=0.1796$; Figure 19 iii).

For combination exposures with *N. virens*, mortality was significantly different between concentration treatments for 0-, 2-, and 4-months of chemical residence time ($p<0.0001$, $p<0.0001$, and $p=0.004209$, respectively; Figure 10). Overall, there was a concentration-dependent increase in mortality. Paired comparisons show that mortality in the highest concentration group (i.e. 2400/400) was significantly greater than the control group at all chemical residence time treatments. At 4-months of chemical residence time the 80/40 group had significantly higher mortality than the control group. No other groups were significantly different than the control group. The reason this data was grouped by chemical residence time was due to a statistically significant interaction between concentration and chemical residence time ($p=0.02595$).

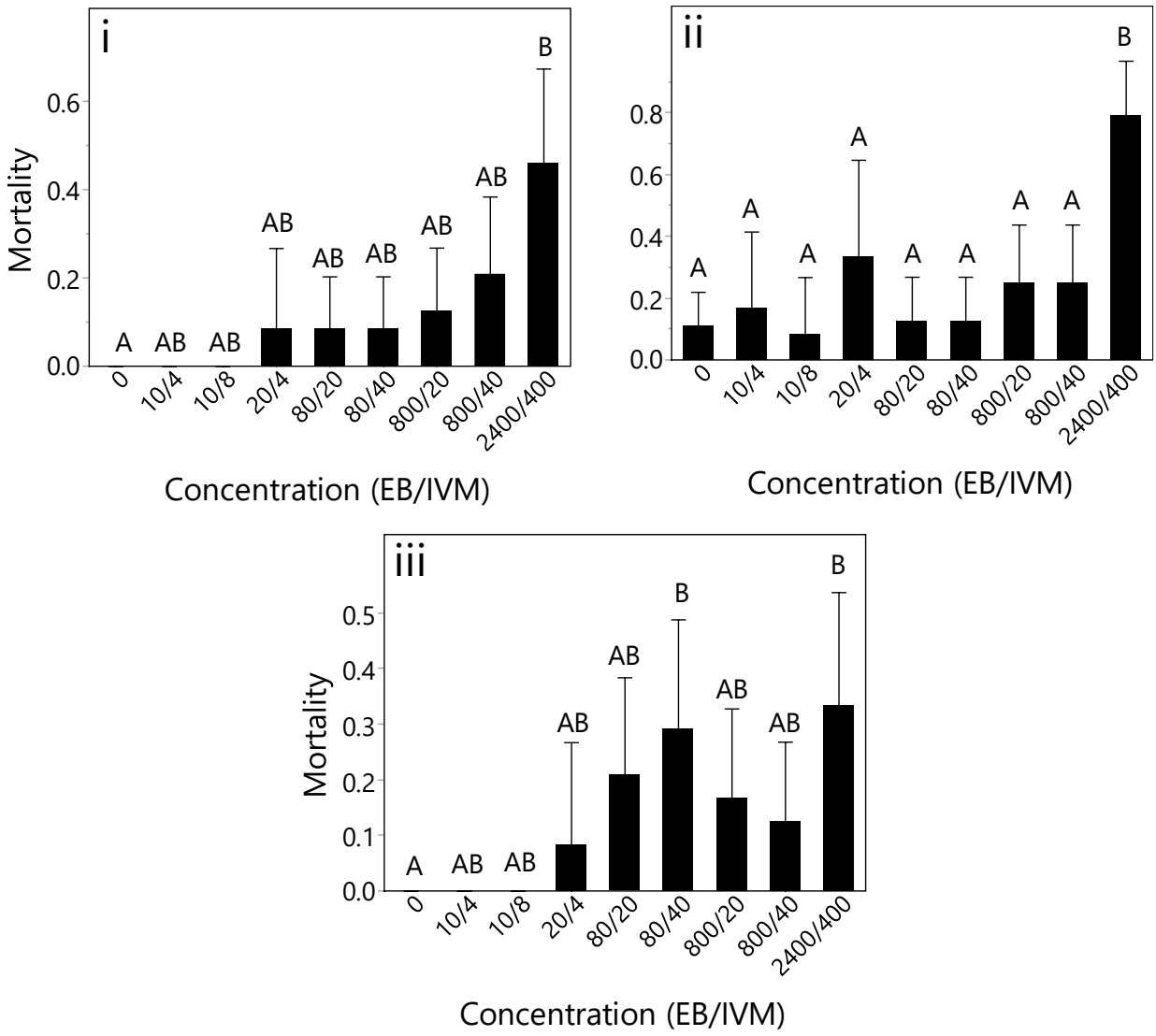


Figure 10: The effect of concentration (µg/kg) on lethal toxicity in *N. virens* exposed to a combination of emamectin benzoate (EB) and ivermectin (IVM). Concentrations are expressed as the concentration of EB / concentration of IVM. The proportion dead (± 95% confidence intervals) is represented for each concentration treatment (i) 0-, (ii) 2-, and (iii) 4-months of chemical residence time. Mortality data is pooled across all organic carbon (OC) treatments. Data grouped by chemical residence time treatments due to a significant interaction between concentration and chemical residence time. Letters above error bars indicate statistical significance; if a letter is shared between groups, they are not statistically different (α=0.05).

2.3.4. Lethal toxicity: the effect of organic carbon

There is some evidence to suggest sediment OC influenced EB-induced mortality in both *E. estuarius* and *N. virens*. For *N. virens*, the mortality proportion, pooled across all EB concentrations, tended to be lower at higher OC content for all chemical residence times (Figure 12). However, this reduction in mortality was not significant in most cases. Since there was a statistically significant interaction between OC and chemical residence time ($p=0.0095$), each factor could not be assessed for their effect on mortality without considering the level of the other factor. Therefore, pairwise comparisons were performed between all 9 chemical residence time and OC combinations, instead of direct comparisons between the 3 OC levels (Figure 12). The mortality proportion in Figure 12 represents the number of dead *N. virens* divided by the number of observations, pooled across all concentrations (from the control to the highest concentration), for each of the 9 chemical residence time and OC treatment combinations. Paired comparisons were made using the binomial logistic regression model described in *Section 2.2.7*. When comparing across OC levels, but within chemical residence time treatments, there are only 2 pairs of treatment groups which are statistically significantly different from each other: the 0-0.5 group has a significantly greater mortality than the 0-1 group ($p=0.0421$), and the 4-0 group has a significantly greater mortality than the 4-1 group ($p=0.0349$). For *E. estuarius*, based on paired comparisons of LC50s, there is less evidence than with *N. virens* exposures, to suggest that OC reduces mortality. When comparing across OC levels, but within chemical residence time treatments, there were only 2 LC50 values which were statistically different from each other: the 2-0.5 group had a significantly higher LC50 value compared to that for the 2-0 group (Figure 11 i, ii, iii). To summarize, OC's protective effect against EB-induced lethal toxicity is not particularly pronounced in *N. virens*, and even less so in *E. estuarius*.

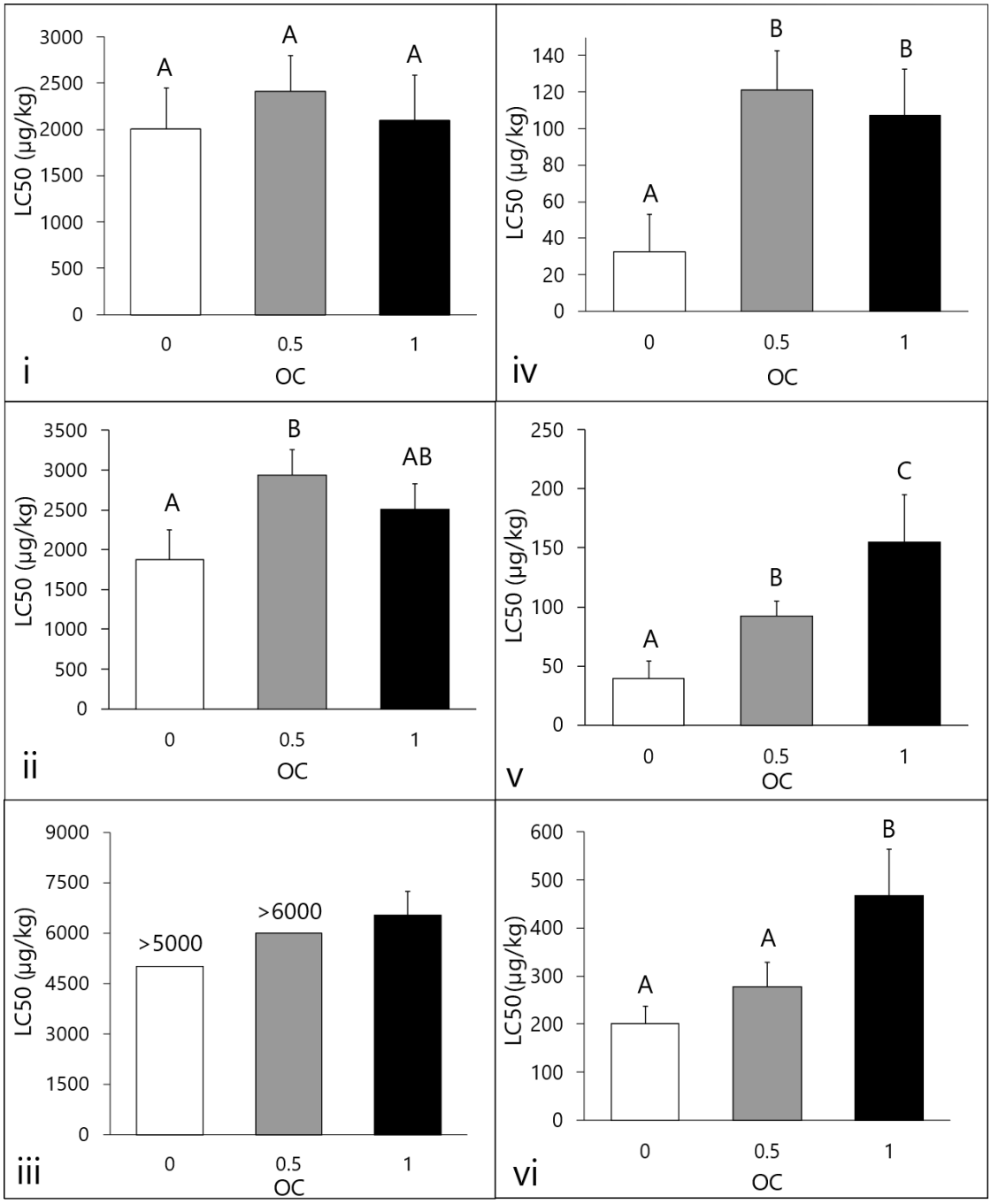


Figure 11: The effect of organic carbon (OC) on lethal toxicity in *E. estuarius* for each chemical residence times in single-chemical exposures. Lethal toxicity is represented by 48-h LC50 values (using nominal concentrations) in µg/kg w.w. sediment. The 48-h LC50 values for emamectin benzoate are shown for each OC level (0-, 0.5-, and 1-OC) for (i) 0-, (ii) 2-, and (iii) 4-months of chemical residence time. The 48-h LC50 values for ivermectin are shown for each OC level for (iv) 0-, (v) 2-, and (vi) 4-months of chemical residence time. The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Error bars represent 95% confidence intervals. Letters above error bars indicate statistical significance; if a letter is shared between groups, they are not significantly different from each

other (Bonferroni adjusted α=0.00172). In cases where the LC50 estimate is greater than the highest concentration tested (i.e. non-estimable), this is indicated above the bars (e.g. panel iii, 0- and 0.5-OC).

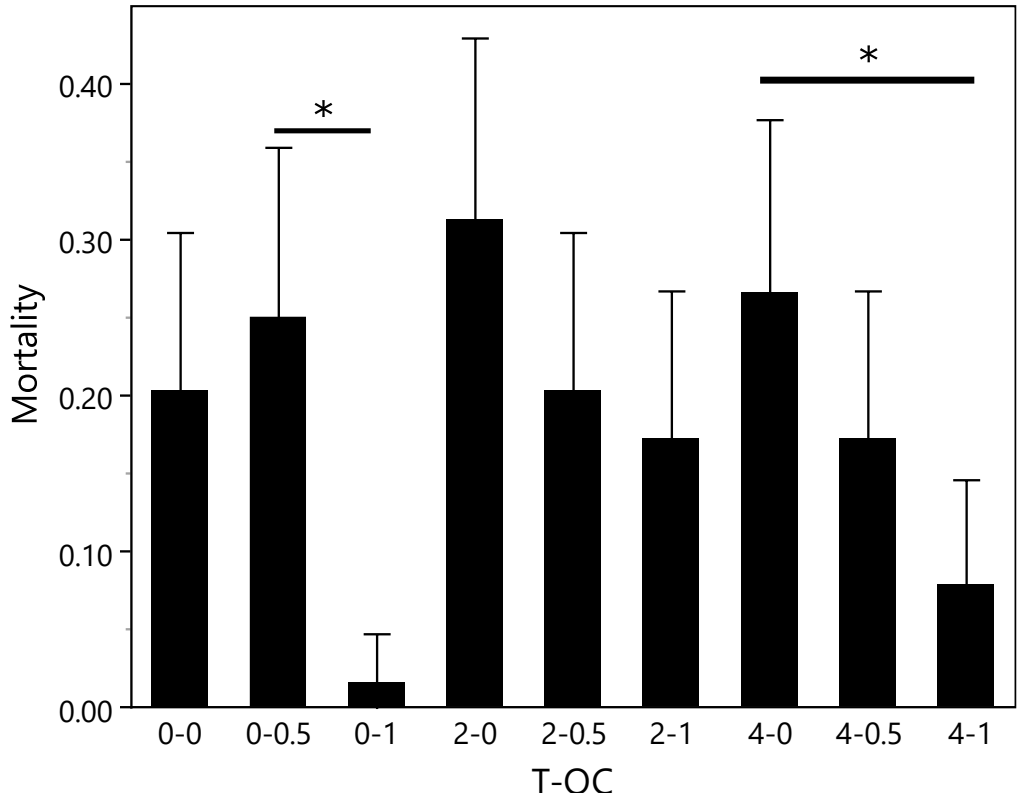


Figure 12: Summary of effects of organic carbon (OC) and chemical residence time (months) on mortality in *N. virens* exposed to emamectin benzoate. Mortality, expressed as a proportion (± 95% confidence intervals), is shown for each chemical residence time and organic carbon (T-OC) combination (e.g. 2-0 corresponds to 2 months of chemical residence time and 0-OC sediment). Mortality data is pooled across all concentrations for each T-OC treatment group. The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Due to a significant interaction between OC and chemical residence time, the effects of these 2 factors on mortality cannot be assessed on their own. * Statistically significant difference in mortality, only between these two T-OC groups ($p<0.05$).

IVM-induced lethal toxicity was significantly reduced in the two highest OC levels (0.5- and 1-OC), relative to the lowest OC level (0-OC) in both *E. estuarius* and *N. virens*. For *N. virens*, OC did not significantly interact with chemical residence time or concentration (p=0.11428 and 0.09301, respectively). Therefore, the main effect of OC on mortality can be assessed on its own

without consideration of chemical residence time or concentration. There was a decrease in mortality from 0- to 1-OC (Figure 13). The mortality proportion in Figure 13 shows the number of dead *N. virens* divided by the number of observations, pooling data across all chemical residence time (0- to 4-months) and concentrations (control to highest), for each OC level. The 0-OC group had significantly higher mortality than the 0.5- and 1-OC groups ($p=0.0336$ and 0.0001, respectively). The latter two groups were not significantly different from each other ($p=0.1408$). For *E. estuarius*, when comparing across OC levels, but within chemical residence times, the LC50 values for IVM at the 0-OC level were significantly lower than those at either the 0.5- or 1-OC level, or both, depending on the chemical residence time (Figure 11 iv, v, vi). The 1-OC group's LC50 values ranged between 2.3- to 3.9-fold higher than the 0-OC group depending chemical residence time. The 0.5-OC group's LC50 values ranged between 1.4- to 3.7-fold higher than the 0-OC group depending on chemical residence time. At 0- and 2-months of chemical residence time, the 0-OC group had a significantly lower LC50 than the 0.5- and 1-OC groups. At 4 months of chemical residence time, the LC50 of the 0-OC group was significantly lower than the LC50 of the 1-OC group, but not significantly different than the LC50 of the 0.5-OC group. Overall, evidence demonstrating OC's protective effect against lethal toxicity is much more convincing in IVM exposures compared to EB exposures.

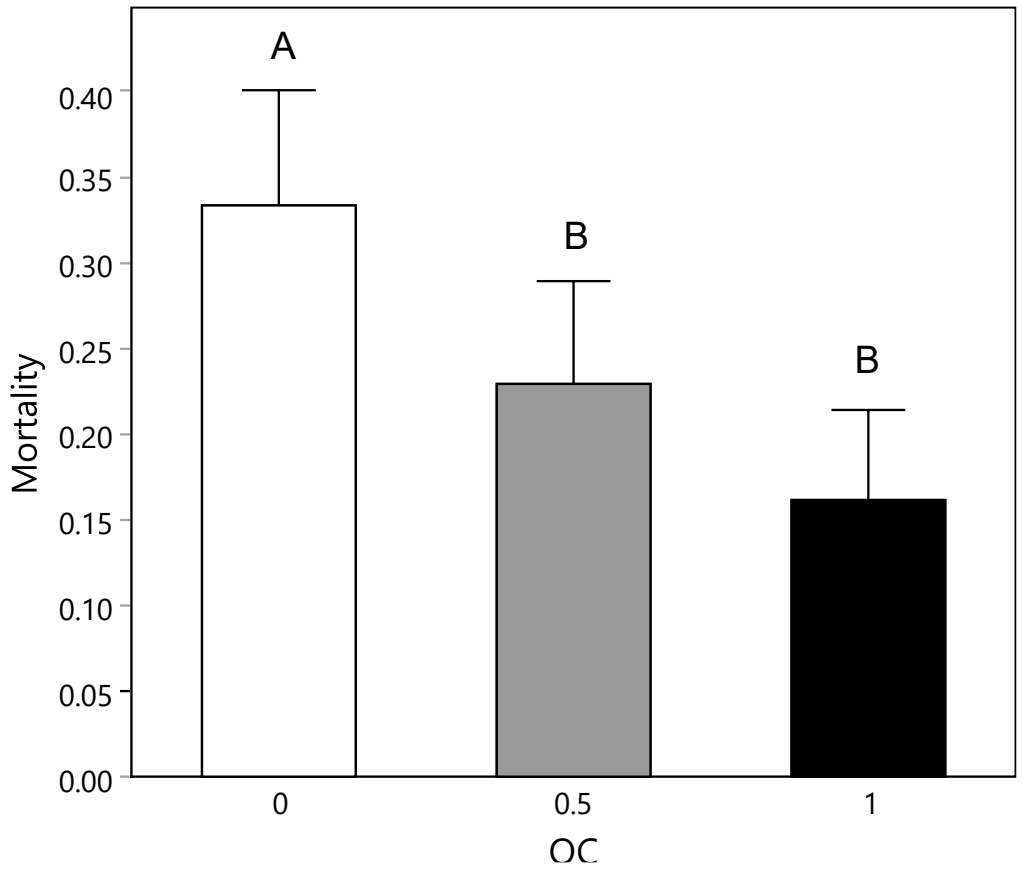


Figure 13: The effect of organic carbon (OC) (0-, 0.5-, and 1) on lethal toxicity after 10 d exposures to ivermectin in *N. virens.* The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Mortality is represented as a proportion for each OC treatment. Mortality data is pooled across all chemical residence time and concentration treatments for each OC level. Error bars represent 95% confidence intervals. Letters above error bars indicate statistical significance; if a letter is shared between groups, they are not statistically different (α=0.05).

OC mitigated lethal toxicity in combination exposures for both *E. estuarius* and *N. virens*. In *E. estuarius*, OC's effects could only be evaluated for a subset of 3 combination concentrations as explained in *Section 2.2.7* (Figure 14). Due to a significant interaction between OC and chemical residence time, OC's effects were evaluated at each chemical residence time, separately. It is evident that at 0- and 2-months of chemical residence time, OC reduces lethal toxicity (Figure 14). At 0 months of chemical residence time, mortality in the 0-OC group was significantly greater than in the 1-OC group at both the 50/5 and 1000/25 combination concentrations (EB/IVM); the 0-OC group also had a significantly greater mortality than the 0.5-OC group at the 50/5 concentration (Figure 14 i). At 2 months of chemical residence time mortality in the 0-OC group was significantly greater than both the 0.5- and 1-OC groups (Figure 14 ii). Due

to a lack of significant lethal toxicity for any treatment group at 4-months, OC's effects on toxicity cannot be determined at this chemical residence time treatment (Figure 14 iii). In *N. virens* combination exposures, OC had a highly significant effect on mortality proportion ($p<0.0001$; Figure 15). There was significantly lower mortality in the 1-OC group than the 0- and 0.5-OC groups ($p<0.0001$ and $p=0.0334$, respectively). The overall trend is a significant decrease in lethal toxicity with increasing OC in *N. virens* and *E. estuarius* exposures, as seen in the IVM single chemical exposures.

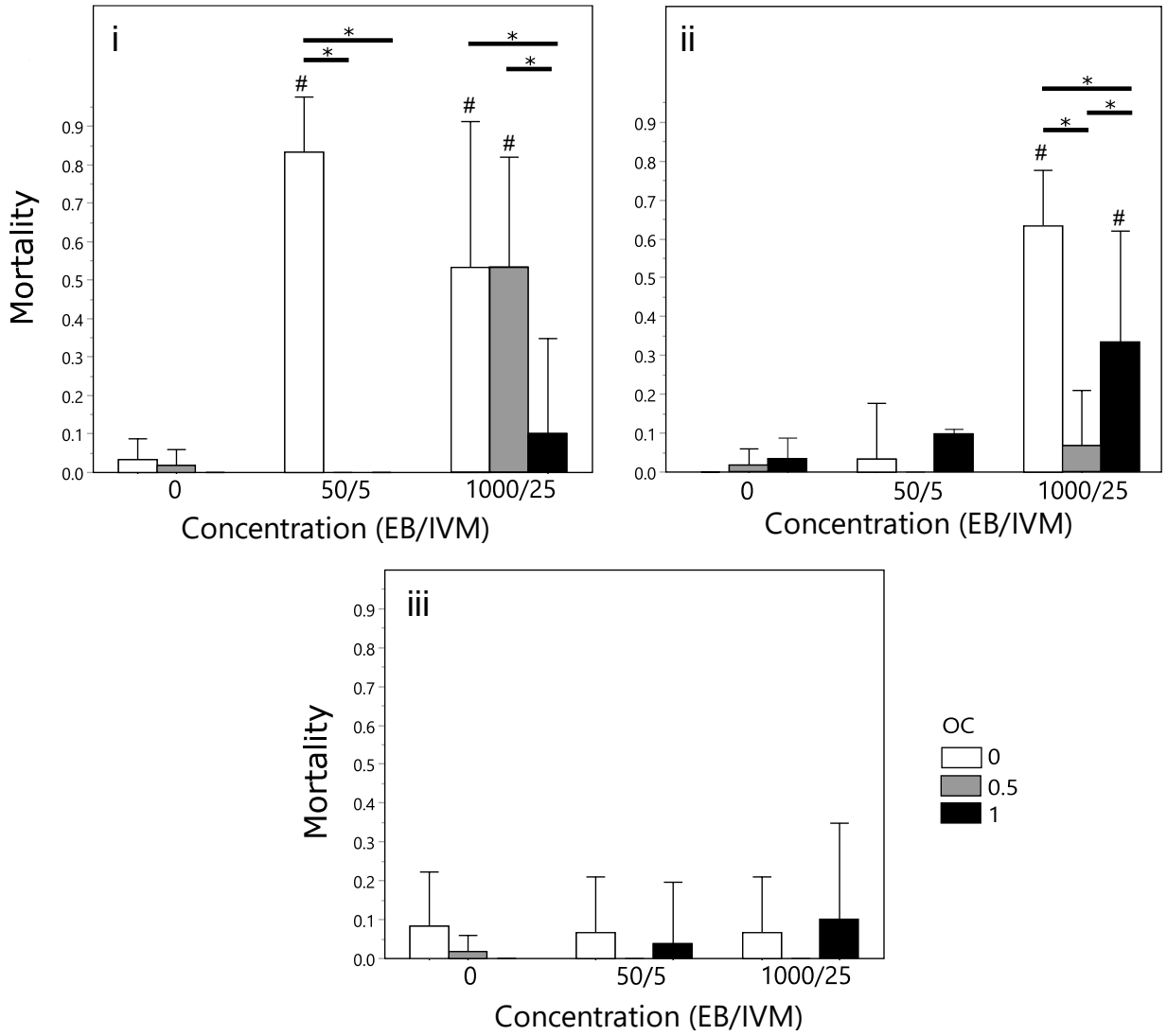


Figure 14: the effect of organic carbon (OC) on lethal toxicity in *E. estuarius* exposed to a combination of emamectin benzoate (EB) and ivermectin (IVM) at (i) 0-, (ii) 2-, and (iii) 4-months of chemical residence time. Concentration is in µg/kg (EB/IVM). Bars represent the mean proportion dead (± 95% confidence intervals). The 3 OC levels (0-, 0.5-, and 1-OC) are represented by bars of different colors on a grayscale (refer to figure legend). The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively.

*Significant differences between OC groups at each concentration are represented are represented by lines above error bars (Tukey HSD, α=0.05). Hashtags (#) denote statistical differences from the control of the same OC treatment.

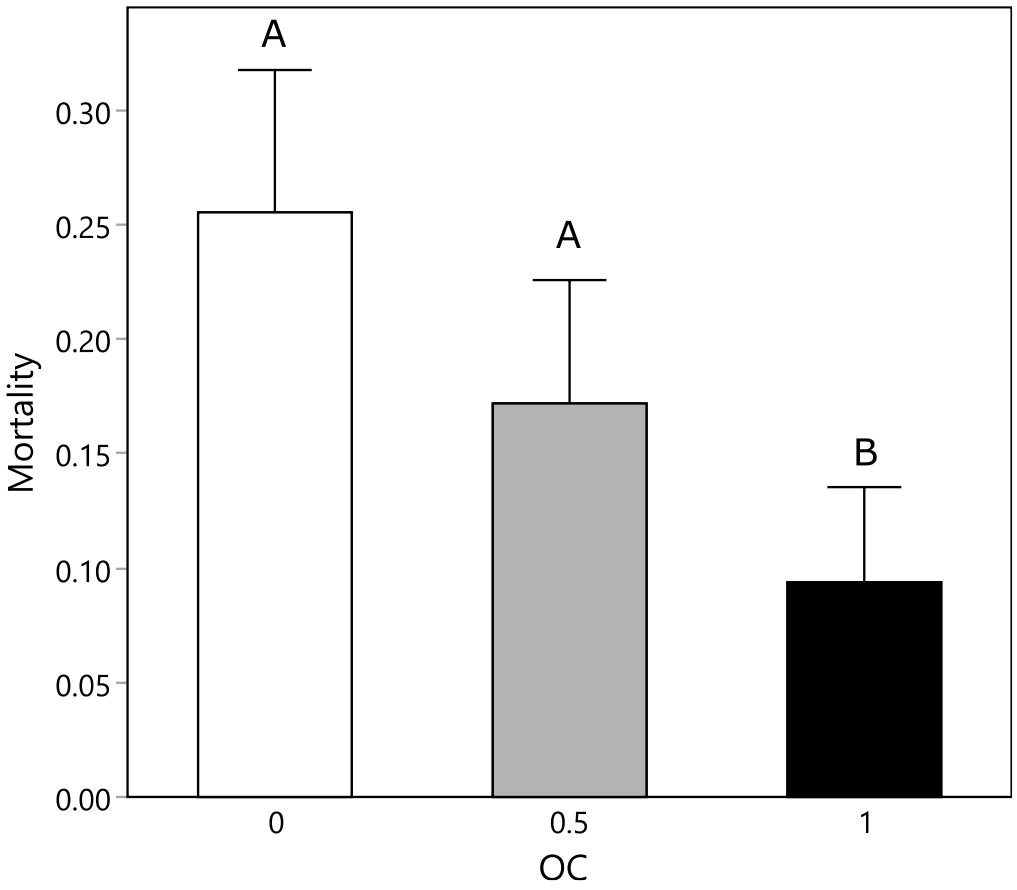


Figure 15: The effect of organic carbon (OC) on lethal toxicity in *N. virens* exposed to a combination of emamectin benzoate and ivermectin. Mortality is expressed as a proportion (± 95% confidence intervals) for each OC treatment (0-, 0.5-, and 1-OC). The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Mortality data is pooled across all concentration and chemical residence time treatments. Letters above error bars indicate statistical significance; if a letter is shared between groups, they are not statistically different (α=0.05).

2.3.5. Lethal toxicity: the effect of chemical residence time

The effect of chemical residence time on EB-induced mortality differed between *E. estuarius and N. virens*. For *N. virens*, chemical residence time did not influence mortality; when comparing across chemical residence time and within OC levels, no significant difference was

found between any groups (Figure 12). On the other hand, based on 48-h LC50 values for *E. estuarius*, 4 months of chemical residence time was associated with a significant reduction in EB-induced mortality (Figure 16 i, ii, iii). At 4 months of chemical residence time, there was an increase in LC50 values relative to the 0- and 2-month chemical residence time groups; these latter two groups were not significantly different at any OC treatments. When comparing across chemical residence times, but within OC treatments, there were two pairs of groups that were significantly different: at 1-OC, the 4 months of chemical residence time treatment had a 3.1- and 2.6-fold greater (both significant) LC50 value than both the 0- and 2-month groups groups, respectively. Within the 0- and 0.5-OC levels, a statistical comparison could not be made with the 4-month group due to the LC50 values being non-estimable (>5000 and >6000 µg/kg, respectively). However, within the 0-OC group, the 4-month chemical residence time treatment had an LC50 value that is > 2.5- and > 2.7-fold larger than the 0- and 2-month groups, respectively. Within the 0.5-OC group, the 4-month treatment had an LC50 value that is > 2.5- and > 2.1-fold larger than the 0- and 2-month groups, respectively. Therefore, the overall trend is a significant decline in lethal toxicity at 4 months of chemical residence time for *E. estuarius*.

The effect of chemical residence time on IVM-induced mortality also differed between *E. estuarius* and *N. virens* exposures. For *E. estuarius* the trend remained the same as the EB exposures: there was a significant decline in IVM's lethal toxicity at 4 months of chemical residence time (Figure 16 iv, v, vi). When comparing across chemical residence times, but within OC levels, no significant differences were found between the 0- and 2-month groups. However, for all OC levels, the 4-month group had significantly higher LC50 values than the 0- and 2-month groups. For example, within the 0-OC group, the 4-month group had a significantly greater LC50 than both the 0- and 2-month groups; this was also the case for the 0.5- and 1-OC groups. The difference between the LC50 values at 4 months of chemical residence time compared to 0- and 2-months, within OC levels, ranged between 2.3- to 6.2-fold. On the other hand, for *N. virens*, the trend differed between EB and IVM exposures: for IVM exposures, chemical residence time had a highly significant effect on mortality ($p<0.0001$), with 2-months of chemical residence time being associated with a significantly higher mortality than both the 0- and 4-month groups ($p<0.0001$ and $p=0.0003$, respectively; Figure 17). Mortality in the 0- and 4-month groups were not significantly different ($p=0.3023$). Since there was a significant interaction between chemical residence time and concentration ($p=0.0002$), the effect of chemical residence time cannot be generalized to all concentrations of IVM. To summarize, in *E. estuarius*, only after 4 months of chemical residence time in EB- and IVM-exposures was lethal toxicity significantly mitigated. On the other hand, in *N. virens*, lethal toxicity was unaffected by chemical residence time of EB

exposures, but for IVM exposures 2 months of chemical residence time was associated with a significant increase in lethal toxicity relative to the 0- and 4-month groups.

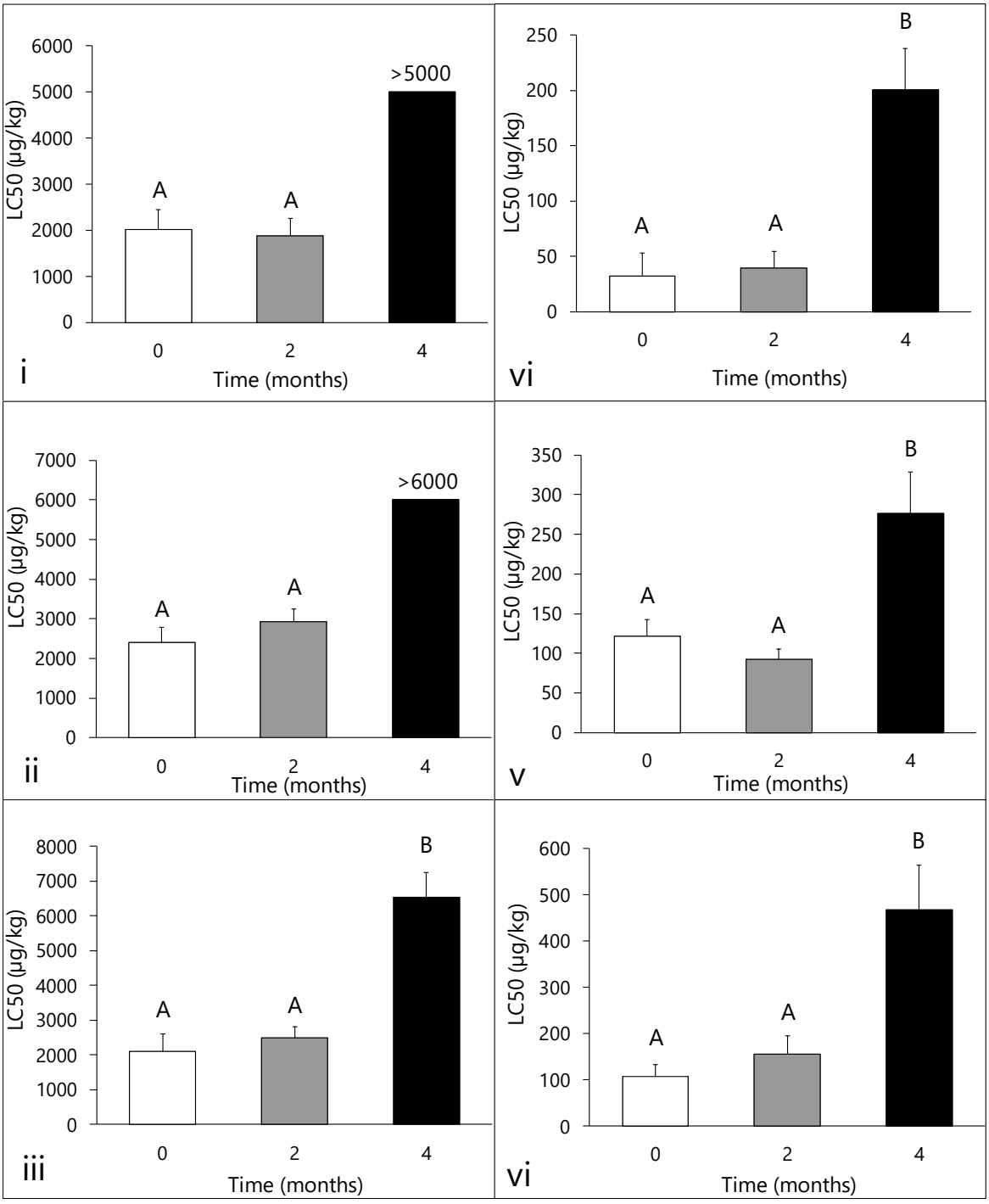


Figure 16: The effect of chemical residence time (months; x-axis) on lethal toxicity in *E. estuarius* for each organic carbon (OC) level in single-chemical exposures. Lethal toxicity is represented by

48-h LC50 values (using nominal concentrations) in µg/kg w.w. sediment. The 48-h LC50 values for emamectin benzoate are shown for each chemical residence time (0-, 2-, and 4-months) for (i) 0-, (ii) 0.5-, and (iii) 1-OC treatments. The 48-h LC50 values for ivermectin are shown for each chemical residence time for (iv) 0-, (v) 0.5-, and (vi) 1-OC treatments. The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Error bars represent 95% confidence intervals. Letters above error bars indicate statistical significance; if a letter is shared between groups, they are not significantly different from each other (Bonferroni adjusted α=0.00172). In cases where the LC50 estimate is greater than the highest concentration tested (i.e. non-estimable), this is indicated above the bars (e.g. panel i and ii, 4-months of chemical residence time).

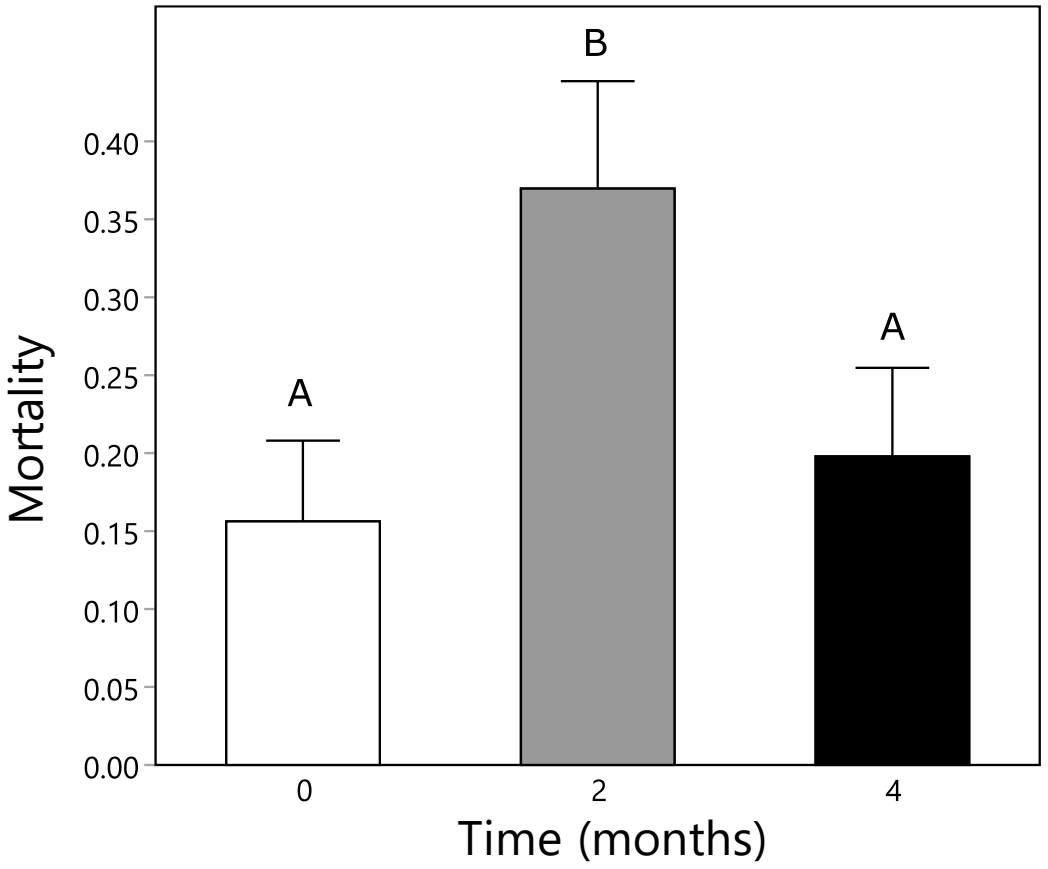


Figure 17: The effect of chemical residence time (0-, 2-, and 4-months) on lethal toxicity after 10 d of exposure to ivermectin in *N. virens*. Mortality expressed as a proportion, pooled across organic carbon (OC) and concentration treatment levels, is shown for each chemical residence time treatment. Error bars represent 95% confidence intervals. Letters above error bars indicate statistical significance; if a letter is shared between groups, they are not statistically different (α=0.05).

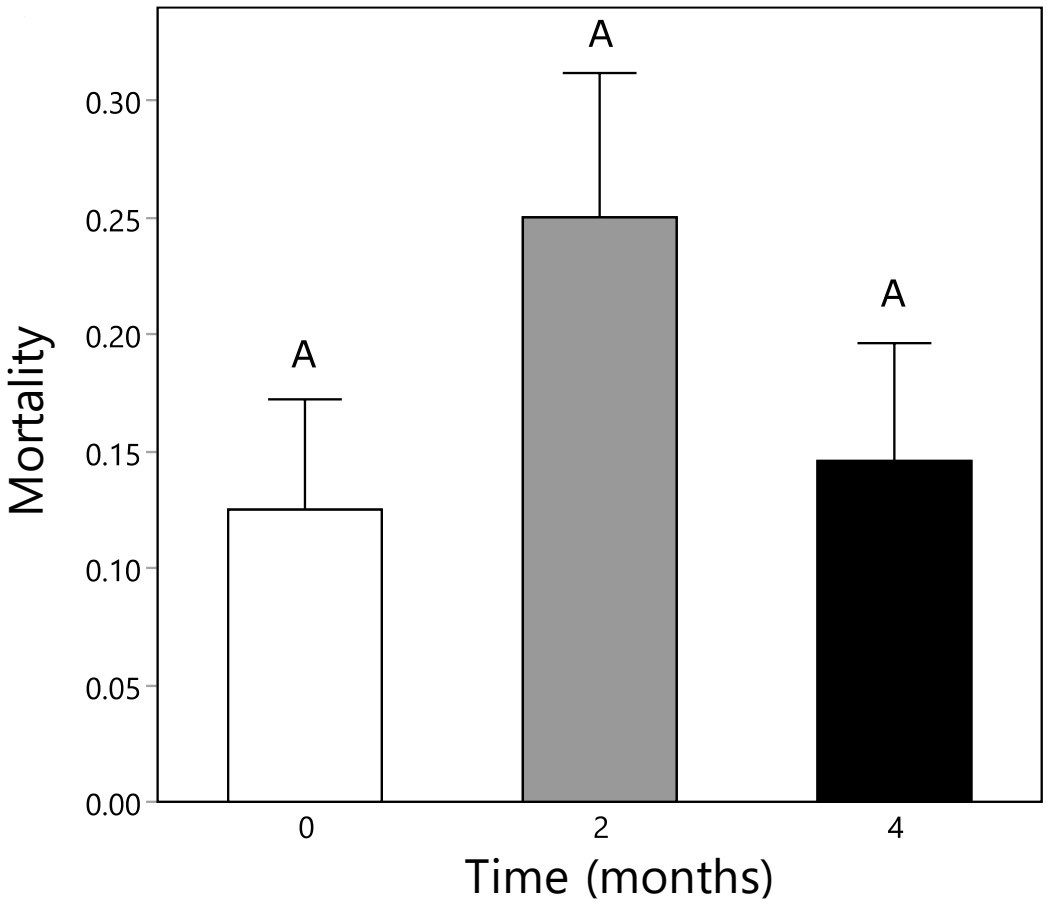


Figure 18: The effect of chemical residence time on lethal toxicity in *N. virens* after 10 d exposures to a combination of emamectin benzoate and ivermectin. Mortality, expressed as a proportion, is shown for each chemical residence time treatment (0-, 2-, and 4-months). Mortality data is pooled across all concentrations and organic carbon (OC) treatments. Error bars represent 95% confidence intervals. Letters above error bars indicate statistical significance from pairwise comparisons; if a letter is shared between groups, they are not statistically different (α=0.05).

In *E. estuarius* combination exposures, the main effect of chemical residence time on mortality could not be determined since there was a significant interaction between chemical residence time and treatment concentrations for all 3 OC levels ($p<0.0001$ for all 3). However, at 0-, 0.5-, and 1-OC there exists a significant difference in mortality between chemical residence time treatments in 5, 4, and 6 out of the 8 combination concentrations, respectively (Figure 19). This suggests that chemical residence time influenced mortality in the combination exposures. The overall trend appears to be that avermectin-induced mortality decreased as chemical residence time increased, showing a pronounced difference in mortality at 4 months of chemical residence time relative to the 0- and 2-month groups; this was a similar finding to single chemical exposures with *E. estuarius*.

For *N. virens*, chemical residence time had a significant effect on lethal toxicity in combination exposures (p=0.00072; Figure 18). The interaction between chemical residence time and concentration was not statistically significant (p=0.051); therefore, these results can be generalized to all concentration treatments. Two months of chemical residence time was associated with the highest mortality compared to the 0- and 4-months of chemical residence time treatments. However, pairwise comparisons between chemical residence time treatments did not reveal any statistically significant differences.

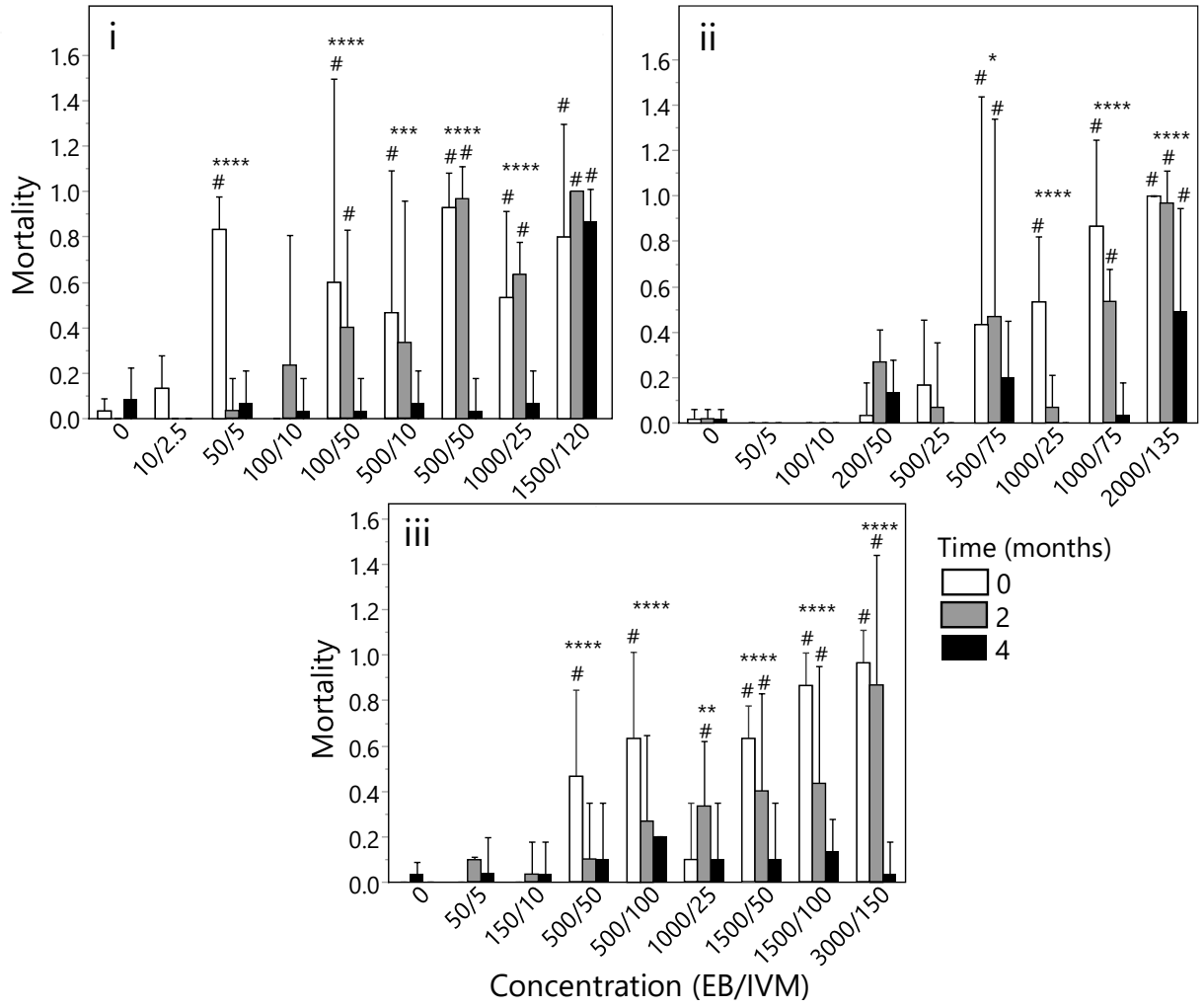


Figure 19: The effect of chemical residence time and concentration on lethal toxicity in combination exposures with *E. estuarius* at the 3 organic carbon (OC) levels: (i) 0-, (ii) 0.5-, and (iii) 1-OC. The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Concentrations (emamectin benzoate / ivermectin) are in µg/kg (w.w.). Mortality is expressed as a proportion. Data points represent mean mortality proportion (± 95% confidence intervals). Bar shadings (white, gray, or black) correspond to different chemical

residence time treatments (0-, 2-, and 4-months; refer to figure legend). Asterisks (*) indicate statistical difference between chemical residence time treatments within the same concentration treatment (*p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001). Hashtags (#) indicate statistical difference from the control group within the same chemical residence time.

2.3.6. Sublethal toxicity: concentration-response analysis

Avermectin exposure was inconsistently associated with reduced light-avoidance in *E. estuarius*. For EB single-chemical exposures with *E. estuarius*, 3 out of 9 time-OC treatment combinations showed a significant concentration-dependent decrease in light-avoidance (Figure 21 i, ii, iii and Table B.*1*). For IVM exposures, 5 out of 9 time-OC treatment combinations showed a significant concentration-dependent decrease in light-avoidance (Figure 21 iv, v, vi and Table B.*1*). Due to the poor correlation between concentration and altered light-avoidance, EC50 values were not extracted from curves generated for each chemical residence time and OC treatment combination in EB and IVM exposures (R^2 values given in Figure 21).

For most chemical residence time and OC treatments, avermectin exposure significantly reduced the ability of *N. virens* to burrow (Figure 20). For EB exposures with *N. virens*, 7 out of 9 combinations of chemical residence time and OC showed a significant concentration-dependent decrease in an organisms ability to burrow (Figure 20): at 0 months of chemical residence time, the 0-, 0.5-, and 1-OC groups all showed significant concentration-dependent decreases in an organism's ability to burrow (p=0.0006, <0.0001, and =0.0005, respectively); at 2 months of chemical residence time, the 0- and 0.5-OC groups showed significant concentration-dependent decreases in an organism's ability to burrow (p=0.0109 and 0.0050, respectively), while the 1-OC group did not (p=0.1062); at 4 months of chemical residence time, the 0- and 0.5-OC groups showed significant concentration-dependent decreases in an organism's ability to burrow (p=0.0140 and 0.0022, respectively), while the 1-OC group did not (p=0.6164). For IVM exposures with *N. virens* all 9 combinations of chemical residence time and OC showed significant concentration-dependent decreases in an organism's ability to burrow (Figure 20). Overall, there is clear evidence for EB- and IVM-concentration-dependent decreases in burrowing ability in *N. virens*.

In *E. estuarius*, there is no clear concentration-dependent trend in light-avoidance upon combined exposures to both avermectins (Figure 27 and Figure 28). For the 0- and 0.5-OC groups there was a significant statistical interaction between treatment concentration and chemical

residence time (p=0.0006 and p=0.0308, respectively; Figure 27). Therefore, concentration's effect on light-avoidance behavior was evaluated for each chemical residence time separately. For the 0-OC group, a significant difference between the mean light-avoidance existed between concentrations at the 0-, 2- and 4-months of chemical residence time treatments (p=0.0007, 0.0003, 0.0178, respectively; Figure 27 i). However, for all 3 chemical residence times, the mean light-avoidance did not significantly differ from the control group for any of the treatment concentrations (Figure 27 i). For the 0.5-OC group, a significant difference between mean light-avoidance existed between concentrations at the 0-months of chemical residence time treatment (p=0.0002), but not at the 2- or 4-months of chemical residence time treatments (p=0.0629 and 0.1049, respectively; Figure 27 ii). However, the mean light-avoidance did not significantly differ from the control group for any treatment concentration within 0-months of chemical residence time. Due to the lack statistically significant difference in light-avoidance from control groups within the same chemical residence time treatment at all treatment concentrations, there is no evidence for concentration-dependent changes in light-avoidance at 0- and 0.5-OC.

In the 1-OC treatment, concentration and chemical residence time did not interact (p=0.0782; Figure 28). Therefore, main effects of these factors could be evaluated on their own. Concentration did have a significant effect on light-avoidance (p=0.0010; Figure 28). Furthermore, two of the higher concentration combinations (500/100 and 1500/50) were significantly greater than two of the lower concentration combinations (50/5 and 150/10) (Tukey HSD; $p<0.05$). However, no concentrations were significantly different than the control group (Tukey HSD; $p<0.05$), therefore there is no evidence to suggest a concentration-dependent change in light-avoidance in the 1-OC treatment. Overall, there is no clear concentration-dependent trend of increasing or decreasing light-avoidance in any of the 3 OC treatments.

In *N. virens* combination exposures, concentration had a highly significant effect on burrowing behavior ($p<0.0001$). There is a clear concentration-dependent trend of increasing proportion unburrowed as concentration increases (Figure 23). No significant interaction was detected between concentration and OC (p=0.7232) or chemical residence time (p=0.3846). Therefore, the overall effect of concentration does not depend on the levels of chemical residence time or OC.

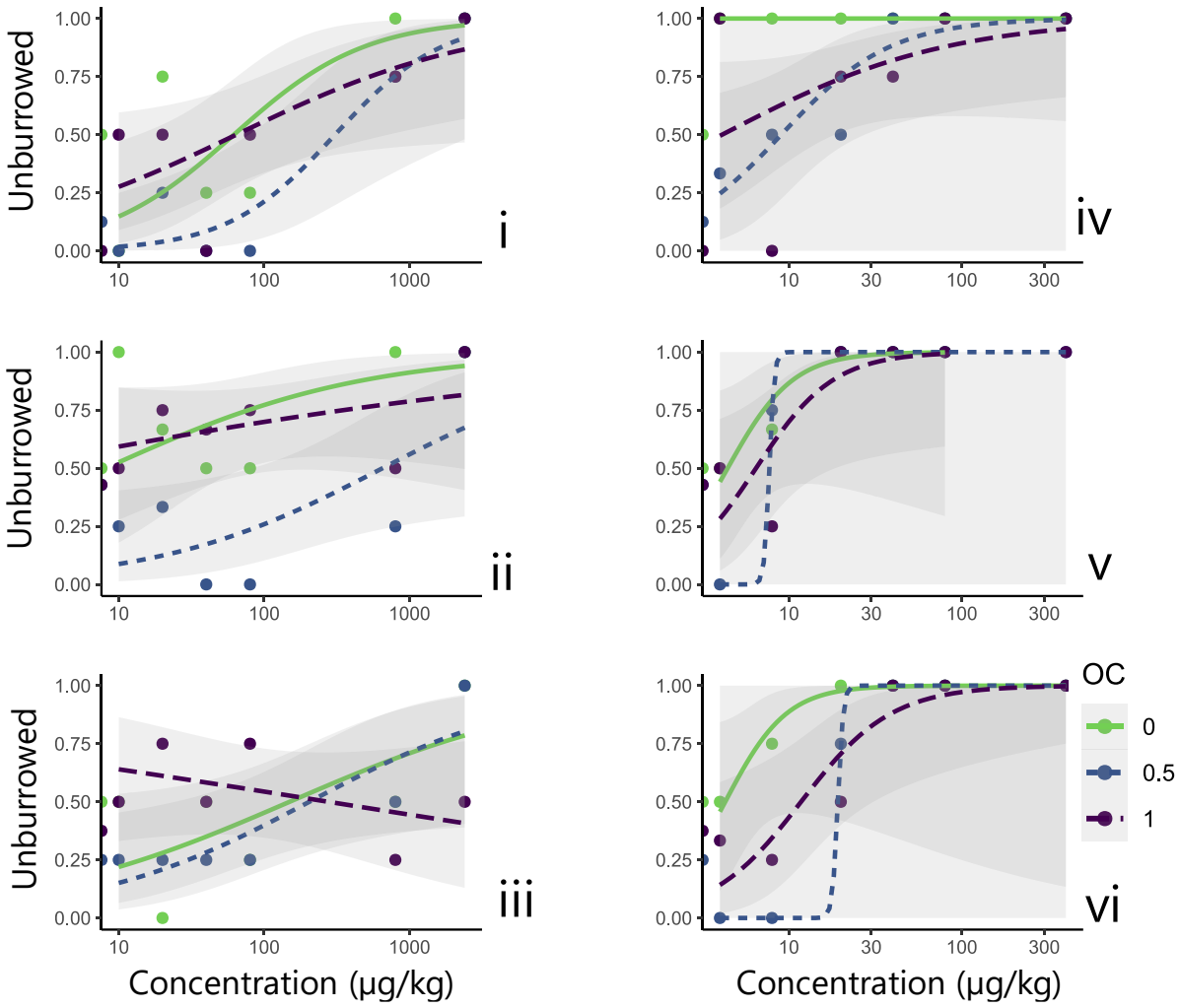


Figure 20: *N. virens* emamectin benzoate concentration-response curves for burrowing behavior in all 3 organic carbon (OC) treatments (refer to figure legend) at (i) 0-, (ii) 2-, and (iii) 4-months of months of chemical residence; ivermectin concentration-response curves at (iv) 0-, (v) 2-, and (vi) 4-months of chemical residence time. The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Concentration is in µg/kg (w.w. sediment). Each data point represents the observed proportion unburrowed. Lines represent binomial logistic regression models fitted to data. Shaded grey regions represent 95% confidence bands of logistic regression curves.

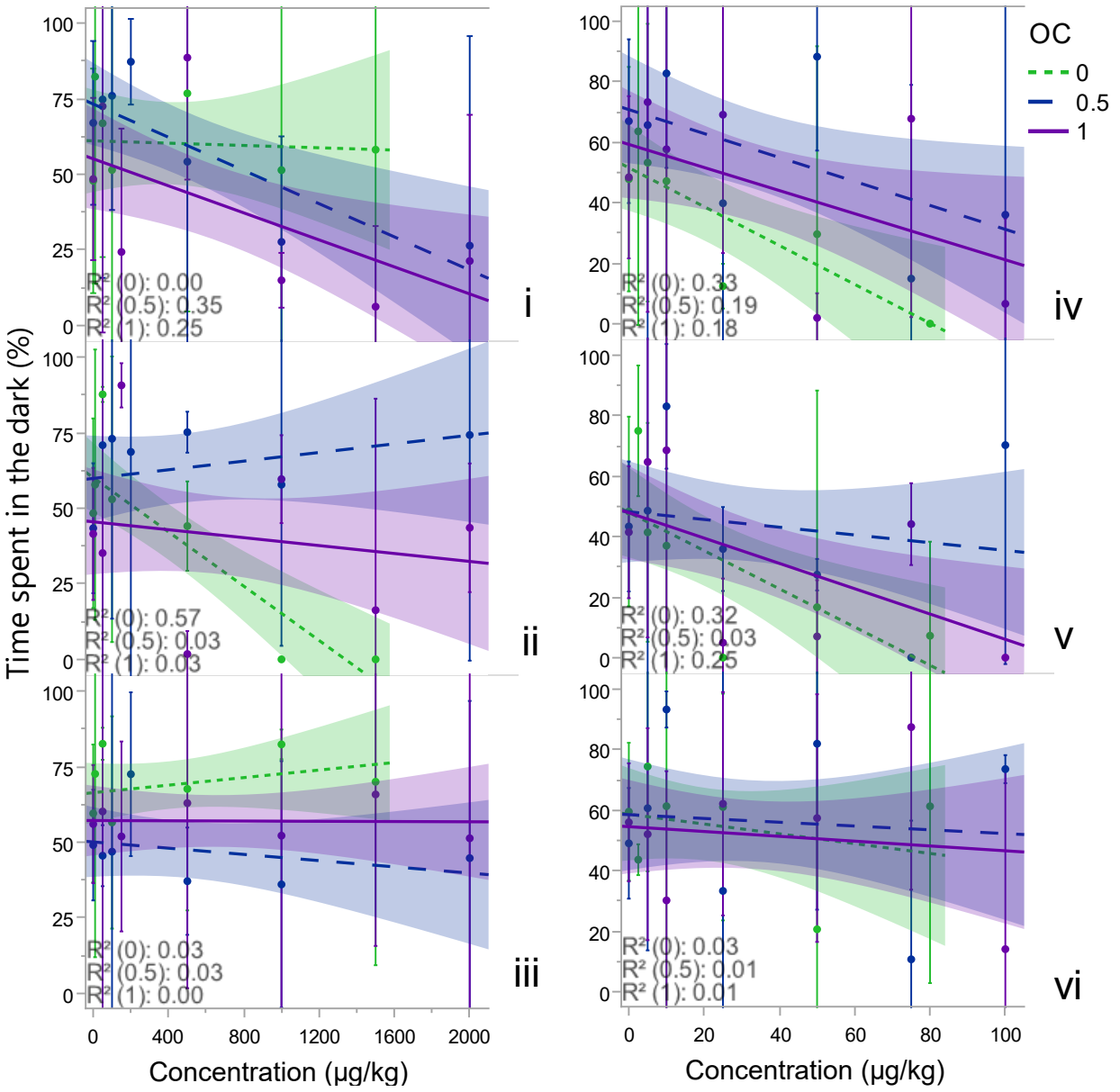


Figure 21: The effect of organic carbon (OC) on *E. estuarius* concentration-response curves for light-avoidance behavior in single-chemical exposures to emamectin benzoate (EB) (i, ii, iii) and ivermectin (IVM) (iv, v, vi). Light avoidance is represented by the percent of time spent in the dark during the light-avoidance behavioral assay. Linear plots are shown for all OC levels (see figure legend) for EB at (i) 0-, (ii) 2-, and (iii) 4-months of chemical residence time; plots for IVM are shown for all OC levels at (iv) 0-, (v) 2-, and (vi) 4-months of chemical residence time. The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Data points represent means (± 95 % confidence intervals). R^2 values for each OC level are provided at the bottom left part of each of the six panels. (E.g. R^2 (0) for panel 'i' represents the R^2 for the linear fit for the EB concentration response curve at 0-OC and 0-months of chemical residence time). ANOVA p-values for associated linear fits are provided in Table B.1 (*Appendix B*).

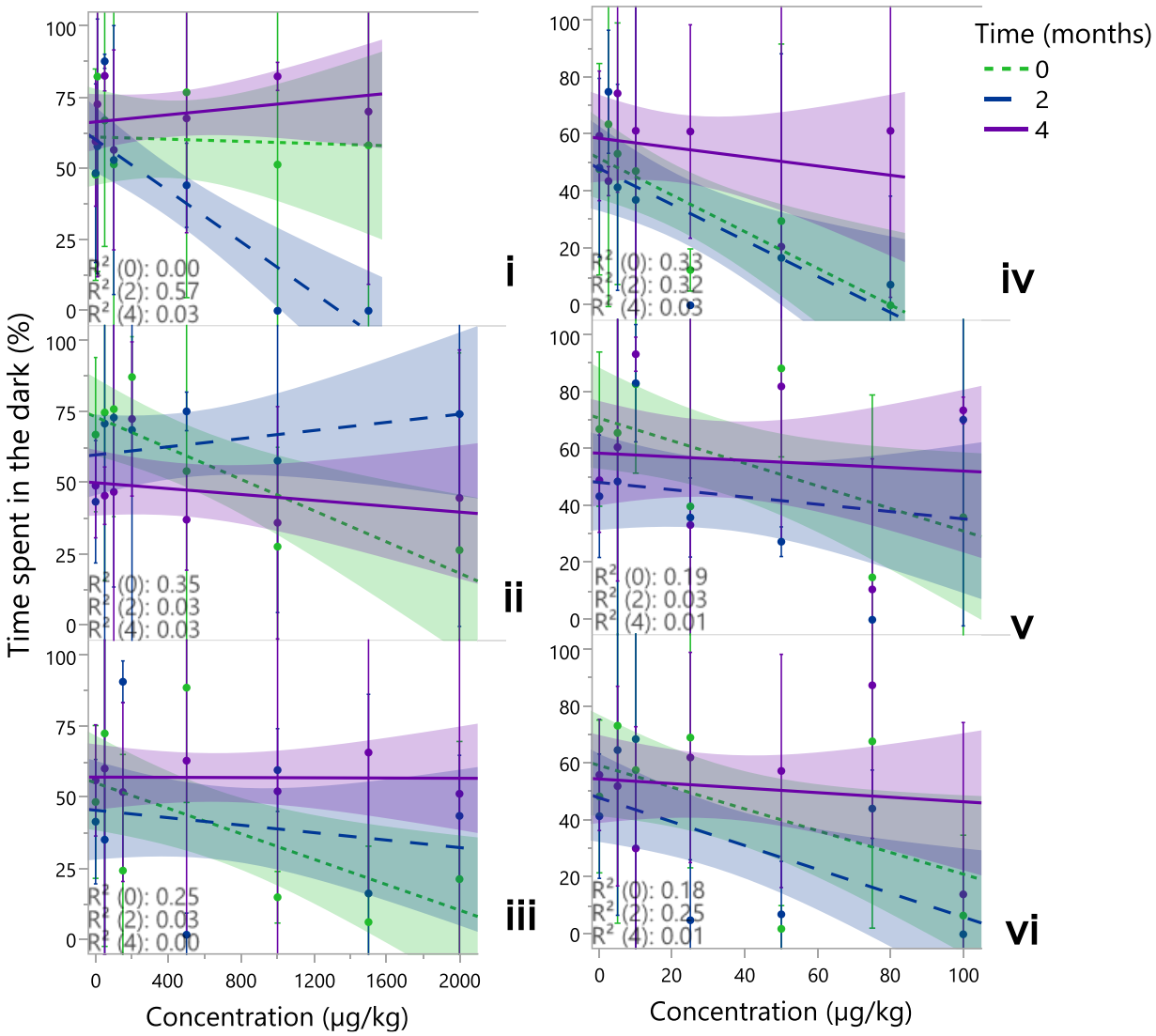


Figure 22: The effect of chemical residence time on emamectin benzoate (EB) (i, ii, iii) and ivermectin (IVM) (iv, v, vi) concentration-response curves for *E. estuarius* light-avoidance assays. Light avoidance is represented by the percent of time spent in the dark (mean ± 95 % confidence intervals) during the light-avoidance behavioral assay. Linear plots are shown for each of the 3 chemical residence times (0-, 2-, and 4-months; refer to figure legend) for EB at the 3 different organic carbon (OC) levels of (i) 0-, (ii) 0.5-, and (iii) 1-OC; plots for IVM are shown for each chemical residence time at (iv) 0-, (v) 0.5-, and (vi) 1-OC. The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. R^2 values for each chemical residence time treatment are provided at the bottom left part of each of the six panels. (E.g. R^2 (2) for panel 'i' represents the R^2 for the linear fit for the EB concentration response curve at 2-months of chemical residence time). ANOVA p-values for associated linear fits are provided in Table B.1 (*Appendix B*).

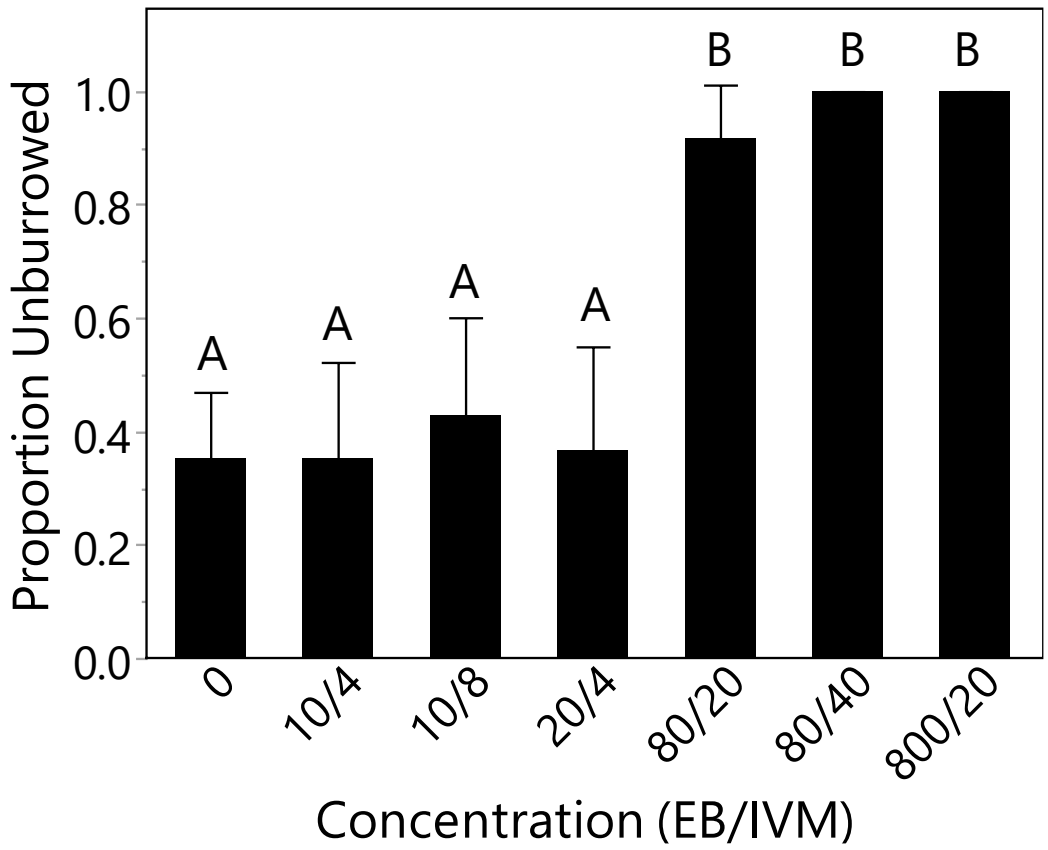


Figure 23: The effect of concentration (emamectin benzoate (EB) / ivermectin (IVM); in µg/kg) on burrowing behavior in exposures where *N. virens* were exposed to a combination of EB and IVM. The proportion of organisms not burrowed after 15 minutes of observation are shown for each concentration. Data is pooled across organic carbon (OC) and chemical residence time treatments for each concentration level. Error bars represent 95% confidence intervals. Letters above error bars indicate statistical significance from pairwise comparisons; if a letter is shared between groups, they are not statistically different (α=0.05).

2.3.7. Sublethal toxicity: the effect of organic carbon

There is evidence to suggest that OC mitigates EB-induced sublethal toxicity in *N. virens* and *E estuarius*. In *E. estuarius* single-chemical exposures, OC's effect on the steepness of the concentration-response curve's negative slope was used to determine how OC influenced sublethal toxicity: if increasing OC made the negative concentration-response slope shallower, it decreased sublethal toxicity; if it made the negative concentration-response slope steeper, it increased sublethal toxicity. In *E. estuarius*, OC affected the concentration-response relationship between EB concentration and light-avoidance at 2 months of chemical residence time; the slopes

of the concentration-response curves are not equal between OC levels (p=0.000718; Figure 21). Pairwise comparison between slopes reveals that the slope of the 0-OC curve is significantly steeper than both the 0.5- and 1-OC groups (p=0.0005 and 0.0099, respectively). The latter two OC groups were not significantly different from each other (p=0.4431). However, at 0- and 4-months of chemical residence time, OC did not significantly affect the slope of the concentration-response curves (p=0.194 and 0.526, respectively). Although, at 4 months of chemical residence time, none of the OC treatments showed a significant concentration-dependent decrease in light-avoidance (Table B.1 in the *Appendix B*). Therefore, toxicity was not observed over the concentration range tested. Due to the absence of toxicity at 4 months of chemical residence time for all 3 OC levels, OC's effect on toxicity should not considered relevant. Overall, depending on chemical residence time, increasing sediment OC content significantly reduced EB-induced sublethal toxicity across the range of concentrations tested.

In *N. virens*, OC's protective effect against EB-induced sublethal toxicity was only observed at the intermediate OC level (0.5-OC). Since there was no statistical interaction (p=0.2933) between OC and chemical residence time, the main effects of OC could be evaluated without considering the level of chemical residence time. OC had a significant effect on *N. virens* ability to burrow (p=0.00093; Figure 24 i), with the intermediate OC level (0.5-OC) being associated with the lowest proportion of unburrowed *N. virens*; EB-induced interference of burrowing ability in *N. virens* was only significantly offset at 0.5-OC (p=0.0105), but not at 1-OC (p=0.3885), relative to the 0-OC group. It should be noted that since OC and concentration have a statistically significant interaction (p=0.0139), the main effect of OC cannot be generalized to all concentrations. Therefore, the magnitude and direction of effect of OC is similar at all EB concentrations tested. Thus the 0.5-OC treatment significantly decreasing the proportion of unburrowed *N. virens* is not the case at all EB concentrations tested. Overall, an intermediate (0.5-OC) treatment of OC significantly offsets EB-induced deficits of burrowing behavior, relative to the 0-OC treatment, but this does not occur at the high-OC (1-OC) treatment.

The protective effect of OC against sublethal toxicity of IVM was only evident in *N. virens*, but not *E. estuarius*. In *E. estuairus*, the slopes of the concentration-response curves for the 3 OC levels were not significantly different at 0-, 2-, or 4-months of chemical residence time (p=0.597, 0.142, 0.932, respectively; Figure 21). This indicates that OC did not have a significant effect on the concentration-response relationship between IVM concentration and light-avoidance. However, as mentioned previously (for EB exposures), since there is no evidence of toxicity at 4 months of chemical residence time, OC's effects on toxicity should not be considered for this

chemical residence time. On the other hand, in *N. virens* IVM exposures, OC had a highly significant effect on burrowing behavior (p=0.00016). The 0.5- and 1-OC groups significantly reduced IVM-induced interference on burrowing behavior relative to the 0-OC group (p=0.0005 and 0.0038, respectively; Figure 24 ii). There was a similar reduction in the proportion unburrowed at the two higher OC levels, relative to the 0-OC group. OC did not interact with either chemical residence time (p=0.3195) or concentration (p=0.1327), therefore the results can be generalized to all concentration levels and chemical residence time treatments. This means that OC's effect on burrowing behavior was similar for all chemical residence time and concentrations tested. Thus, 0.5- and 1-OC significantly increased burrowing ability relative to 0-OC, regardless of the IVM concentration or chemical residence time tested, in *N. virens*.

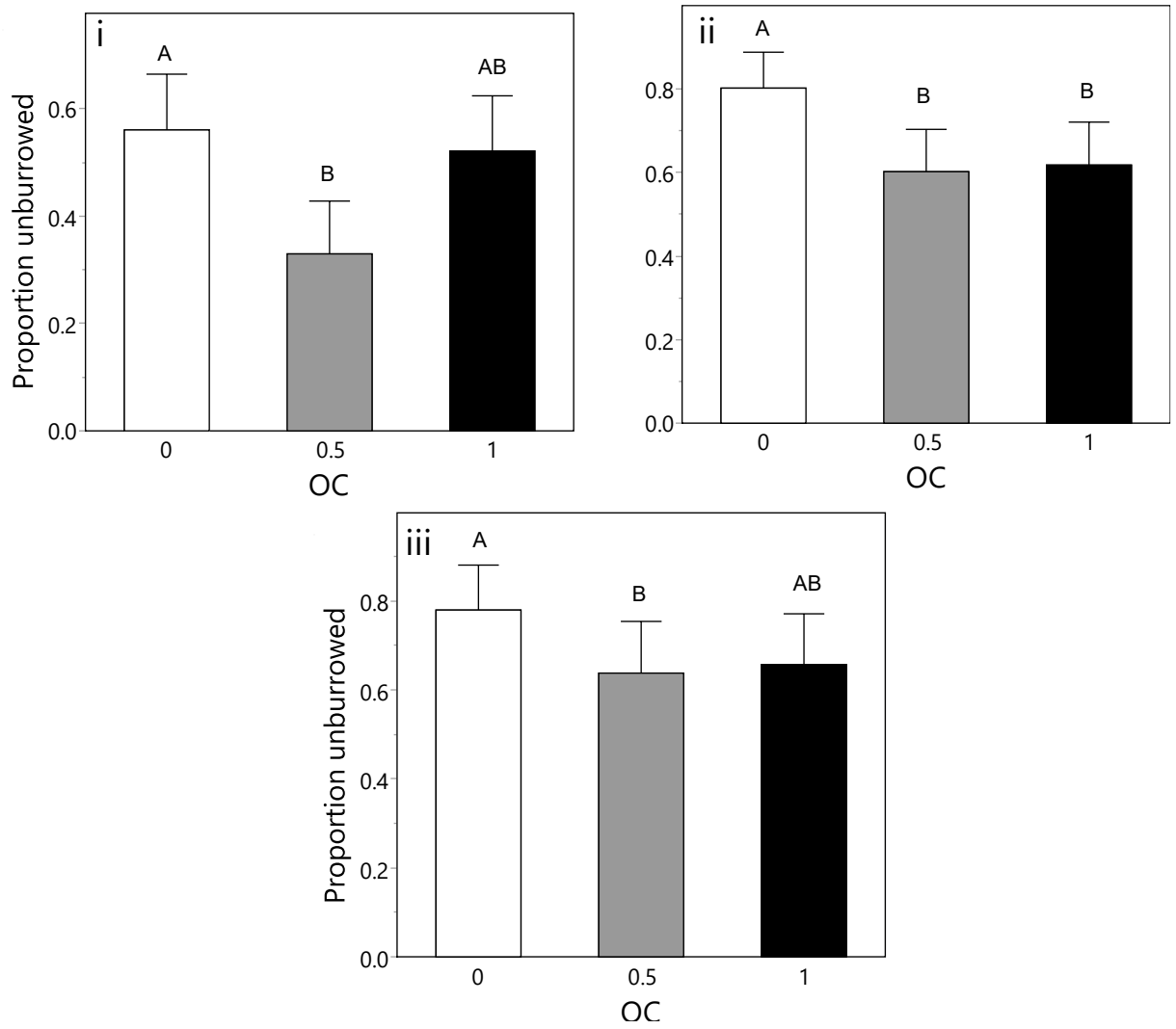


Figure 24: The effect of organic carbon (OC) on sublethal toxicity (burrowing behavior) in *N. virens* exposed to (i) emamectin benzoate (EB), (ii) ivermectin (IVM), and (iii) a combination of both EB and IVM. The proportion unburrowed after 15 minutes of observation is shown for each OC level

(0-, 0.5-, and 1-OC). The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Burrowing data is pooled across all chemical residence times and concentrations for each OC level. Error bars represent 95% confidence intervals. Letters above error bars indicate statistical significance; if a letter is shared between groups, they are not statistically different (α=0.05).

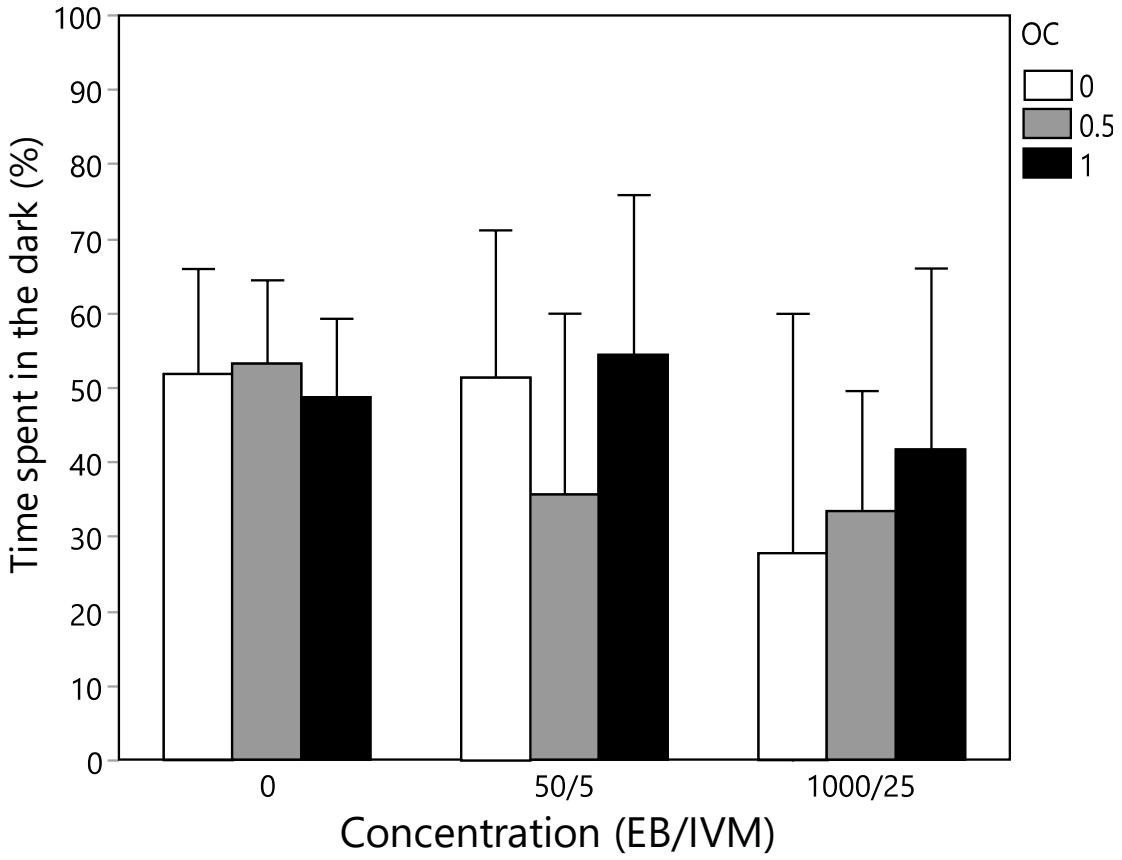


Figure 25: The effect of organic carbon (OC) on sublethal toxicity in *E. estuarius* combination exposures in a subset of 3 combination concentrations (emamectin benzoate / ivermectin; in µg/kg). Light-avoidance is represented by percentage of time spent in the dark during 12-min observation period of the light-dark assay. Bars represent mean percentage of time spent in the dark for each OC level (refer to figure legend). The 0-, 0.5-, and 1-OC treatments used sediments with total OC contents of < 0.05, ~0.2, and 0.42%, respectively. Data is pooled across chemical residence time.

In *E. estuarius* combination exposures, there is no evidence to suggest that OC had a significant effect on sublethal toxicity. In *E. estuarius* combination exposures, OC did not significantly affect light-avoidance behavior based on data from a subset of 3 combination concentration of EB and IVM (p=0.548; Figure 25). The 2-way ANOVA showed no significant interaction between concentration and OC (p=0.510), therefore OC's effect on light-avoidance was not assessed at each concentration separately, unlike with mortality data presented at the end of *Section 2.3.4*.

In *N. virens* combination exposures, OC had a significant effect on burrowing behavior (p=0.0105; Figure 24 iii). The 0.5-OC group had a significantly lower proportion of unburrowed *N. virens* than the 0-OC group (p=0.0152). Pairwise comparisons found no other statistically significant differences: burrowing in the 1-OC group was not significantly different than the 0-OC group (p=0.057). No statistically significant interactions were detected between OC and chemical residence time (p=0.2735) or concentration (p=0.7234). Therefore, the main effect of OC can be generalized to all levels of these 2 factors. This means that OC had a similar effect (i.e. 0.5-OC having a lower proportion unburrowed than the 0-OC group) at all concentrations and chemical residence times tested. Overall, it appears the 0.5-OC treatment mitigates sublethal toxicity in *N. virens*, but the 1-OC treatment does not, relative to the 0-OC treatment; This is the same trend that was observed for the single-chemical EB exposures with *N. virens*. While in *E. estuarius*, OC has no effect on sublethal toxicity.

2.3.8. Sublethal toxicity: the effect of chemical residence time

In *E. estuarius* EB exposures, there is some evidence that chemical residence time significantly affects the relationship between concentration and light-avoidance. However, no consistent trend of increasing chemical residence time decreasing sublethal toxicity was found (Figure 22). In *E. estuarius* single-chemical exposures, chemical residence time's effect on the steepness of the concentration-response curve's negative slope was used to determine how chemical residence time influenced sublethal toxicity: if chemical residence time made the negative concentration-response slope shallower, it decreased sublethal toxicity; if it made the negative concentration-response slope steeper, it increased sublethal toxicity. Chemical residence time significantly affected the slope of the concentration-response curves at 0- and 0.5-OC (p=0.00059 and 0.00880), but not at 1-OC (p=0.1165) (Figure 22 i, ii). For the 0-OC group, at

2 months of chemical residence time the concentration-response curve has a significantly steeper negative slope than at 0- and 4-months (p=0.0060 and 0.0009); the latter two treatments did not have significantly different concentration-response slopes (p=0.8174). Given the steeper (negative) concentration-response slope, this suggests 2 months of chemical residence time significantly increases sublethal toxicity, while 4 months does not affect toxicity, relative to 0 months of chemical residence time. On the other hand, for the 0.5-OC group, the concentration-response curve for the 0-month group had a significantly steeper negative slope than the 2-month group (p=0.0069), but not the 4-month group (p=0.5064); while the latter two chemical residence time treatments did not have significantly different slopes (p=0.1133). Therefore, in this case, 2 months of chemical residence time significantly reduces toxicity, while 4 months does not affect toxicity, relative to 0 months of chemical residence time. Overall, there is no consistent trend as to whether increasing chemical residence time reduces toxicity. In contrast, for IVM exposures, chemical residence time had no significant effect on the slope of the concentration-response curves, at 0-, 0.5-, or 1-OC (p=0.1698, 0.3648, 0.2728, respectively; Figure 22 iv, v, vi).

In *N. virens* EB exposures, chemical residence time had no effect (p=0.1037) on burrowing behavior. (Figure 26 i) There was a statistically significant interaction between chemical residence time and concentration (p=0.00456), therefore these results cannot be generalized to all concentration levels. This means that chemical residence time's lack of effect of burrowing behavior may not occur at all concentrations tested. Two months of chemical residence time appears to be associated with the highest proportion of unburrowed *N. virens*, despite the lack of statistical significance of this trend. Similarly, for IVM-exposures, chemical residence time did not have a significant effect on burrowing behavior of *N. virens* (p=0.3207; Figure 26 ii). Chemical residence time did not have a significant interaction with either concentration (p=0.6121) or OC (p=0.3195); therefore, the lack of effect of chemical residence time on burrowing behavior can be generalized to all levels of these two factors. To summarize, chemical residence time did not significantly alter the ability of *N. virens* to burrow upon single-chemical exposure to either EB or IVM.

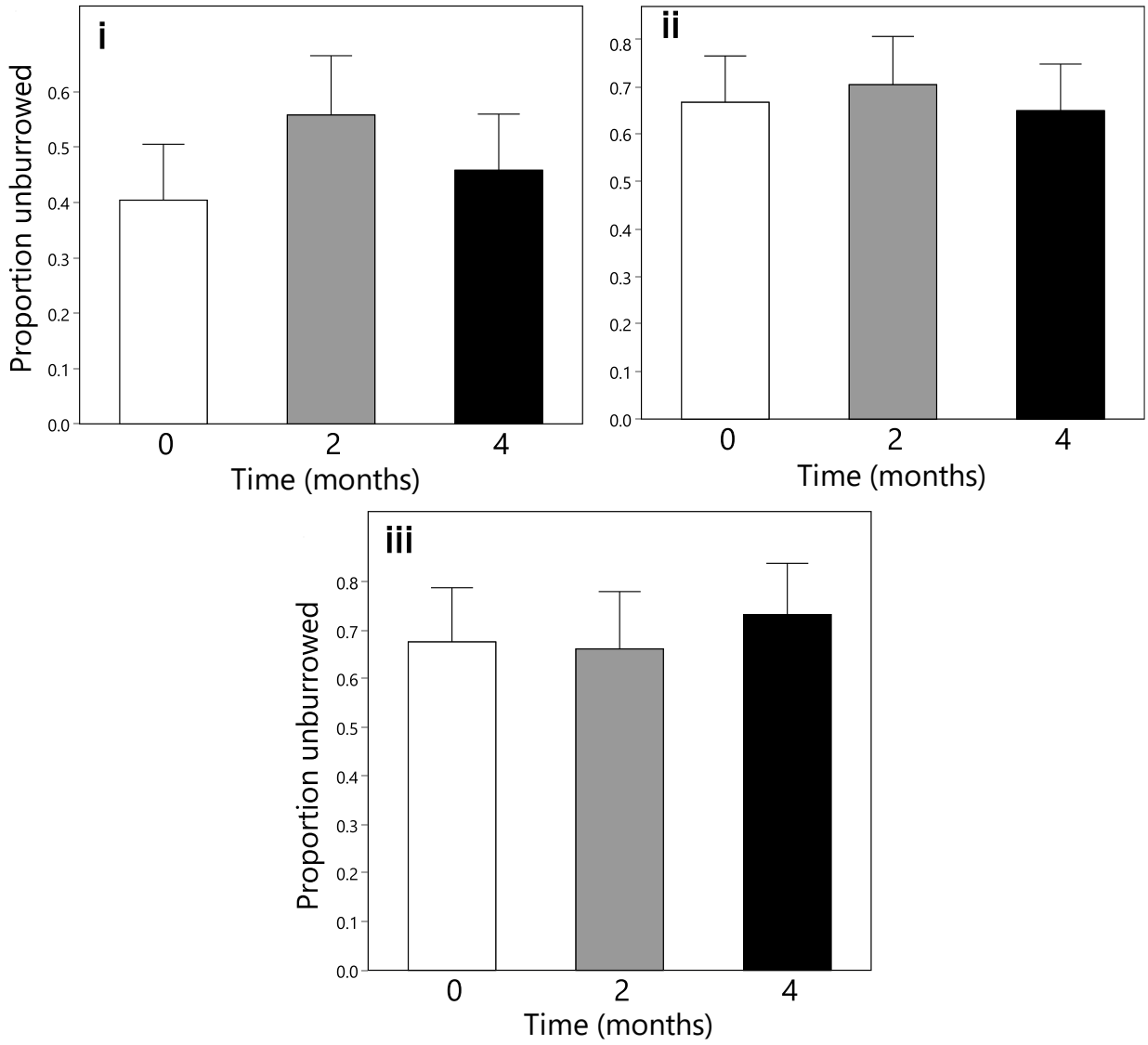


Figure 26: The effect of chemical residence time on burrowing behavior of *N. virens* after 10 d exposures to (i) emamectin benzoate (EB), (ii) ivermectin (IVM), or (iii) a combination of EB and IVM. The proportion of *N. virens* unburrowed (± 95% confidence intervals) after the 15-minute observation period is shown for each chemical residence time treatment (0-, 2-, and 4-months). Burrowing data is pooled across all organic carbon (OC) and concentration levels for each chemical residence time treatment. Error bars represent 95% confidence intervals.

In *E. estuarius* combination exposures, the whole model main effects of chemical residence time on mortality could not be determined for the 0- and 0.5-OC groups since there was a significant interaction between chemical residence time and treatment concentrations for these OC treatments levels (p=0.0006 and p=0.0308, respectively; Figure 27). However, based on one-way ANOVAs conducted at each concentration combination treatment, there is little

evidence to suggest chemical residence time influenced the light-avoidance endpoint (Figure 27). At 0-OC, there exists a significant difference between chemical residence time treatments at 3 out of 7 (including the control) combination concentrations of EB and IVM (Figure 27 i). At 0.5-OC a significant difference exists between chemical residence time treatments only at 1 out of 6 combination concentration treatments (Figure 27 ii). Furthermore, a clear trend as to whether chemical residence time increases or decreases light-avoidance is not evident. In the 1-OC treatment, concentration and chemical residence time did not interact (p=0.0782; Figure 28). Therefore, main effects of these factors could be evaluated on their own. For this OC treatment, chemical residence time did not have a significant effect on light-avoidance (p=0.0595; Figure 28 ii). Overall, there is a lack of evidence to suggest chemical residence time affected light-avoidance behavior at any level of OC.

Chemical residence time did not have a significant effect on burrowing behavior in *N. virens* exposed to a combination of EB and IVM (p=0.2037; Figure 26 iii); the same result was observed in single-chemical exposures for *N. virens*. Chemical residence time did not statistically interact with either OC (p=0.2735) or concentration (p=0.3846), therefore chemical residence time's lack of effect can be generalized to all levels of these other two factors. In summary, combination exposures with *N. virens* and *E. estuarius* did not demonstrate chemical residence time affecting sublethal toxicity.

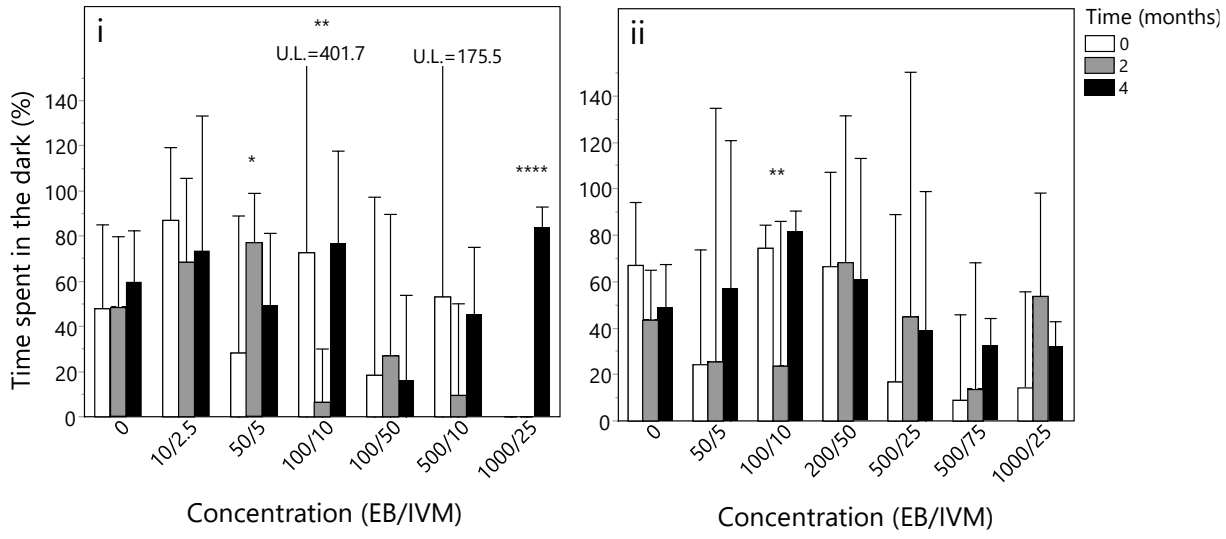


Figure 27: The effects of chemical residence time and concentration (emamectin benzoate (EB) / ivermectin (IVM); in µg/kg) on light-avoidance behavior in *E. estuarius* after 48 h exposures to a combination of EB and IVM for the (i) 0- and (ii) 0.5-OC organic carbon (OC) treatments. The 0- and 0.5-OC treatments used sediments with total OC contents of < 0.05 and ~0.2%, respectively.

Light avoidance is quantified as percentage of time spent in the dark during the light-dark assay. Data points represent the mean percent of time spent in the dark (± 95% confidence intervals). Different chemical residence times are represented by different bar shadings: 0- (white), 2- (gray), and 4-months (black) (refer to figure legend). Asterisks indicate statistical significance between chemical residence time treatments within the same treatment concentration (ANOVA: *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001). No concentration treatments were significantly different than the control (0) treatment within the same chemical residence time treatment (Tukey HSD, α=0.05). The upper limit (U.L.) of the error bars are denoted for concentration treatments 100/10 and 500/10 in panel i.

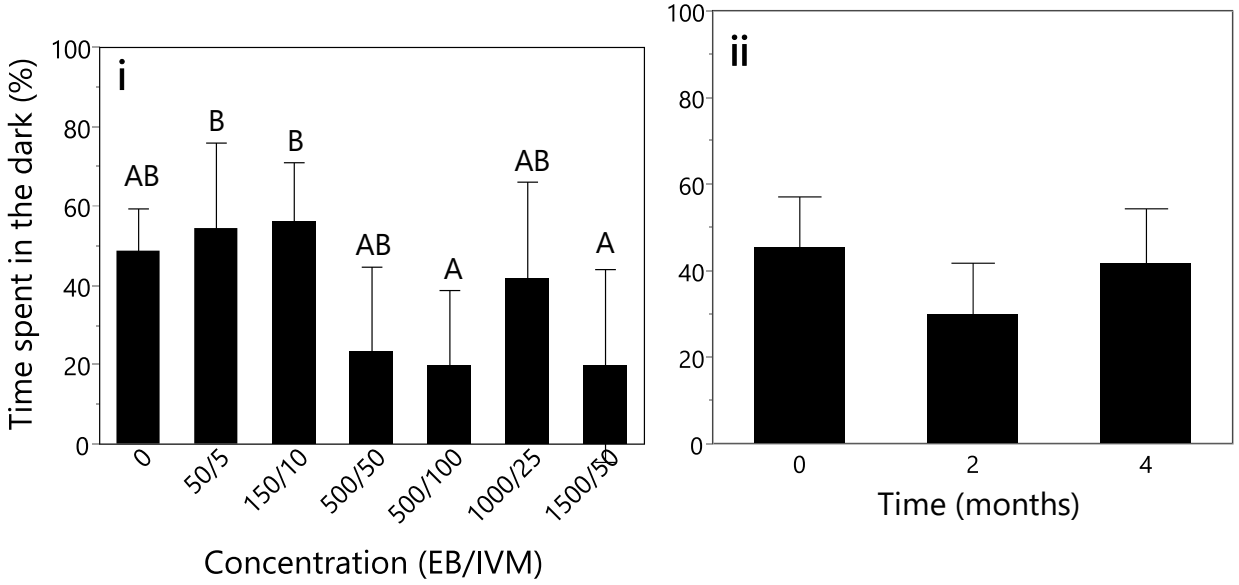


Figure 28: The effect of concentration (emamectin benzoate (EB) / ivermectin (IVM); in µg/kg) (i) and chemical residence time (ii) on light-avoidance in *E. estuarius* for EB and IVM combination exposures at the 1-OC organic carbon (OC) treatment which used sediment with a total OC content of 0.42%. Light avoidance is quantified as the percent of time spent in the dark. Data points represent the least square means of percentage of time spent in the dark (± 95% confidence intervals) for various (i) concentrations and (ii) chemical residence times (0-, 2-, and 4-months). Values are pooled across all chemical residence times for each concentration in (i), and across all concentrations for each chemical residence time in (ii). Letters above error bars indicate statistical significance from pairwise comparisons; if a letter is shared between groups, they are not statistically different (Tukey HSD, α=0.05).

2.4. Discussion

2.4.1. Acute lethal toxicity of avermectins

For EB and IVM single-chemical exposures, significant concentration-dependent increases in lethal toxicity were seen in acute 48-h and 10-d exposures in *E. estuarius* and *N. virens*, respectively. For most chemical residence time and OC co-treatments, the generation of sigmoidal concentration response curves fit the data well and allowed for relatively precise LC50 estimates. Conversely, mortality in *N. virens* was not high enough at the highest concentrations tested to achieve equally precise LC50 estimates.

It is challenging to draw comparisons between the acute lethality (LC50) values for *E. estuarius* in the present study to those reported in literature due to differences in exposure duration and/or the species tested. In the present study, at 0 months of chemical residence time, the 48-h LC50 values for *E. estuarius* ranged between 2009 and 2413 µg/kg (w.w.), depending on OC treatment, in EB exposures. Kuo et al. (2010) found that in *E. estuarius*, the 10-d LC50 for EB was 185 µg/kg (w.w.). This is highly comparable to another species of amphipod, *Corophium volutator*, which has reported 10-d LC50 values for EB of 193 and 153 µg/kg (w.w.) (Lumaret et al. 2012, Mayor et al. 2008). Relative to the present study, a direct comparison cannot be drawn due to the difference in exposure duration. However, given that the present study used 48 h exposures, much shorter than the 10 d exposures used in the aforementioned studies, it is reasonable that the LC50 values in the present study are higher (by an order of magnitude) than values reported in those studies. For IVM exposures in the present study, the 48-h LC50 values for *E. estuarius* at 0 months of chemical residence time ranged between 32.4 and 121 µg/kg (w.w.), depending on OC treatment. Davies et al. (1998) found that in the amphipod *C. volutator*, the 10-d LC50 was 180 µg/kg d.w. That LC50 value is higher than in the present study, despite the exposure duration being substantially longer. Therefore, it appears likely that *E. estuarius* is more sensitive to IVM than *C. volutator*.

For *N. virens*, at 0 months of chemical residence time, the 10-d LC50 values for EB exposures ranged between 7107.1 and >9600 µg/kg w.w., depending on OC treatment. No reported toxicity concentration descriptors (e.g. EC/LCx, NOEC, or LOEC) exist for EB in *N. virens* in the literature. However, *Hediste diversicolor*, another closely related marine polychaete species of the same family (Nereidiae), had a 10-d LC50 of 1368 µg/kg w.w. (Mayor et al. 2008). Another

study done with a more distantly related polychaete, *Arenicola marina* (Order: Capitellida), reported a 10-d LC50 of 111 µg/kg w.w. for EB (Lumaret et al. 2012). Therefore, it appears *H. diversicolor* and *A. marina* are more sensitive to EB than *N. virens*. It is possible that since *H. diversicolor* is much smaller than *N. virens*, it is subject to a higher internal dose to a given sediment concentration of EB. An average individual wet weight for *H. diversicolor* is approximately 0.30 g (Bergstrom et al. 2019), while *N. virens* typically weigh approximately 3 g (wet weight) (Clos 2014). On the other hand, *A. marina* adults are approximately 4 g (Chennu et al. 2015), which is comparable to *N. virens*. Therefore, body weight discrepancy is not likely to play a major role in the observed differences in sensitivity to EB between these two species. Altogether, there are no direct comparisons that can be drawn to literature LC50 values for *N. virens* at an exposure length of 10 d, due to a difference in the study species used.

Due to an absence of IVM sediment exposure data with *N. virens* in the literature, comparison of LC50 values in the present study are made to other annelid species. In the present study, at 0 months of chemical residence time, IVM's 10-d LC50 values for *N. virens* ranged between 2694 and 4753 µg/kg w.w., depending on OC treatment. The polychaete *A. marina* was found to have a 10-d LC50 of 23 µg/kg d.w. (Grant and Briggs 1998). This is more than two orders of magnitude lower than the 10-d LC50 for IVM in *N. virens*. Therefore, *N. virens* is less sensitive to IVM compared to *A. marina*. Meanwhile, the annelid *Lumbriculus variegatus* (Oligochaeta) was reported to have a 28-d LC50 for IVM of 6440 µg/kg d.w. Since this LC50 estimate is higher than those found in the present study even though the exposure duration is almost 3-fold longer, *N. virens* is likely more sensitive to IVM than *L. variegatus*. Overall, it appears annelids are highly variable in their sensitivity to avermectin toxicity.

The present study showed that IVM had a higher toxic potency than EB. For example, in *E. estuarius*, the LC50 values for EB are more than an order of magnitude higher than the values for IVM. The higher relative potency of IVM compared to EB has previously been seen in other studies. For example, the 48-h LC50 values of EB and IVM for *D. magna* were reported to be 1.0 µg/L (Lumaret et al. 2012) and 0.025 µg/L (Halley et al. 1989), respectively. Similarly, in the species *A. marina*, the 10-d LC50 values for EB and IVM were reported to be 23 µg/kg d.w. (Grant and Briggs 1998) and 111 µg/kg w.w. (Lumaret et al. 2012), respectively. In both species, IVM is approximately 5-fold more potent than EB based on comparison of LC50 values. Additionally, in *L. macrochirus* (bluegill sunfish), the reported 96-h LC50 values for EB and IVM are 180 µg/L (OPP 2000) and 4.8 µg/L (Halley et al. 1989), respectively, more than one order of magnitude apart; this is comparable to the potency difference between EB and IVM found in the present

study. However, some studies have found EB and IVM to have comparable potency. For example, the 10-d LC50 for IVM-exposed *C. volutator* reported by Davies et al. (1998) of 180 µg/kg d.w. is highly comparable to the 10-d LC50 of 153 µg/kg w.w. for EB in this species (Mayor et al. 2008). Daoud et al. (2018) also reported comparable toxicity between EB and IVM in *Homarus americanus*: a 10-d LC50 of 250 µg/kg w.w. for EB, and a 10-d LC50 of 212 µg/kg w.w. for IVM. Therefore, the relative difference in toxic potency between EB and IVM may be species-specific.

The differences in toxic potencies between macrocyclic lactones – the family of drugs that avermectins belong to – may be related to their varying abilities to inhibit the family of ATP-binding cassette (ABC) transporters called P-glycoproteins (P-gp) (Prichard et al. 2012). The ABC transporters are a highly evolutionarily conserved superfamily of proteins involved in multi-xenobiotic resistance in both invertebrates and vertebrates (Higgins et al. 1992, Jeong et al. 2017). These transporters actively pump xenobiotics out of cells, aiding in their excretion (Smital et al. 2000). A variety of xenobiotics are known to inhibit the functioning of P-gp (Jeong et al. 2017); macrocyclic lactones are one such class of xenobiotics (Lespine et al. 2011, Prichard et al. 2012). The inhibition of P-gp by macrocyclic lactones can therefore exacerbate the toxicity of these compounds. There are differences between different macrocyclic lactones in their capacity to inhibit P-gp functioning as demonstrated *in vitro* with Chinese hamster ovary cells (Prichard et al. 2012). For example, moxidectin is a significantly less potent inhibitor of P-gp function than IVM, eprinomectin, abamectin, selamectin, and doramectin (Prichard et al. 2012). However, moxidectin is not an avermectin; it is a milbemycin. Among the avermectins (IVM, eprinomectin, abamectin, and selamectin), there appears to be comparable potency for inhibiting P-gp function (Prichard et al. 2012). Additionally, in a study conducted with *in vitro* membrane preparations of *Sf*9 cells (derived from *Spodoptera frugiperda*) overexpressing P-gp, EB appears to have a comparable potency (albeit slight lower) to IVM in inhibiting P-gp; the IC50 (50% inhibition of P-gp ATPase activity) values were approximately 8 and 5 µM for each compound, respectively (Igboeli et al. 2012). Therefore, it appears that P-gp inhibition may be similar amongst avermectins and not explain their varying toxic potencies. Conversely, in another study, IVM has been shown to be a more potent inhibitor of P-gp than EB in hepatocytes of *Oncorhynchus mykiss* (rainbow trout) (Kennedy et al. 2014). Additionally, IVM was shown to be significantly more toxic than EB when assessing swimming performance in *O. mykiss* (Kennedy et al. 2014). Furthermore, the co-treatment with a chemo sensitizing agent which inhibits P-gp activity (Cyclosporin A) exacerbated EB toxicity to a much greater extent than IVM (Kennedy et al. 2014); this further reinforces the notion that EB does not inhibit P-gp to as great an extent as IVM. Overall, there are mixed results regarding the degree to which P-gp inhibition explains varying toxic potencies among

avermectins. However, in *O. mykiss*, the greater inhibition of P-gp displayed by IVM compared to EB, appears to provide an explanation for the difference in toxic potencies of these 2 compounds.

Another reason for the differences in toxic potencies among macrocyclic lactones may be related to their varying potency in increasing the activation of ligand-gated chloride channels, their proposed molecular targets. For example, in *C. elegans*, it was shown that the chloride ion current generated by glutamate-gated chloride channels upon separate exposures (at the same concentrations) to 3 avermectin analogs and milbemycin D was significantly positively correlated with the lethal toxicity of these 4 macrocyclic lactones (quantified as the LD95) (Arena et al. 1995). These 4 compounds listed from highest to lowest chloride current potentiation value and lethal toxicity were as follows: IVM > 4'-epi-acetylamino-4'-desoxy-4a(2-imidazolylcarbonyloxy)-avermectin B1a > 13-Epi-avermectin B1a > milbemycin D (Arena et al. 1995). Conversely, when expressing *Caligus rogercresseyi* isoforms of the glutamate-gated chloride channels in *Xenopus laevis* oocytes, Cornejo et al. (2014) found that EB and IVM showed a similar potency in activating glutamate-gated chloride channels: the EC50 (50% maximum observed increase in chloride current) was 202 and 181 nM for these 2 compounds, respectively. Therefore, the potency to activate ligand-gated chloride channels may not explain the different toxic potencies between EB and IVM.

Typically, chemicals which have the same molecular target, like EB and IVM, tend to exert toxicity additively (Borgert et al. 2004). Toxic interaction between 2 chemicals can fall into the following 4 categories: additivity, antagonism, potentiation, and synergism. Additivity is when the resulting toxicity of combined exposure to 2 chemicals is equal to their combined individual toxicities; these chemicals exert their toxicity independently of one another. Antagonism is when combined exposure to 2 chemicals results in toxicity that is significantly less than what would be expected if these 2 chemicals exerted their toxic effects additively. Potentiation is when 1 chemical, at a concentration that would not result in significant toxicity if acting alone, significantly exacerbates the toxicity of another chemical when these 2 chemicals are delivered in combination. Synergism is when 2 chemicals exacerbate each other's toxic effects so that the resulting toxicity of a combined exposure to these 2 chemicals is significantly greater than if these chemicals exerted their toxicity additively. In the present study, combination exposures to EB and IVM appeared to additively exert their lethal toxicity in both *E. estuarius* and *N. virens*. For example, in *E. estuarius*, at 0 months of chemical residence time and 1-OC, mortality is approximately 90% at a combined exposure of 1500 and 100 μg/kg of EB and IVM, respectively. Single chemical exposures to these same concentrations of EB and IVM resulted in approximately

30% and 50% mortality, respectively. Therefore, the combined toxicity of EB and IVM appears to be additive. Similarly, in *N. virens*, the highest concentration of combined exposure to EB and IVM (2400 and 400 μg/kg, respectively) resulted in approximately 50% mortality at 0 months of chemical residence time, which was significantly higher than the control group. Single chemical exposures at these same concentrations of EB and IVM, resulted in approximately 25% mortality for each of these chemicals. Overall, EB and IVM appear to act additively when delivered in combination.

2.4.2. Acute sublethal toxicity of avermectins

The tendency to move towards (positive) or away from (negative) a light source has been termed phototaxis (Kohler et al. 2018). Phototaxis has previously been investigated in crustaceans exposed to neuromodulating substances (Fossat et al. 2014, Guler and Ford 2010, Hamilton et al. 2016). For example, in the marine amphipod *Echinogammarus marinus*, there was a significant concentration-dependent decrease in light-avoidance after 3-week exposures to serotonin concentrations ranging from 0.01 to 10 μg/L (Guler and Ford 2010). However, for fluoxetine-exposed *E. marinus*, there was a non-monotonic trend where an intermediate concentration of 0.1 μg/L caused a significant reduction in light-avoidance, while other concentrations (0.01, 1, and 10 μg/L) did not result in light-avoidance behavior that was significantly different than unexposed controls. Another study showed that *Pachygrapsus crassipes* (striped shore crab) exposed to 5 and 25 mg/L of fluoxetine for 15 min sought out dark conditions significantly less than unexposed controls: controls exhibited negative phototaxis, with approximately 67% of time spent in dark conditions, while those exposed to 5 and 25 mg/L of fluoxetine did not show a preference for light or dark conditions, spending approximately 50% of their time in dark conditions (Hamilton et al. 2016). In contrast, *Procambarus clarkii* (crayfish) injected with serotonin (5 μg/g) was found to significantly increase light-avoidance relative to an unexposed control group that exhibited a preference for dark conditions. Therefore, exposure to neuro-modulating compounds appears to alter an aquatic invertebrate's phototactic behavior, but in which way it does this (i.e. positively or negatively) is perhaps species-specific.

In the present study, light-avoidance was assessed in *E. estuarius*, a marine amphipod that naturally spends most of its time burrowed in sediment, under low-light conditions. Therefore, in addition to previous research on phototactic behavior in invertebrates, the normal light avoidance/burrowing behavior of a benthic organism like *E. estuarius* suggested itself as a

potential behavioral endpoint that might be useful in toxicity tests (F.Lin personal communciation). Following bioassays, it was found that the mean percent of time spent in test chambers in the dark for unexposed (control) *E. estuarius* was typically 50% under conditions where the organisms had access to both light and dark. Therefore, unexposed *E. estuarius* did not appear to show a natural preference for low-light conditions. This is not consistent with reported findings in literature where the baseline behavior for other marine invertebrates is to exhibit negative phototaxis, which has been shown in the following test species: *Gammarus pulex*, *E. marinus*, *P. clarkii*, and *P. crassipes* (Fossat et al. 2014, Guler and Ford 2010, Hamilton et al. 2016, Kohler et al. 2018). Regardless, the effects of exposure on light-avoidance could still potentially be determined in this design since the exposure to EB and IVM can result in avermectin-induced locomotory depression in invertebrates (in extreme cases flaccid paralysis) (Lankas et al. 1997, Lumaret et al. 2012, Lunt 1991, Song et al. 2016). Therefore, in the present study's design, once initially placed in the light, *E. estuarius* may be less able to seek out low-light conditions if exposed to avermectins, due to locomotory depression. In invertebrates, avermectins bind to glutamate-gated chloride channels and ionotropic GABA receptors on nerve and muscle cells, resulting in an influx of chloride ions at the postsynaptic membrane (Batiha et al. 2020, Lumaret et al. 2012, Song et al. 2016, Wolstenholme 2010). This hyperpolarizes the postsynaptic membrane, preventing propagation of action potentials, resulting in dampened signal transmission of motor impulses. This is thought to be the reason for the observed flaccid paralysis that occurs in avermectin-exposed invertebrates (Arena et al. 1995, Lumaret et al. 2012, Song et al. 2016). In aquatic invertebrates, numerous studies have shown locomotory deficits upon exposure to EB (Daoud et al. 2018, McBriarty et al. 2018, Raja et al. 2020, Willis and Ling 2003) and IVM (Daoud et al. 2018, Ding et al. 2001, Bundschuh et al. 2016, Garric et al. 2007). For example, acute exposure to IVM was shown to result in paralysis in 7 species of aquatic invertebrates (Bundschuh et al. 2016); the most sensitive species was *D. magna* with a 48-h EC50 (immobilization) of 0.59 μg/L, while the least sensitive, *T. tubifex*, had a 24-h EC50 (immobilization) of 1961 μg/L.

However, in the present study, a concentration-dependent reduction in light-avoidance only occurred inconsistently for single-chemical exposures, and this trend was not observed at all in combination exposures. Therefore, there was little evidence for an impaired ability for *E. estuarius* to seek out low-light conditions. Perhaps, to see clear evidence for sublethal toxicity with the light-avoidance assay, exposures to higher concentrations of avermectins would be necessary. Also, an increase in the number of replicates would be warranted, especially given the high variability in response: for example, unexposed (controls) *E. estuarius*, which generally

showed the lowest variability in percent time spent in the dark, tended to have 95% confidence intervals which spanned approximately 50% of the response range (e.g., 50% ± 25%). Certain treatments exposed to EB and/or IVM were even associated with 95% confidence intervals which spanned the entire response range. It is also possible that the *E. estuarius* experienced stress in the bioassay test chamber since they were introduced into an environment without sediment, which in natural circumstances would be available to them. Perhaps this would make them exhibit exploratory behavior in the test chamber, with the biological imperative of seeking out sediment to burrow into. This could explain why controls did not exhibit negative phototaxis. Thus, the effect of avermectins on *E. estuarius* light-avoidance behavior in a stress-inducing artificial environment, like the bioassay chamber, might not be extrapolated to a natural environment. On the other hand, negative phototaxis has been shown to be more pronounced in stressed organisms, as demonstrated with the freshwater crustacean *P. clarkii* (Fossat et al. 2014). However, this may be species specific, especially considering *P. clarkii* is not an organism that burrows in sediment, unlike *E. estuarius*. Interestingly, many treatment groups showed an increase in light-avoidance relative to the control at lower concentrations (Figure 21). It is possible that at low concentrations of avermectins, *E. estuarius* becomes sensitized to light, and avoids it, while at higher concentrations, they become subject to the locomotory depression that accompanies avermectin toxicity. This would provide a reason as to why the concentration-response trend might be non-monotonic (i.e. the slope of the concentration-response curve changes directions within the concentration range tested), which would make it difficult to find a concentration-dependent trend of decreasing light avoidance. However, the suggestion of a non-monotonic concentration-response curve for light-avoidance is speculative, with no clear evidence, and would require further investigation.

Burrowing behavior of *N. virens* is an ecological relevant endpoint. The inability to burrow makes *N. virens* more prone to predation, which reduces its overall fitness and natural survival rates. The disruption of burrowing behavior and an associated increased natural mortality in *N. virens*, has ecological implications. Burrowing of benthic fauna, like *N. virens*, disturbs sediment and water (bioturbation) in the benthic zone, which plays a critical role in the aeration of sediment porewater and overlying water at the sediment-water interface, and in nutrient cycling/redistribution (Aller 1988, Biles et al. 2002, Gautreau et al. 2020, Krantzberg 1985, Mermillod-Blondin and Rosenberg 2006). Burrowing activity promotes aeration in porewater and overlying water by ventilating water and reducing surficial sediment consolidation in the benthic zone (Gautreau et al. 2020, Pearson 2001). Therefore, large populations of *N. virens* could drive widescale oxygenation of the benthic zone in marine ecosystems preventing development of

hypoxic/anoxic conditions in sediment porewater and overlying water, making it a habitable environment for most aquatic species. In fact, ventilation rate of water by *N. virens* has been reported to be about 86 mL water/g worm / h (Miron et al. 1994); this equates to approximately 21 L of water moved within a 24 h period for a large specimen of this species (McBriarty et al. 2018). Bioturbation is also essential for the redistribution of inorganic and organic nutrients within an aquatic ecosystem, potentially making them more widely available for other species, including microbes, and preventing nutrients from concentrating to deleterious levels (Biles et al. 2002, Gautreau et al. 2020, Mermillod-Blondin and Rosenberg 2006). For example, distribution of OM by bioturbation in sediment can support microbial communities that mineralize OM (Mermillod-Blondin and Rosenberg 2006); mineralization of OM provides inorganic nutrients to facilitate algal growth (Biles et al. 2002). Additionally, even distribution of OM prevents excessive nutrient loading in sediment, which would otherwise result in anoxic conditions due to excess microbial activity (Suess 1979). Overall, burrowing of benthic fauna, like *N. virens*, in aquatic ecosystems is important for the survival of this species itself, and consequently the beneficial ecosystem processes that it contributes to.

Sublethal avermectin toxicity was clearly demonstrated in the present study using the burrowing endpoint in *N. virens* exposures. Declining burrowing success in *N. virens* was significantly associated with increasing concentrations of avermectins in single-chemical and combination exposures. Typically, baseline (unexposed controls) failure to re-establish burrows within 15 minutes was approximately 50%, and then peaked at 100% upon exposure to approximately to ≥ 1000 μg/kg of EB and between 10 to 100 μg/kg of IVM. Locomotory function is crucial for coordination of burrowing in *N. virens*. The observed avermectin-induced reduction in burrowing ability may be attributed to the neurotoxicity of avermectins and their deleterious effects on locomotory function. Significant disruptions of burrowing in *N. virens* have previously been shown with exposure to concentrations of EB (formulation: Slice®) at 171 μg/kg (w.w. sediment), with nearly 0% (out of 50 organisms) burrowed after a 30 d exposure period, compared to > 80% were burrowed in control groups (McBriarty et al. 2018). This EB concentration (171 μg/kg) is approximately 5-fold higher than maximal concentrations (35 μg/kg) measured in surface sediment at the edge of active salmonid net pens, 2 to 3 weeks following the first day of a typical 7-d Slice® treatment regimen in BC, Canada (DFO 2011b). Therefore, environmentally relevant concentrations of EB in sediment near (within 100 m) salmonid aquaculture operations, which actively treat sea lice with Slice®, have the potential to adversely affect burrowing behavior in *N. virens* and more sensitive benthic fauna.

2.4.3. The effect of organic carbon on toxicity

In the present study, sediment OC significantly mitigated lethal and sublethal avermectin-induced toxicity in *N. virens*, and lethal toxicity in *E. estuarius*, although this effect was less pronounced in EB exposures relative to IVM and combination exposures. The lack of effect of OC on sublethal toxicity in *E. estuarius* may be the result of little or no quantifiable effect of avermectin toxicity on light-avoidance behavior in the present study; this was discussed in *Section 2.4.3*. OM, measured as OC, is a well-established toxicity modifying factor. Humic substances that make up a large portion of OM may have bound to EB and/or IVM, reducing their bioavailability and thus their toxicity. Environmental contaminants can bind to humic substances by a variety of chemical interactions including the following: hydrogen/ionic/covalent bonding, hydrophobic interactions, π-π stacking, and Van der Waals forces (Bollag et al. 1992, Bollag and Meyers 1992, Steinberg 2003). Since EB and IVM are relatively hydrophobic contaminants, it is likely that these two compounds bind with molecules of humic substances in the sediment primarily through hydrophobic interactions, Van der Waals forces, and π-π stacking (Steinberg 2003). Hydrophobic contaminants have a high affinity for non-polar regions of humic substances, particularly the aromatic rings (Moeckel et al. 2013, Steinberg 2003).

Avermectins have been previously described as having an affinity for OM in sediment, making them relatively immobile (Gruber et al. 1990, Halley et al 1993, Krogh et al. 2008, Lumaret et al. 2012). However, evidence for this is inconsistent. For example, the distribution coefficient values (K_d: the ratio of the concentration of sediment-bound compound to concentration of unbound/dissolved compound) of EB and abamectin have been shown to not correlate with sediment OC content (Mushtaq et al. 1996, Novotny et al. 2020). The K_d values of EB were found to have no relationship with sediment OC when testing 4 different sediments ranging from 0.03 to 1.07% TOC (Mushtaq et al. 1996). The K_d values of abamectin were found to have no significant correlation with sediment OC when testing 5 different sediments (at 2 different depths) with a TOC ranging from 0.50 to 6.66% TOC (Novotny et al. 2020). On the other hand, the K_d value of IVM increased from 227 to 333, with an increase in sediment OM content from 2.5 to 3.9% (Halley et al. 1989), indicating that sediment-associated OC may be involved in adsorbing the contaminant to sediment. Although only 2 different sediments were tested in this case preventing adding uncertainty to conclusions drawn. Furthermore, other physicochemical characteristics also differed between the different sediments tested in their study, such as particle size distribution,

pH, and cation exchange capacity (Halley et al. 1989). Furthermore, Krogh et al. (2008) combined their own data with the data found in Halley et al. (1989) and found no correlation between K_d values of IVM and OM content, when using data from 5 different sediment ranging in OM content from 0.1 to 4.9%. On the other hand, the K_d of avermectin B1a has been shown to have a clear positive correlation with sediment OC content; 3 different sediments with 0.1, 2.1, and 4.8 % TOC content were tested for K_d values which were 17.4, 80.2, and 147, respectively (Gruber et al. 1990). Overall, it is evident that avermectins tightly bind to soil/sediment (indicated by their high K_d) values. However, their affinity for OC is not necessarily a main contributor to this and may depend on the avermectin and/or other physicochemical characteristics of the sediment.

The bioavailability of IVM has previously been shown to decrease with increasing sediment OC. Slootweg et al. (2010) found that there was a significant negative correlation between bioaccumulation of IVM in *L. variegatus* (an aquatic oligochaete) and sediment OC content. They found that the bioaccumulation factors (BAF; the ratio of the concentration of compound in organism tissue to the concentration in of the compound in sediment) were 4.5, 2.93, 2.09, 0.58, and 0.12, for sediments with % TOC values of 0.62, 1.96, 2.86, 3.67, and 20.6%, respectively. This reflects a reduction in bioavailability with increasing sediment OC content. This is consistent with the decrease in IVM toxicity at higher OC treatments observed in the present study, although the range of sediment OC tested in Slootweg et al. is much higher than in the present study. The effect of OC on the bioavailability/toxicity of other avermectins, including EB, has not been previously investigated, so whether this trend also applies to other avermectins is unclear.

Studies have shown toxicity mitigating effects of sediment OC in aquatic sediment exposures involving organic contaminants other than avermectins (Cano et al. 1996, Harwood et al. 2013, Meador et al. 1997, Tsui and Chu 2004); these studies found significant increases in LC/EC50 values in the highest OC treatments relative to the lowest OC treatments. This difference ranged between a factor of 1.6 to 5.7-fold. However, several of these studies used a high-OC treatment of >2% TOC (Cano et al. 1996, Harwood et al. 2013, Tsui and Chu 2004), much higher than the present study, making it difficult to draw a comparison to the present study. For example, in a toxicity study with bifenthrin, Harwood et al. (2013) found that a sediment TOC of 4.43% resulted in a significant reduction in toxicity when compared to a TOC of 0.56% in *H. azteca* and *Chironomus dilutus;* this resulted in a 5.7- and 2.2-fold increase in the LC50s in these organisms, respectively. On the other hand, Meadeor et al. (1997) used sediment OC treatments what were much more comparable to the present study. They found that 0.59% sediment TOC

was enough to cause a significant decrease in tributyltin-induced mortality in *Rhepoxynius abronius* (Amphipoda) when compared to sediment with a TOC of 0.12%. In addition, they found that sediment with a TOC of 0.87% was associated with a significant decrease in tributyltin-induced mortality in another species, *Armandia brevis* (Polychaeta), relative to sediment with a TOC of 0.30%. For both species tested in their study, the difference between OC content of the low and high TOC treatments was comparable to that of present study. Therefore, this is consistent with the findings in the present study that a high-OC treatment of 0.42% TOC can result in a significant reduction in toxicity relative to a low-OC treatment of < 0.05% TOC.

However, reductions in toxicity cannot definitively be attributed to TOC content since many other sedimentary characteristics can also alter a contaminant's observed toxicity. Sediment characteristics which can potentially act as confounding variables in sediment toxicity assessments can be categorized as follows: (1) persistent physical characteristics; (2) persistent chemical characteristics; and (3) non- or less-persistent chemical characteristics (Word et al. 2005). Persistent physical characteristics include the following: grain size/angularity, sediment consolidation, and water retention capacity. Persistent chemical characteristics include the following: TOC, total organic nitrogen (TON), sediment matrix-associated minerals, exogenous pollutants, and woody debris. Non- or less- persistence chemical characteristics include ammonia, salinity, pH, and sulfides. It is possible that some of these sediment characteristics could have contributed to variability in the findings for exposures involving EB and IVM for *E. estaurius* and *N. virens*. Particle size distribution is an excellent example of this, as higher OC-content typically co-occurs with smaller grain sizes.

Particle size distribution (i.e. grain size) is a well-recognized sediment characteristic linked to modifying toxicity (Campana et al. 2013, Lapota et al. 2000, Strom et al. 2011). In the present study, sediment characteristics that were measured include the following: particle size distribution, TOC, total organic matter (TOM), and pH. There was a clear difference between the 1-OC and 0-OC sediment with regards to particle size distribution; a grain size of < 0.063 mm (silt) composed approximately 20% and 5% of these two types of sediments, respectively (Refer to *Appendix A*, Figure A.1 and Figure A.2). The smaller grain size of the 1-OC sediment could have a toxicity mitigating effect, as has been seen in previous studies (Strom et al. 2011). For example, when controlling for sediment TOC content, Strom et al. (2011) found that toxicity was negatively correlated with grain size in both *Spisula trigonella* and *Tellina deltoidalis*. When comparing copper toxicity in sediment with a silt content of 100% to that of 25%, the LC50 values were 4.3- and 4.9-fold higher in the former treatment group, for both species, respectively. Conversely,

smaller grain size has also been linked to deleterious effects in organisms such as amphipods (Lapota et al. 2000). For instance, it can impede amphipods from burrowing, resulting in stress (Lapota et al. 2000). This was unlikely the case in the present study given that toxicity was found to decrease in the 1-OC sediment. It may also be worth noting, that grain size, TOC, sulfides, ammonia are all typically correlated to one another. Smaller grain size provides more surface area for OM to adsorb to, which in turn harbors sulfide- and ammonia-producing microbes. Therefore, in a natural setting these factors should be seen to co-occur, and one may be predictive of the others. This means that parsing these factors apart may be unnecessary from the standpoint of predicting toxicity in environmentally relevant conditions. Instead, it may be more helpful to view these factors as a tetrad of factors that modify toxicity in sediment exposures.

Another relevant physicochemical characteristic that may affect toxicity of sediment is its inorganic constituents (e.g. sediment matrix-associated minerals). It has been shown that avermectins can form complexes with inorganic components in sediment (Krogh et al. 2008, Litskas et al. 2011). For example, IVM can form adducts with cations, such as ammonium and sodium, as has been shown with liquid chromatography–mass spectrometry (Ali et al. 2000). It has also been suggested that IVM may bind to calcium ions, which was inferred by Krogh et al. (2008) based on a 3-fold decrease in K_d value with 5-fold decrease in concentration of $CaCl_2$ solution used for adsorption testing. Litsaks et al. (2011) found a strong correlation (r^2=0.87) between cation-exchange-capacity (CEC; log transformed) and K_d values, when pooling data on 3 different avermectins. In their study they pooled data for IVM (Krogh et al. 2008), abamectin (Gruber et al. 1990), and their own study on eprinomectin, combining for a total of 9 different sediments with varying CEC (0.328 to 39.2 mols of electric charge per 100 g sediment). In addition to this, they also found that when pooling data for IVM and eprinomectin, there was a strong correlation (r^2=0.95) between varying Cu (range: 0.04 to 27 mg/kg) and Fe (range: 0.09 to 852 mg/kg) content for 6 different soils. Therefore, it is possible that the association between avermectins and inorganic components of soil/sediment may play as important a role in sediment adsorption processes as hydrophobic interactions with sediment-associated OM. In fact, Litskas et al. (2011) found no correlation (r^2<0.5) between K_d values and sediment OM content (range: 0.1 to 4.8%) when pooling data for 3 avermectins from 9 different types of sediment (in the same manner as mentioned above). Thus, their study suggests that interactions with inorganic components of sediment may play a bigger role in the adsorption of avermectins to the sediment matrix, than hydrophobic interactions with sediment OM. Altogether, it remains uncertain to what extent adsorption to sediment can be attributed to OM or other physicochemical characteristics of sediment. However, it is clear that many sediment characteristics play a role in how tightly

avermectins bind to sediment, and therefore perhaps their bioavailability. This means the reduced avermectin toxicity seen in the high-OC treatment relative to the low-OC treatment in the present study may not be solely attributed to avermectins binding to OM, but instead to a multitude of sediment characteristics, some of which are co-occurring as explained in the above paragraph.

Unlike in IVM and combination exposures, EB exposures showed a less pronounced and consistent protective effect of OC on lethal and sublethal toxicity in both species in the present study. Perhaps the most notable example of this is that with *E. estuarius*, OC did not affect EB-induced lethal toxicity, based on LC50 value comparisons, while in IVM exposures there were clear increases in LC50 values with increasing OC content. These results would suggest that perhaps EB binds less strongly to humic substances in OM, compared to IVM. However, EB and IVM share many chemical characteristics and are highly similar in their chemical structures. Although, as mentioned previously, the affinity to OC may depend on the avermectin. Therefore, it is a possible explanation for the discrepancy in OC's effect on EB *v.* IVM toxicity.

Another explanation for the less pronounced effect OC had on EB-induced toxicity, is that Slice® is largely composed of two carbohydrates: maltodextrin (47.4%) and cornstarch (50.07%) (Bright and Dionne 2005). Together these make up 97.5% of the Slice® formulation (Bright and Dionne 2005). These two carbohydrates can be considered a source of fresh OM (Thurman 1985). Therefore, even the 0-OC groups would have had a detectable amount of OM present in the form of maltodextrin and cornstarch when Slice® is used to spike this sediment. For example, the highest EB concentration tested for the 0-OC group in *E. estuarius* exposures was 5000 µg/kg, which corresponded to the addition of 0.25 g of Slice® to 100 g of sediment. After addition of Slice® this would have introduced an additional 0.24% of OM in the form of maltodextrin and cornstarch. As a rule of thumb, a conversion factor of 0.58 to convert OM to OC is typically applied in the context of soil OM; this is known as the van Bemmelen factor (Wang et al. 2016). Therefore, after applying this conversion factor, this addition at the highest EB concentration would correspond to an addition of approximately 0.14% TOC to the 0-OC sediment. However, the 1-OC treatment would also experience an increase in sediment TOC, like the 0-OC treatment. For example, the highest concentration test in the 1-OC treatment in *E. estuarius* exposures was 8500 µg/kg, which corresponded to adding 0.43 g of Slice® to 100 g of sediment. This corresponds to an additional 0.24% of OC incorporated into the sediment, adding to the 0.42% already present in the 1-OC treatment, for a total of 0.66% TOC in the 1-OC sediment. Therefore, the high-OC treatment experiences an increase in OC content as well, which would perhaps offset the effect of introducing additional OC in the low-OC treatment. On the other hand, if the presence of just a

small amount of sediment TOC (such as 0.14%) is sufficient to bind to the same number of molecules of EB or IVM and prevent toxicity just as effectively as a larger amount of sediment OC (such as 0.66%), then the amendment of the 0-OC sediment with a small amount of OC could reduce toxicity to a similar extent as the 1-OC sediment; this assumes that the avermectin-OC binding potential is similar between a TOC of 0.14% and 0.66%, perhaps due to a lack of saturation of OC-binding sites in the 0.14% treatment. This would confound the results and may explain why the effect of OC on toxicity was not as evident in EB exposures as it was in IVM exposures.

It is speculation that OC being introduced in the form of carbohydrates found in the Slice® formulation may have confounded the results, since the source of OC may not be a form that readily binds to EB. However, it is plausible that the type of formulation, or whether a formulation is used at all, can play a major role in OC's effect on toxicity in sediment toxicity testing with non-metal contaminants. For example, Tsui and Chu (2004) highlighted the importance of knowing the type of formulation that is used when it comes to sediment OC influencing toxicity. Their study investigated the effect of sediment OC on glyphosate toxicity, in 2 different formulations: Roundup® and Roundup Biactive®. The only difference between these 2 formulations is the surfactant used. When conducting 48 h toxicity tests with *Ceriodaphnia dubia*, they found that a sediment TOC of 2.10% caused a significant 3.1-fold increase in LC50 value when compared to the lowest TOC of 0.0% for Roundup® exposures. On the other hand, for Roundup Biactive® exposures they found that a TOC of 2.10% corresponded to a 1.3-fold decrease (non-significant) in the LC50 value when compared to the 0.0% TOC treatment. This demonstrates the importance of considering which formulation is used when conducting toxicity tests. It should also be noted that it was observed in the present study that after 4 months of incubation, sediment spiked with significant amounts of Slice® had the presence of a slick black material, with a sulfurous odor. This likely indicates the thriving of sediment microbes, resulting in an anaerobic environment where sulfate-reducing bacteria produce hydrogen sulfide (H_2S), which may be responsible for the sulfurous odor (Muyzer and Stams 2008). With the abundance of microbial activity, it is possible these 2 carbohydrates could have also been biotransformed by sediment microbes into other types of OM molecules. Overall, although speculative, the introduction of OC in the form of carbohydrates found in the Slice® formulation may explain the discrepancy in the results regarding OC's effect on toxicity between purified IVM exposures versus Slice® exposures.

Since salmonid aquaculture uses Slice®, one could argue that the 2 carbohydrates found in this formulation would also factor into benthic fauna EB-exposure scenarios underneath salmon

farms, and therefore the laboratory results may reflect results in the field. But this toxicity-mitigating effect of OC in Slice® might not be extrapolated from a closed system (i.e. a jar with spiked sediment) in a laboratory to a more complex open system like a salmon farm. For example, on a salmon farm, EB in uneaten pellets treated with Slice® may separate from the carbohydrates found in the formulation. These carbohydrates are generally more water soluble, and could then be influenced by ocean currents, and not end up underneath the open net pen at all. Meanwhile, EB would sink to the benthic zone due to its much lower water solubility then maltodextrin and cornstarch found in Slice®. Similarly, if Slice®-treated pellets are ingested by farmed fish, the carbohydrates and EB may separate in the gastrointestinal tract of the treated fish, whereby EB would be excreted by treated fish into the environment and end up in the sediment not accompanied by the 2 carbohydrates in the formulation. Altogether, the potential of the carbohydrates in the Slice® formulation to mitigate toxicity (confounding the results regarding sediment OC's effect on EB toxicity) in the laboratory setting may not translate to what would occur in the field. Therefore, naturally occurring sediment OC may have a bigger impact on EB toxicity underneath a salmon farm, then is suggested by the present study. Furthermore, underneath salmon farms, sediment TOC can be as high as approximately 9% (Brown et al. 1987). The present study does not provide insight as to EB toxicity that would occur at these levels of sediment OC.

2.4.4. The effect of chemical residence time on toxicity

In *E. estuarius* exposures, 4 months of chemical residence time appeared to have significantly reduced the bioavailability of both avermectins, and consequently, acute lethality in this treatment group. Chemical residence time may affect how tightly bound a contaminant is to sediment and its associated OM (Clark and Choppin 1990, Nam and Kim 2002) and/or result in the contaminant gradually migrating and sequestering in pockets of sediment pores that are inaccessible to biota (Alexander 2000, Conrad et al. 2002, Nam and Kim 2002). The latter mechanism is well recognized, and results in a reduction in a contaminant's physical availability with increasing chemical residence time.

The reduction in bioavailability with increasing chemical residence time has previously been observed with persistent organic pollutants in terrestrial and aquatic environments (Conrad et al. 2002, Chung and Alexander 1998, Kukkonen and Landrum 1998, Landrum et al. 1992,

Leppanen and Kukkonen 2000, Menchai et al. 2008, Reid et al. 2000, Taylor et al. 2019, White et al. 1999). For example, when normalized to the physically available fraction, the bioavailability (measured as the change in BAF) of pyrene to *L. variegatus* (an aquatic oligochaete) decreased by approximately 58% over the course of 220 d of chemical residence time (Conrad et al. 2002). Since this normalized value only considers the physically available pool of the contaminant, this calculated decrease in bioavailability is attributed to factors other than a reduction in physical availability. Additionally, the chemical extractability, representing the physically available fraction, of pyrene decreased by 50% over the course of 220 d of chemical residence time (Conrad et al. 2002). The compounded effect of the reduction in physical availability of the contaminant and factors unrelated to physical availability resulted in an overall 70% reduction in pyrene body burden in *L. variegatus*, representing the overall reduction in bioavailability (Conrad et al. 2002). In the same species, You et al. (2009) found that the bioavailability of permethrin to *L. variegatus* decreased significantly by 65% when comparing 7 d *v*. 90 d chemical residence time in one of the sediment types tested. However, in the other sediment type tested, they found a non-significant 1% decline in bioavailability. Therefore, it appears sediment physicochemical characteristics play a role in chemical residence time's effect on bioavailability. Kukkonen and Landrum (1998) found that 13 months of chemical residence time in aquatic sediments resulted in a 42% decline in the bioavailability of benzo(a)pyrene in exposures with *Diporeia spp.*, a benthic amphipod. Additionally, phenanthrene has been observed to experience a 38% reduction in bioavailability after 30 d of soil aging (relative to 3 d of aging), in *Eisenia foetia* (earthworm) (White et al. 1999). Furthermore, after 365 d of chemical residence time, Menchai et al (2008) found that the physical availability of dichlorodiphenyltrichloroethane (DDT) decreased by between 86 to 93%, depending on soil type; in their study, physical availability was measured using a semi-permeable membrane device containing cod liver oil. Altogether, there is substantial evidence for significant reductions in bioavailability occurring upon 30 d or more of sediment-contaminant contact time in organic contaminants.

The reduction in bioavailability of a contaminant due to contact time with sediment has previously been characterized as an initially rapid process. A large portion of reduced bioavailability occurs within the first day of contact with sediment (Conrad et al. 2002). For example, Conrad et al. (2002), found that the bioavailability (normalized to physical availability) initially decreased by approximately 30% after 1 day of contact time, afterwards remaining relatively constant over the course of 70 d. However, when measured after 220 d, there was an additional notable (28%) decrease in bioavailability. This led the authors to conclude changes in bioavailability – in this case normalized to physical availability – of sediment-associated

contaminants follow a 3-stage process involving (1) an initial rapid decrease, (2) a plateau, and (3) a further decrease.

The reduction in physical availability appears to be a slower, more gradual process than aspects of bioavailability unrelated to physical availability of a contaminant. Conrad et al. (2002) found a 10% drop in physical availability after 70 d of pyrene-sediment contact time. However, after 220 d, there was a substantial drop in physical availability to around 50% of initial values. This appears to be the only study that differentiates the effects of physical availability from factors unrelated to physical availability of a contaminant, when investigating chemical residence time's effect on bioavailability. Most studies measure overall bioavailability. For instance, Kukkonen and Landrum (1998) found that 90% of the decrease in bioavailability of benzo(a)pyrene over the course of 13 months of chemical residence time, occurred within the first week. Indeed, it has been recognized that a biphasic reduction in bioavailability with increasing chemical residence time is typical: a rapid initial reduction in bioavailability, followed by a more gradual reduction in bioavailability (Reid et al. 2000). For example, Mah et al. (2012) found that the bioavailability to *E. fetida* (earthworm) of soil-assocaited phenanthrene and pyrene decreased substantially (approximately 40% and 25%, respectively) after the first 15 d of chemical residence time. This was followed by a steady and more gradual decline in bioavailability by an additional 50% and 30%, respectively, over the next 135 d of chemical residence time. In the present study, perhaps a large reduction in bioavailability of avermectins occurred rapidly prior to introduction of organisms, whereby the 0-month treatment group – in which avermectins spent approximately 12 h in contact with sediment prior to the introduction of organisms for toxicity testing – experienced similar reductions in avermectin bioavailability as the 2-month group. Meanwhile, perhaps the 4-month treatment group was subject to a further gradual decrease in bioavailability of the avermectins, resulting in the significant change in mortality associated with this treatment group in *E. estuarius*, compared to the other 2 treatment groups.

In their study, Conrad et al. (2002) confirmed that pyrene had not degraded over the course of the 220 d study, therefore it could be concluded that chemical degradation was not responsible for the observed reduction in pyrene body burden. This highlights an important limitation in the present study's design, whereby the roles of chemical residence time and chemical degradation in the altered toxic response cannot be parsed since chemical degradation was not measured. Therefore, it may be erroneous to conclude that longer avermectin-sediment contact times reduced their bioavailability which was ultimately responsible for the observed reduction in mortality of *E. estuarius* at 4 months of chemical residence time. Instead, this may

just as easily be explained by degradation of EB and IVM. Laboratory studies suggest that the degradation half-life (DT_{50}) of IVM ranges between 93 to 240 d when residing in a soil-feces mixture at 22^{o}C in the dark (Halley et al. 1989). The DT_{50} of EB is comparable to IVM, ranging between 164 to 404 d in marine sediment (Benskin et al. 2006, Bright and Dionne 2005). Therefore, after 4 months of incubation at 4^{o}C in the dark, it is plausible that EB and IVM may have significantly degraded, thus explaining the decrease in lethal toxicity observed relative to the 0- and 2-months of chemical residence time groups. Therefore, the confounding effect of chemical degradation of EB and IVM should be considered and is a possible explanation for the observed outcome; however, both chemical residence time and chemical degradation may have contributed to the reduction in mortality at 4 months of chemical residence time. Although it is not clear how much EB and IVM would have degraded during incubation at 4^{o}C in the dark, as was the case in the present study. To address this, future studies should use measured concentrations instead of nominal concentrations.

In *N. virens*, it is unclear why the degradation of EB and IVM, as discussed above, did not reflect a significant reduction in toxic response (and therefore, presumed bioavailability) after 4 months of incubation with sediment, as it did in *E. estuarius* exposures. Instead, this suggests that significant chemical degradation of EB and IVM did not occur after 4 months of chemical residence time. Perhaps it is possible that EB and IVM concentration remained relatively constant, but the bioturbation generated by *N. virens* triggered its desorption from sediment. This is consistent with the fact that *N. virens* is a much bigger organism, capable of disturbing sediment to a much greater extent than *E. estuarius*. Therefore, due to less bioturbation in *E. estuarius* exposures, perhaps EB and IVM remained relatively immobile, sequestered in inaccessible pockets of the sediment matrix. This might explain the reduction in mortality observed after 4 months of chemical residence time in *E. estuarius*. In contrast, in *N. virens* exposures, bioturbation may have liberated avermectins from inaccessible parts of the sediment matrix, allowing them to become bioavailable to *N. virens*, resulting in similar mortality across all chemical residence times.

Differences in feeding behavior between *N. virens* and *E. estuarius* may have also contributed to the difference between the effect of chemical residence time on avermectin toxicity in each species. Since *N. virens* ingests sediment (qualitative observation in present study; Macdonald and Ingersoll 2003, Vismann 1990), absorption of sediment-associated contaminants is likely primarily through an oral route of exposure. Although, *E. estuarius* is thought to be a deposit feeder (USEPA 1994), like *N. virens*, therefore it is also likely exposed to sediment-associated contaminants *via* an oral route of exposure. Nevertheless, it is possible that *E.*

estuarius is exposed to a lesser extent via an oral route of exposure, and instead more through its integument (dermal exposure), when compared to *N. virens*. If, after avermectin-sediment contact time, absorption of avermectins only decreases for the dermal route of exposure, but remains constant for the oral route of exposure, this could explain the discrepancy in chemical residence time's effect on toxicity between these 2 test species. In this case, *N. virens* would absorb avermectins primarily *via* the oral route of exposure, so absorption might remain relatively constant across all chemical residence time treatments. Meanwhile, *E. estuarius* would absorb avermectins to a lesser extent as soon as their absorption decreases through the dermal exposure route with increasing chemical residence time. In fact, absorption of sediment-associated persistent hydrophobic contaminants has previously been shown to decline more rapidly with increasing chemical residence time *via* a dermal route of exposure compared to an oral route of exposure. Leppanen and Kukkonen (2000) showed that *L. variegatus* capable of feeding accumulated pyrene and benzo(a)pyrene between 2- to 9-fold faster (depending on which chemical and the length of contaminant-sediment contact time) relative to those which were unable to feed. The observed decline in pyrene bioaccumulation rate due to increasing contaminant-sediment contact time (up to 34 d) was less pronounced in feeding worms. The rate of bioaccumulation of pyrene in feeding worms was approximately 2-fold higher than non-feeding worms during 0 to 6 d of pyrene-sediment contact time; meanwhile it was approximately 9-fold higher in feeding worms relative to non-feeding worms during 28 to 34 d of pyrene-sediment contact time. Therefore, it is possible that sediment contact time has less of an effect on absorption through an oral route of exposure. However, this result was not observed with benzo(a)pyrene, and therefore may be chemical-dependent. To summarize, a difference in feeding ability, and therefore route of exposure, between the 2 test species in the present study may explain the difference in chemical residence time's effect on avermectin toxicity.

For chemical residence time to exacerbate lethal toxicity as it did in the IVM exposures with *N. virens*, 2 scenarios can be considered: (1) IVM bioavailability increased after 2 months of chemical residence time; (2) IVM was biotransformed into a more toxic form. It is unlikely that avermectin bioavailability would have increased with increasing contaminant-sediment contact time, as all studies previously done with persistent organic contaminants indicated that bioavailability either decreases or remains the same (and does not increase) with increasing contaminant-sediment contact time (Conrad et al. 2002, Chung and Alexander 1998, Kukkonen and Landrum 1998, Landrum et al. 1992, Leppanen and Kukkonen 2000, Menchai et al. 2008, Reid et al. 2000, Taylor et al. 2019, White et al. 1999). No evidence was found in the literature for chemical residence time increasing bioavailability of sediment-associated organic contaminants.

Instead, it is perhaps possible that IVM was metabolized by sediment microbes into more potent toxic metabolites. For example, in the case of EB, the desmethyl metabolite of EB is 10-fold more potent than EB itself (Kuo et al. 2010). However, IVM is not known to have metabolites which are more toxic than the parent compound (Halley et al. 1989). For instance, the monosaccharide and aglycone metabolites of IVM have been shown to be much less toxic to *D. magna*, with 48-h LC50 values of 0.4 and 17 μg/kg, respectively, compared to the 48-h LC50 value of 0.025 μg/kg for the parent compound (Halley et al. 1989). Furthermore, Slootweg et al. (2010) found that after a 28 d IVM exposure with *L. variegatus* in a sediment-water system, no biotransformation products were found in sediment or *L. variegatus* tissue. However, of the small proportion of IVM and its metabolites in the water column (7% of the initial IVM added to the system), a large proportion (83%) of this was comprised of 2 metabolites, the rest (17%) being parent compound. Based on the results of Prasse et al. (2009), these were likely the aglycone and monosaccharide metabolites of IVM: this study also found that these metabolites only reside in the water column, and not sediment. Given that IVM metabolites are less toxic, and only represent a small fraction (approximately 6%; Slootweg et al. 2010) of an entire aerobic sediment-water system, the presence of these metabolites does not explain the increased mortality in *N. virens* seen after 2 months of chemical residence time.

Chapter 3. Conclusion and future directions

3.1. Conclusion

In conclusion, the present study finds novel evidence for marine surface sediment OC and sediment aging exerting a significant protective effect against sublethal and lethal toxicity of avermectins. The toxicity-mitigating effects of OC were discovered in both marine benthic invertebrate test species, *N. virens* and *E. estuarius*. Generally, this effect was less pronounced in EB exposures relative to IVM and combination exposures. On the other hand, the effect of sediment aging on avermectin toxicity showed interspecies difference. Generally, four months of contaminant-sediment contact time significantly reduced toxicity of both avermectins in *E. estuarius*. Chemical decay and/or a decrease in bioavailability of both avermectins are likely responsible for this trend. However, 4 months of chemical residence time had no effect on toxicity in *N. virens*. Therefore, the toxicity-ameliorating effects of sediment aging are species specific.

3.2. Recommendations for use of avermectins in aquaculture

Sediment TOC is particularly relevant to aquaculture since overcrowded net pens tend to result in a significantly higher sediment TOC content underneath and in the immediate vicinity of aquaculture enclosures. For example, in salmonid net-pens in BC, Canada, Sutherland et al. (2000) found that the carbon flux adjacent to the salmon farms was 2-fold higher when compared to control sites. This may increase the TOC within 3 m of the edge of salmon pens by greater than 2-fold, in comparison to the baseline TOC of the surrounding sediment (Brown et al. 1987). However, this increased TOC is only observed up to 15 m from the edge of the salmon pen. Therefore, the dispersal distance for avermectins delivered on a salmon farm should also be considered since the elevated sediment TOC content may only be present within a relatively small radius around a finfish farm. Furthermore, the high sediment OC treatment in this study may not have reflected the conditions underneath a salmon farm. Marine sediment can have a TOC content as high as 9.35% underneath salmon farms in BC (Brown et al. 1987). Therefore, it is possible that at these levels, OC would influence avermectin-induced toxicity to an even greater extent. The extent to which avermectins persist and remain bioactive underneath salmon farms

is another important consideration, which could determine temporal spacing of avermectin treatments on salmon farms. For example, EB has been shown to persist at detectable levels (3 μg/kg; limit of detection: 0.12 μg/kg) in sediment 1.5 years after a standard 1-week administration of Slice® on salmon farms (DFO 2011b).

In conclusion, based on the present study's findings, it is advisable to consider the sediment TOC percent of the benthic zone when regulating or assessing the environmental risk of avermectin sea lice chemotherapeutant usage. Even a TOC content of < 1% (and all other associated sediment characteristics) appears to be sufficient to significantly mitigate toxicity of avermectins, as seen in the present study. Additionally, appropriate temporal spacing of treatment administration of avermectins should be exercised as these are relatively persistent compounds when residing in sediment. This is evident by their relatively high DT_{50} values in sediment. Chemical residence time may mitigate avermectin toxicity; however, this was species-specific, and although 4 months significantly reduced toxicity in *E. estuarius*, this did not occur with *N. virens*. Therefore, generalizations should not be made regarding toxicity reductions due to chemical residence time across species. To be conservative, salmonid aquaculture operations should space the administration of avermectins by more than 4 months (a more specific recommendation would require testing on longer chemical residence time treatments), since no change in toxicity was observed after 4 months of chemical residence time in *N. virens*. It is worth noting also that the extent to which chemical decay of avermectins was involved in the reduction in toxicity seen in *E. estuarius* also remains unknown in the present study. However, the degree to which chemical residence time *v.* chemical degradation contribute to the observed toxicity reduction may not be important since the combination of both gives an idea as to the sum of effects of both mechanisms: this is ultimately what is important when considering an exposure scenario at a given site. Similarly, for sediment TOC, frequently co-occurring sediment characteristics (e.g. smaller particle size) may contribute to toxicity reduction. Therefore, the extent to which TOC is solely responsible for the observed toxicity reduction is debatable. However, it is the sum of effects of all these co-occurring sediment characteristics that takes precedence when considering the toxicity-ameliorating characteristics of any given sediment.

3.3. Recommendations for future studies

The present study takes the initial steps in understanding the effects of 2 toxicity modifying factors, namely sediment organic carbon and chemical residence time, that are relevant to the application of avermectin sea lice chemotherapeutants in salmonid aquaculture net pens. However, there remain data gaps to address with regards to the environmental risk/impact of EB and IVM on salmonid farms, and the importance of toxicity modifying factors for deriving site-specific ecotoxicity guidelines. Firstly, it would be worth designing studies which make a more direct measure of bioavailability and/or gain a more detailed mechanistic understanding of changes in bioavailability of avermectins with alterations in toxicity modifying factors. Secondly, it would also be useful to conduct population level studies of benthic fauna near salmonid aquaculture sites – and perhaps comparing sites with varying characteristics – to evaluate whether avermectin sea lice chemotherapeutant usage is ecologically harmful; therefore, *in situ* studies on ecological impact would be advisable. Thirdly, using environmentally relevant exposure concentration and longer exposure durations may also affect how OC and chemical residence time affect toxicity, and therefore these may be worth investigating. Finally, it may be important to test the effects of avermectin-toxicity in other species of benthic fauna to widen the database on the relative sensitivities of aquatic species to avermectins.

The bioavailability of avermectins with contaminant-sediment contact time in aquatic sediment exposures has not been well investigated. While this study does address the toxicity (indirectly measuring bioavailability) that occurs at varying sediment aging and OC co-treatments, it does not directly measure bioavailability. For example, most bioavailability studies with persistent organic pollutants measure the BAF or other tissue burden parameters of contaminants, to understand how much a contaminant is still absorbed upon varying conditions. Additionally, to get mechanistic insight into how these 2 factors affect bioavailability, measuring the concentration of organic- and inorganic-avermectin adducts using analytical chemistry techniques may be helpful. For example, one could measure the level of avermectin adducts compared to unbound avermectin and compare that with changes in bioavailability. This would help determine whether bioavailability may be attributed to the formation of organic- or inorganic-adducts, and to what extent organic v. inorganic adducts play a role in changes in bioavailability of avermectins. Furthermore, with regards to chemical residence time's effect on bioavailability, one could investigate to what extent the decrease bioavailability is the result of sequestration of contaminants into physically inaccessible parts of the sediment matrix. To achieve this, one could

use harsh chemical extraction techniques, to estimate the physically available fraction of avermectins. This would provide a more detailed understanding as to the mechanism involved in changes in bioavailability with increasing chemical residence time. Overall, future studies would be advised to build on the present research by elucidating the bioavailability alterations of avermectins that occur with varying OC and chemical residence time, or other toxicity modifying factors, in the context of sediment exposures with benthic fauna. This would be invaluable for site-specific assessment of avermectin sea lice chemotherapeutant usage.

Investigating the ecological impact of avermectin usage on aquaculture with population level studies is challenging, but necessary. If no population level effects can be discerned from population level studies of a wide variety of species from all trophic levels, then the ecological effects of avermectin usage on aquaculture may be negligible. Without large scale, multi-species ecological effects testing, it remains difficult to determine the ecological impact of avermectin usage as sea lice chemotherapeutants on salmon farms. Additionally, it may be helpful to collect sediment from underneath aquaculture operations and conduct laboratory testing with that sediment. Site-specific sediment toxicity studies could then be conducted, also evaluating the effects of toxicity modifying factors in these studies.

Exposure durations were acute in the present study, and future studies would be advised to conduct sub-chronic and chronic exposure durations; longer durations would reflect more ecologically relevant exposure scenarios, with lower concentrations used. Exposure duration and chemical concentration might even impact the extent to which OC and chemical residence time could alter toxicity. For example, the adsorption dynamics of a compound may change with concentration of a contaminant. Additionally, it may be helpful to conduct toxicity tests with organisms of other taxa, such as sediment-dwelling vertebrates (e.g. demersal fish species) in future studies. In general, there is a lack of toxicity literature on sediment exposures with avermectins with sediment-dwelling aquatic vertebrates (Lumaret et al. 2012). Benthic vertebrates may forage on or near the sediment increasing risk of exposure to contaminants which primarily build-up in sediment, like avermectins. Furthermore, when ecological risk assessment is carried out, it is helpful to have data available for a wider array of taxa, as this helps identify particularly sensitive taxa. It is also possible the extent to which OC can be protective may be different across taxa. For example, perhaps feeding habits would affect route of exposure, and this could affect the extent to which sediment OC is protective against avermectin toxicity. Or maybe dermal exposure may be affected by different epidermal casings which vary across taxa.

To summarize, the following investigations are warranted with regards to avermectin sea lice chemotherapeutant usage on salmon farms: (1) gaining mechanistic insights into of how toxicity modifying factors alter bioavailability; (2) conducting environmental impact assessments, characterizing population level effects and gauging the resulting ecological disturbance; (3) using more environmental realistic exposure scenarios such as by using site-specific sediment for toxicity testing, or using lower concentrations for longer exposure durations; (4) investigating toxic thresholds in other species to widen the database, helping in identifying particularly sensitive species. There remain many avenues of research for avermectin usage in salmonid aquaculture.

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Appendix A.

Characteristic	CB	TM
OM (%)	<1.0	2.8
TOC (mg/kg)	< 500	4200
pH	7.3	7.82
Sand/silt/clay	78/15/7	95/3/2

Table A.1. Summarizing analysis of sediment characteristics for centennial beach (CB; 0-OC) sediment and Tofino Mudflats (TM; 1-OC) sediment. OM denotes organic matter (limit of detection is 1.0%); TOC denotes total organic carbon (limit of detection is 500 mg/kg). Sand/silt/clay is the % of each respective grain size (below are graphs showing the distribution).

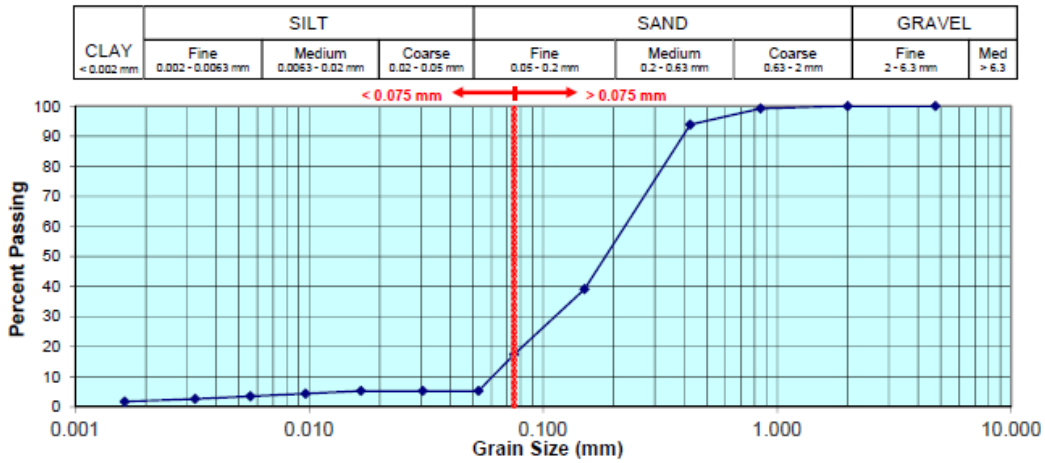

Figure A.1. Graph showing particle-size distribution graph of centennial beach sediment (0-OC).

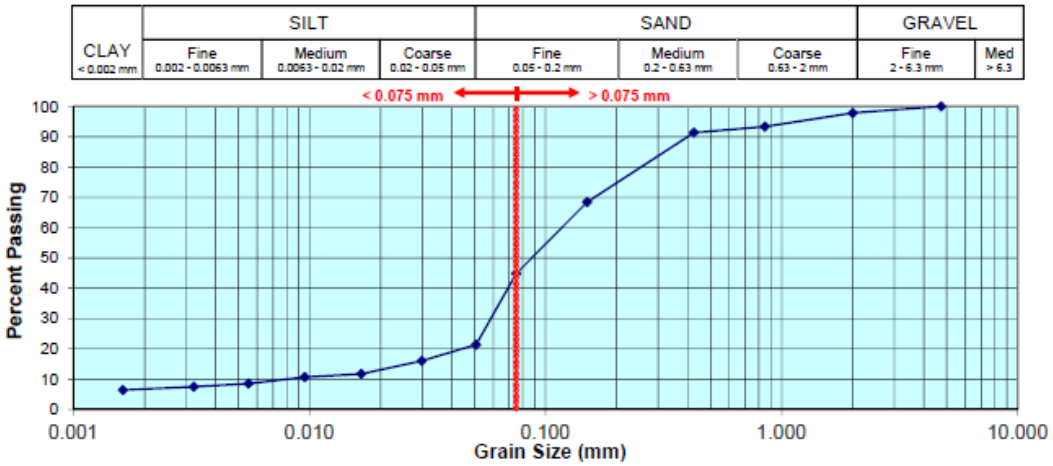

Figure A.2. Graph showing particle-size distribution of Tofino Mudflats Sediment (1-OC).

E. estuarius

Group (T-OC)	Temperature (°C)	pH	DO (%)	Salinity (ppt)
0-0	13.43 ± 0.43	7.07 ± 0.08	83.36 ± 1.30	29.27 ± 0.57
	n=50	*n=32*	*n=50*	*n=50*
0-0.5	13.44 ± 0.18	6.72 ± 0.15	78.06 ± 3.32	29.11 ± 0.58
	n=50	*n=32*	*n=50*	*n=50*
0-1	13.29 ± 0.29	7.22 ± 0.195	73.00 ± 3.72	29.00 ± 0.35
	n=62	*n=56*	*n=62*	*n=62*
2-0	9.48 ± 0.13	-	99.00 ± 1.35	30.56 ± 0.83
	n=12	-	*n=13*	*n=24*
2-0.5	11.38 ± 0.36	-	99.86 ± 0.67	29.83 ± 0.42
	n=55	-	*n=56*	*n=56*
2-1	11.82 ± 0.13	-	98.93 ± 1.09	28.58 ± 0.30
	n=28	-	*n=28*	*n=28*
4-0	8.21 ± 0.19	-	93.27 ± 0.94	31.24 ± 0.79
	n=30	-	*n=30*	*n=30*
4-0.5	9.61 ± 0.44	-	94.79 ± 0.44	28.78 ± 0.51
	n=24	-	*n=24*	*n=24*
4-1	-	-	-	-

Table A.2. Mean values for water quality parameter measurements (± 95% confidence intervals) for 48-hour single-chemical and combination exposures with *E. estuarius*. Data is shown for each of the nine chemical residence time and OC treatment groups (T-OC). Data from measurements before and after exposure are pooled. Dashes refer to missing data.

N. virens

Group (T-OC)	Temperature (°C)	pH	DO	Salinity
0-0	9.41 ± 0.10	7.47 ± 0.14	92.60 ± 0.80	30.12 ± 0.48
	n=20	*n=20*	*n=20*	*n=20*
0-0.5	11.43 ± 0.15	-	82.2 ± 1.74	30.02 ± 0.69
	n=20	-	*n=20*	*n=20*
0-1	9.07 ± 0.11	8.07 ± 0.05	88.46 ± 1.97	29.27 ± 0.45
	n=52	*n=52*	*n=52*	*n=52*
2-0	8.1*	-	100*	30.49 ± 0.56
	n=2	-	*n=2*	*n=14*
2-0.5	9.33 ± 0.15	-	94.63 ± 2.41	28.75 ± 0.34
	n=16	-	*n=16*	*n=16*
2-1	8.65 ± 0.16	-	95.85 ± 1.59	30.24 ± 0.68
	n=37	-	*n=26*	*n=42*
4-0	8.87 ± 0.16	8.06 ± 0.05	89.84 ± 3.87	29.41 ± 0.63
	n=44	*n=44*	*n=44*	*n=44*
4-0.5	8.80 ± 0.05	8.07 ± 0.05	91.03 ± 1.21	29.38 ± 0.53
	n=78	*n=52*	*n=36*	*n=65*
4-1	8.97 ± 0.09	8.02 ± 0.06	87.75 ± 2.73	29.18 ± 0.84
	n=52	*n=20*	*n=52*	*n=50*

Table A.3. Mean values for water quality parameter measurements (± 95% confidence intervals) for 10-day single-chemical and combination exposures with *N. virens*. Data is shown for each of the nine chemical residence time and OC treatment groups (T-OC). Data from measurements before and after exposure are pooled. Dashes refer to missing data. *95% confidence intervals could not be calculated due to insufficient replicates.

Group		*E. estuarius*	*N. virens*
OC group	T (months)	Mortality (%) (95 % CIs)	Mortality (%) (95 % CIs)
0	0	3.3 (-2.1-8.8)	0.0
0.5	0	1.7 (-2.6-6.0)	0.0
1	0	0.0	0.0
0	2	0.0	16.7 (-4.8-38.2)
0.5	2	1.7 (-2.6-6.0)	8.3 (-7.7-24.3)
1	2	3.3 (-2.1-8.8)	8.3 (-7.7-24.3)
0	4	8.3 (-5.6-22.3)	0.0
0.5	4	1.7 (-2.6-6.0)	0.0
1	4	0.0	0.0

Table A.4. Showing mean control mortality of *E. estuarius* (n=6) and *N. virens* (n=12) in each organic carbon (OC) and chemical residence time (T) group. OC is unitless (relative scale). 95% confidence interval (95CI) of mean mortality are given in brackets.

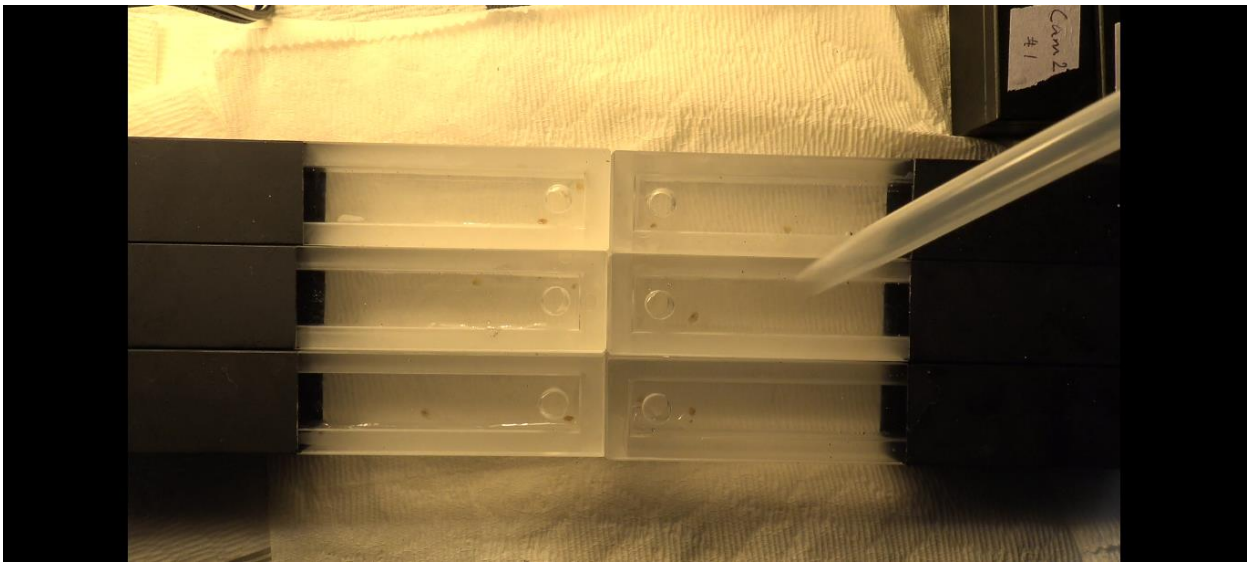

Figure A.3. *E. estuarius* light-dark assay set-up showing one group of 6 light-dark blocks (next to another group of 6, only the corner of one box is shown of the other group of 6 blocks, in the top right corner of the image). Two *E. estuarius* are placed in each light-dark block.

Appendix B.

	p-value	
Time-OC	EB	IVM
0-0	0.8773	0.0036*
0-0.5	0.0025*	0.0348*
0-1	0.0131*	0.0419*
2-0	<0.0001*	0.0049*
2-0.5	0.4021	0.4486
2-1	0.4546	0.0124*
4-0	0.3887	0.4524
4-0.5	0.4577	0.7342
4-1	0.9711	0.608

Table B.1. A list of ANOVA p-values associated with the linear concentration-response fits for EB and IVM exposures with *E. estuarius* for each chemical residence time and OC treatment combination (Time-OC) that are shown in Figure 21 and Figure 22.

Appendix C.

Supplementary Data Files

Description:

The accompanying Excel spread sheets contain raw data from toxicity tests with amphipod and polychaete test species (*Eohaustorius estuarius* and *Neanthes virens*, respectively). These data sheets show mortality and sublethal endpoint data. Refer to data sheet files for definitions/descriptions of each column heading.

Filenames:

AmphipodDataMastersheet.xlsx

PolychaeteDataMastersheet.xlsx