The sublethal toxicity of the sea lice chemotherapeutants SLICE® and Ivermectin to juvenile Starry Flounder (*Platichthys stellatus*)

by

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Abstract

Open net-pen Atlantic Salmon (*Salmo salar*) aquaculture is an important part of Canada's aquaculture industry. SLICE® (a.i.: emamectin benzoate [EMB]) and ivermectin (IVM) are two chemicals used in salmon aquaculture to treat and prevent sea lice infestations that can adversely affect fish health and yield. The sublethal toxicity of these chemotherapeutants on behavioural, physiological and biochemical endpoints was assessed using juvenile Starry Flounder (*Platichthys stellatus*) exposed to treated sediment. Significant concentration-dependent reductions in burst swim performance and aerobic scope were found when fish were exposed to IVM and the combination IVM/EMB. Significant reductions in burrowing behaviour, evidence of avoidance of treated sediment, and significant increases in darkened skin colouration and reduced camouflage ability were also observed for exposures to EMB, IVM and the combination of both at concentrations that may be found in the environment. These findings will lead to developing guidelines for acceptable environmental chemotherapeutant concentrations protective of wild flatfish.

Keywords: aquaculture; sea lice; SLICE®; emamectin benzoate; ivermectin; starry flounder

Dedication

I dedicate this thesis to my wife, Chantel, thank you for your unwavering patience, love and support the entirety of my time getting this degree. I could not have done this without you. I look forward to now supporting you in getting your MBA. Also, to our future child baby King (due June 2023).

I also dedicate it to my mother-in-law Valorie, who passed during the writing of this thesis, and my dog Sage, who was born and passed while completing this degree. I think of you and miss you every day. I wish you both could be here to see what my life is like without a thesis.

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List of Acronyms

α-MSH	α-Melanophore stimulating hormone
AI	Active ingredient
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AS	Aerobic scope
AST	Aspartate aminotransferase
B/B0	Bound sample/maximum bound
BBAMP	Boundary Bay Assessment and Monitoring Program
BBB	Blood-brain barrier
BC	British Columbia
BL	Body lengths
CA	California
CAT	Catalase
CI	Confidence interval
CNS	Central nervous system
DFO	Fisheries and Oceans Canada
EC_{25}	Effective concentration affecting 25% of test organisms
EC_{50}	Effective concentration affecting 50% of test organisms
ELISA	Enzyme-linked immunosorbent assay
EMB	Emamectin benzoate
EQS	Environmental quality standard
GABA	γ-Amino butyric acid
$GABA_AR$	γ-Amino butyric acid A receptor
GPx	Glutathione peroxidase
HPLC	High-performance liquid chromatography
HSI	Hepatosomatic index
IQR	Interquartile range
IVM	Ivermectin

K	Fulton's condition factor
K_n	Relative condition cactor
LC_{50}	Concentration lethal to 50% of test organisms
LDH	Lactate dehydrogenase
Log K_{ow}	The log of the ratio of a chemical concentration in octanol and water at equilibrium at a specified temperature. This value is used as a measure of hydrophobicity.
MCH	Melanin concentrating hormone
MI	Michigan
MIF	Melanization inhibiting factor
MMR	Maximum metabolic rate
MO_2	Oxygen consumption rate (mg O_2 kg^{-1} h^{-1})
MS222	Tricane methanesulfonate
MSF	Melanization stimulating factor
N_2	Nitrogen
NAChR	Nicotinic acetylcholine receptor
O_2	Oxygen
AS	Aerobic scope
Pgp	P-Glycoprotein
$q_{0.2}$	20% quantile
QC	Quebec
REML	Restricted maximum likelihood
ROS	Reactive oxygen species
SEPA	Scottish Environment Protection Agency
SFU	Simon Fraser University
SMR	Standard metabolic rate
SOD	Superoxide dismutase
U_{burst}	Burst swimming rate (BL s^{-1})
VA	Vancouver Aquarium
VIE	Visible implant elastomer
UV	Ultraviolet

WA	Washington State
w/v	Weight/volume
w/w	Weight/weight

Chapter 1. General Introduction

1.1. Aquaculture and Fisheries

Capture fisheries and aquaculture contribute significantly to food and economic security globally, providing primary sources of animal protein, essential nutrients and income (Welcomme et al., 2010). In 2020, capture fisheries and aquaculture combined were the second largest producer of protein derived from animals (13,950 kt; 17%), only behind chicken (15,366 kt) and ahead of beef and pork (FAO, 2023, 2020a, 2020b). In addition to crude protein, food from the sea contains bioavailable micronutrients and essential fatty acids that are not easily found in land-based foods, and thus an important contributor to global nutrition (Hicks et al., 2019). Capture fisheries have been an important sector in global food supply, both economically and for food security, for thousands of years. Farming of aquatic animals has been practiced for over 2,000 years but did not contribute significantly to global meat production until the advent of commercial aquaculture in the twentieth century (Stickney and Treece, 2012). Global aquaculture production has increased rapidly since the 1950s, while the production of capture fisheries has been relatively constant with no trend of increase since the early 1990s (See Fig. 1-1; FAO, 2023). Capture fisheries have also been increasingly unsustainable over time, with an estimated 10% of fisheries unsustainably harvested in 1974, to 35.4% in 2019 (FAO, 2022). While capture fishery production has stagnated and become increasingly unsustainable, aquaculture increased to the point of exceeding capture fisheries production for human consumption in 2016 (See Fig. 1-1; FAO, 2023). Although production of wild capture fisheries is approaching its ecological limits, current mariculture (marine aquaculture) production is far below its ecological limits and could be increased sustainably through policy reforms, technological advancements and increased demand (Gentry et al., 2017). As the global human population continues to increase, with an estimated growth to between 9.2 to 9.5 billion by 2050, and capture fishery production remains constant or even declines, sustainable aquaculture is going to be critical component of the global food supply in the future (Costello et al., 2020; Engle, 2016; FAO, 2023, 2022). Globally, aquaculture is the fastest growing food producing industry, contributing over 82 million tonnes of seafood annually, worth US $250 billion (FAO, 2020b).

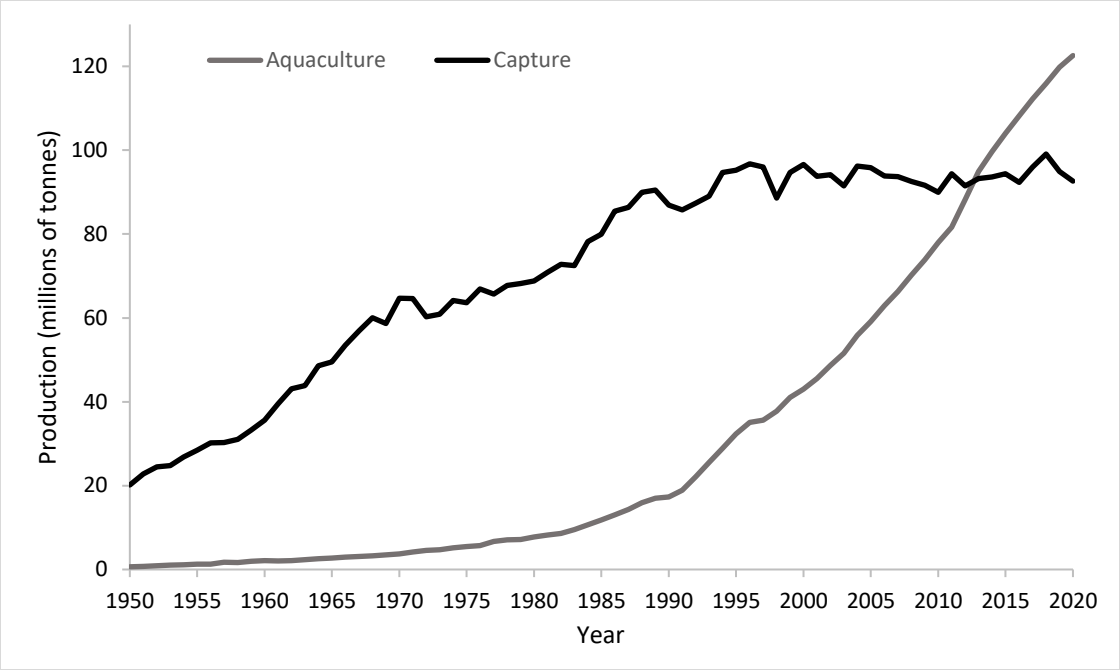

Figure 1-1. World production of capture fisheries and of aquaculture from 1950 to 2020 (Source: FAO, 2023)

1.2. Atlantic Salmon Aquaculture

Finfish are a substantial contributor to global aquaculture production for human consumption at 40%, or 5582 kt, in 2018 (Boyd et al., 2022; FAO, 2023). Within the finfish sector, Atlantic Salmon (*Salmo salar*) aquaculture is the fourth largest producer at roughly 5% or 301 kt in 2018 (Boyd et al., 2022; FAO, 2023). Several nations produce *S. salar* using marine open-net pen aquaculture, with the largest producers in order (See Fig. 1-2) being Norway (1,400,000 t), Chile (787,131 t), the United Kingdom (193,000 t), Canada (120,427 t) and Faroe Islands (88,950 t; FAO, 2023). The production of farmed Atlantic Salmon has risen dramatically in recent decades with 223,892 produced in 1990 to 2,721,876 t in 2020 (FAO, 2023). With improved technology, animal husbandry practices and site selection, the scale of salmon farms has also increased over time. Farms in the 1970s and 1980s raising tens of thousands of fish per farm with farms scattered over a large geographic area, whereas farms today can cover many hectares of nearshore coastal area and raise hundreds of thousands of fish per farm site (Milewski, 2001).

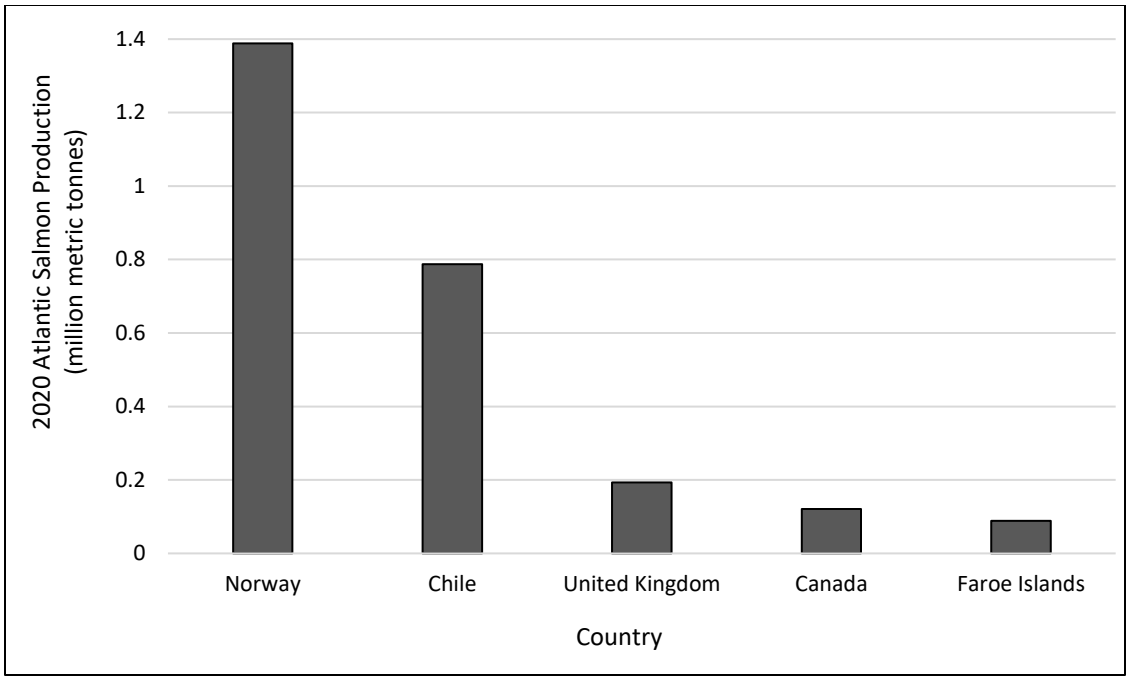

Figure 1-2. World production of Atlantic Salmon from aquaculture in 2020 (Source: FAO, 2023)

In Canada, Atlantic Salmon open-net pen aquaculture is an important industry. Atlantic Salmon mariculture started in the Maritime provinces of Canada, due to sheltered and productive coastal areas well-suited to open-net pen aquaculture operations, and for similar reasons is now in coastal British Columbia (BC), with BC now the largest producer (Statistics Canada, 2022). In Canada in 2021, salmon aquaculture (97% Atlantic Salmon) provided approximately 10,000 jobs which amounted to CAD $146 million in paid salaries (Statistics Canada, 2022). Atlantic Salmon aquaculture in Canada has some of the greatest economic value compared to other species produced using aquaculture, where the value of salmon produced amounted to almost CAD $ 1 billion, with 70% of production coming from BC (Statistics Canada, 2022, 2021a). Most salmon produced with aquaculture is exported to the United States (US), China and Japan (MAL, 2019). Since the mid-2000's BC's farmed *S. salar* production has steadily increased, whereas the production from the Maritime provinces has remained relatively constant (See Fig. 1-3; Statistics Canada, 2022). Atlantic salmon farms in BC have gone from 5 net pen sites in 1984 to 86 active site licenses operated by three multinational Norwegian companies (Mowi Global, Cermaq, Marine Harvest and Grieg Seafood; DFO, 2023). The increase in production in BC since the 1990s is largely responsible for the growth in the industry nationally (See Fig. 1-3). As of June 2021, there were 77 active marine finfish aquaculture

facilities and 34 inactive licensed facilities in BC (DFO, 2021), many of which are in isolated coastal and rural communities. This industry has provided employment opportunities in managing the facilities and fish health, harvesting and processing and economic security for these communities, with partnerships with local First Nations in some cases.

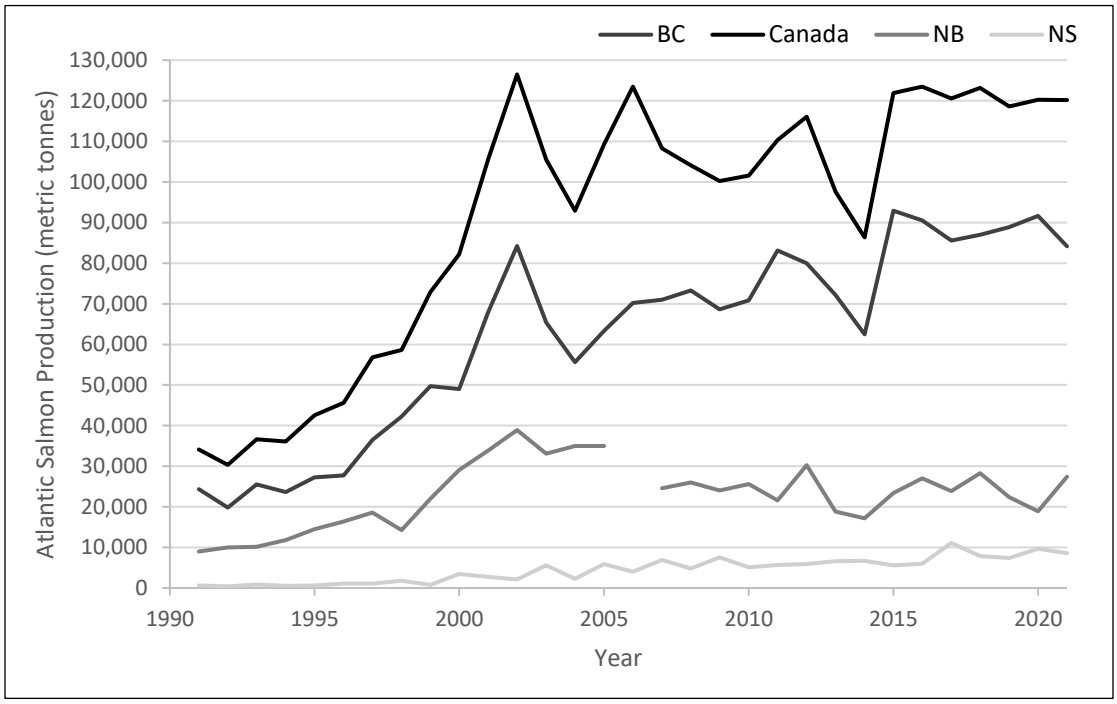

Figure 1-3. Atlantic Salmon production in Canada by year for Canada, British Columbia (BC), New Brunswick (NB) and Nova Scotia (NS).

Open-net pen Atlantic Salmon aquaculture in BC has become a contentious industry with increasing public, scientific and regulatory scrutiny. This scrutiny is largely due to the suspected impact of these aquaculture facilities on wild Pacific salmon, along with other ecological impacts. Potential environmental impacts of open-net pen aquaculture include organic and nutrient enrichment from feed and feces released into the environment, eutrophication, hypoxia, escaped non-native fish, impacts to other large-bodied marine fauna (e.g., seals, whales and birds), unsustainable harvest of wild forage fish species used in the feed, and alteration of the community of primary and secondary producers leading to alterations in the trophic ecology around the net pens (Milewski, 2001; Wang et al., 2013; Weston, 1986; Wildish and Pohle, 2005; Winsby et al., 1996). Another primary

concern associated with open-net pen aquaculture is the altered timing and increased abundance of ecto-parasitic sea lice and the consequential impacts that change in copepod abundance can have. Sea lice and their associated pathogens can spread from Atlantic Salmon farms to wild Pacific salmon, including those at immature life stages (Bateman et al., 2016; Mordecai et al., 2021). Sea lice can also reduce the health and yield of Atlantic Salmon in the net pens (Johnson et al., 2004). To address sea lice infestations as well as secondary infections that can occur due to sea lice parasitism, chemotherapeutic intervention is often used. Release of these chemotherapeutants into the surrounding marine environment both directly and indirectly excreted from the Atlantic Salmon is another environmental concern associated with this method of aquaculture. Toxicants released from open-net pen facilities in this way can include pesticides, antibiotics, anti-fouling agents and heavy metals (Brooks et al., 2002; Milewski, 2001; Weston, 1986).

In response to these concerns, Atlantic Salmon aquaculture licenses in key salmon migration corridors, such as the Discovery Islands region, have not been renewed, and licenses in other areas are receiving shorter-term renewal periods in BC (Cruickshank, 2022). These measures were to allow for review and consultation by Fisheries and Oceans Canada in creating a transition plan toward land-based aquaculture operations rather than open-net pens. The BC Salmon Farmers Association released an independent economic analysis outlining the consequences to BC's indigenous and non-indigenous coastal communities if 79 farming licenses are not reissued by the federal government. The report found BC would lose more than 4,700 jobs, $1.2 billion in economic activity annually and $427 million in GDP if the licenses were not renewed (RIAS Inc., 2022). An independent economic analysis of the viability of salmonid farming using recirculating aquaculture systems (RAS) found that the transition to land-based aquaculture is unlikely to be economically viable and attract investors required for the transition (Counterpoint Consulting, 2022). While there are concerns surrounding the socioeconomic impacts of ending or reducing open-net pen Atlantic salmon aquaculture tenures, there is a valid public and scientific concern based on recent findings (Bateman et al., 2022, 2016; Mordecai et al., 2021) surrounding the potential effects of these farms on wild Pacific salmon due to an increase in sea lice abundance. Thus, further investigation of the effects Atlantic Salmon aquaculture on sea lice abundance and the impacts of that, as well as mitigation measures to reduce sea lice abundance, on wild fauna are warranted.

1.3. Sea lice

There are two species of ectoparasitic lice commonly found on the Atlantic Salmon reared in open-net pens: *Lepeophtheirus salmonis* and *Caligus clemensi*. The difference between the two is that *L. salmonis* is only able to complete its life cycle and produce eggs on salmonids, while *C. clemensi* is a host generalist. Coastal Atlantic salmon farms are the primary source of heavy *L. salmonis* infestations on wild juvenile salmon in the Broughton Archipelago, BC, where most related studies have been completed (Jones and Hargreaves, 2007; Krkosek et al., 2007; Marty et al., 2010; Morton et al., 2008). Sea lice attach to the host skin and feed on the tissue, mucus and blood. Parasitism by ectoparasitic copepods such as sea lice causes skin lesions, osmoregulatory issues, reduce host fecundity, growth and survival; as well, they impair salmonid immune function, whereby sea lice can increase fish susceptibility to other pathogens either directly from sea lice as a disease vector or indirectly from infection of the skin lesions (Barker et al., 2009; Jakob et al., 2011). Pathogens of concern associated with sea lice infection for both wild and farmed salmon include piscine orthoreovirus (PRV) which causes heart and skeletal muscle inflammation (HSMI) in salmonids, infectious salmon anemia virus (ISAV), infectious haematopoietic necrosis virus (IHNV) and salmon leukemia virus (Dill, 2017; Garseth et al., 2013; Wessel et al., 2017).

The presence of Atlantic Salmon farms change the normally cyclical nature of sea lice abundance altering their location, abundance and timing such that it leads to an increase in exposure of sea lice to out-migrating wild juvenile salmonids. Atlantic Salmon farms disrupt migratory allopatry of wild salmon and sea lice, where sea lice abundance increases with adult spawning fish abundance and decreases as they enter their natal streams to spawn and die. This timing normally protects out-migrating juvenile salmon from exposure due to different migration times where the two groups do not interact directly (Bateman et al., 2016). However, the open-net pen Atlantic Salmon farms placed in sheltered coastal waters are within the migration route of the wild salmon which provides a host population that would not normally exist in the fall and winter to retain and grow the sea lice population over the winter months which can then infect out-migrating juvenile salmon in the spring. The presence of reservoir hosts of domestic animal populations can increase the impacts of parasites on wildlife, as they remove the usual density-dependent mechanisms that regulate epizootics (De Castro and Bolker, 2005). Studying the population- or ecosystem-level impacts of an increase in sea lice infestations on Pacific

salmon is technically and logistically challenging due to the broad range of interspecific variability in life history traits including age, migration timing, migration route and freshwater and oceanic residence time. However, studies have assessed the direct effect of sea lice infestation on juvenile salmonid survival for several species, and correlative analyses using salmon spawner abundances have inferred potential population-level impacts. Recent research suggests that infections by *C. clemensi* can reduce the ability of juvenile Sockeye Salmon to compete for food and thus reduces their growth (Godwin et al., 2017, 2015). Direct evidence has been demonstrated for the increased mortality of juvenile salmonids due to sea lice for Chum Salmon (*Oncorhynchus keta*), Pink Salmon (*Oncorhynchus Gorbuscha*), and Coho Salmon (*Oncorhynchus kisutch*; Krkosek et al., 2011, 2006; Morton and Routledge, 2005). Krkosek et al (2011b) used sea lice data on farms and spawner-recruit data for *O. gorbuscha* and *O. kisutch* salmon populations in the Broughton Archipelago compared to nearby regions without farms present and found a negative correlation associated with sea lice abundance on farms and the productivity of both Pink and Coho Salmon. Although there is compelling evidence of the adverse ecological impacts of the presence of Atlantic Salmon farms on wild salmon due to sea lice, adaptive pest management regimes within the farms changing the timing of sea lice treatments to match the outmigration of juvenile salmonids has been shown to have positive conservation outcomes (Peacock et al., 2013).

In addition to the effects on wild salmon, sea lice infestations can have substantial impacts on fish health and welfare and economically for Atlantic Salmon farms. A 2011 economic analysis of the losses incurred by the salmon farming industry due to sea lice by Abolofia et al (2017) found that the losses from typical sea lice infestations with pest management strategies in place would be 9% of farm revenue, and in 2011 US $600 M in damages were incurred by the industry globally. The Canadian aquaculture industry spent CAD $37 M on therapeutants in 2021, many of which were applied to open-net pen Atlantic Salmon farms (Statistics Canada, 2021). Due to the risk to wild salmon, the health of farmed salmon, and the potential economic impacts of sea lice infestations, a robust pest management regime with good husbandry practices are required, including pesticide treatments targeting sea lice.

1.4. Sea lice management

The permanent presence of open-net pen salmon farms in sheltered coastal areas leads to a host reservoir for sea lice year-round. In response to the risks associated with sea lice infestations, strict management strategies have been implemented in BC including improved animal husbandry, required monitoring of sea lice and mortalities as well as reporting of health status and inventory of fish to DFO on a monthly basis (DFO, 2017). A regulatory threshold of 3 motile sea lice per fish permits implementation of management procedures to provide immediate control of infestations. The aquaculture industry relies heavily on the use of chemotherapeutants to reduce sea lice parasitic loads either with in-feed, or bath treatments. Additionally, farms in Canada are required to report the timing and quantity of all chemotherapeutant applications to treat sea lice (Hamoutene et al., 2022).

There are two modes of application of pesticides used to treat sea lice infestations in Atlantic Salmon aquaculture, one where the chemotherapeutant is applied to the fish feed and the other where the fish are treated by immersion in an isolated bath with the therapeutant added. In Canada, the in-feed treatments are controlled by the Veterinary Drugs Directorate under the *Food and Drugs Act*, which provides regulations associated with antibiotic use, and the topical bath treatments are managed through the *Pest Control Products Act* through the Pest Management Regulatory Agency (DFO, 2019; ENV, 2019). Administration of chemotherapeutants in Atlantic Salmon aquaculture facilities required a prescription form a licensed veterinarian. Depending on physical and chemical properties of the pesticide used and, the mode of application, the chemotherapeutant is ultimately released into the water column and may become associated with sediment in the benthic environment around the net pens. Anti-sea lice chemotherapeutants lack the specificity of some agriculture pesticides used in terrestrial applications, and due to this and the direct release into the surrounding environment, there are concerns associated with their effect on non-target marine fauna in the benthic and pelagic environments.

A broad range of chemotherapeutants have been used in open-net pen aquaculture to treat salmonids for sea lice including organophosphates, pyrethroids, chitin synthase inhibitors, hydrogen peroxide and avermectins. Many of these therapeutants have are no longer in use or not renewed for use in Canada due to low efficacy, the development of resistance by sea lice or low therapeutic indexes, where the treatment can be harmful to the fish being administered therapeutant. The Canadian Aquaculture

Activities Regulations require that each licensed marine finfish farm reports on usage of antibiotics and pest control products (DFO, 2020a). It also requires that every measure to mitigate sea lice infestations must be attempted prior to the application of chemotherapeutants. Several alternatives are currently being used in a trial or research application including the strategic placement of a culture of filter feeders within a net pen to filter the pelagic microscopic early-life stage of sea lice (Trigo and Mondéjar, 2020), as well as lumpfish used as a cleaner fish for mechanical removal of the sea lice (Imsland et al., 2018; McEwan et al., 2018). Both alternatives to chemotherapeutant use have shown promising results to date for managing sea lice infestations on Atlantic Salmon farms. Chemotherapeutants currently approved for use in Canada include the bath pesticides: Salmosan® (A.I. azamethiphos [AZA]) and Interox Paramove 50® (A.I. hydrogen peroxide [HP]), and the in-feed drugs: SLICE® (A.I. emamectin benzoate [EMB]), IVOMEC® (A.I. ivermectin [IVM]), and Calcide® (A.I. teflubenzuron; DFO, 2020b). Only SLICE® is approved for use to treat sea lice infestations in BC and Interox Paramove 50® is approved on a site-by-site basis, with IVM discontinued in British Columbia after the year 2000 and only used in Atlantic Canada (Chang et al., 2022). The most commonly applied anti-sea lice chemotherapeutants in Canada in recent years has been EMB and AZA, followed by HP and IVM, where the chemotherapeutants are often applied in sequence or several or all are used at a single site in a given year, though only in Atlantic Canada (Chang et al., 2022; DFO, 2020b; Hamoutene et al., 2022). IVM is mostly used to treat smolts due to the slow elimination from tissues to avoid presence in commercially-ready fish tissue, and both avermectins (i.e., EMB and IVM) were applied at some of the same sites in certain years (Hamoutene et al., 2022). The focus of the current study is on the avermectin-type sea lice chemotherapeutants and their use in Canada.

1.5. Avermectins

Avermectins are macrocyclic lactones isolated from the fermentation broth of the soil actinomycete, *Streptomyces avertmitilis*. They are either produced directly by *S. avermitilis* or generated through semisynthetic modifications (Fisher and Mrozik, 1989; Stevens and Breckenridge, 2001). Included in this avermectin group are abamectin and emamectin benzoate, which are used as insecticides, and ivermectin, which is sold for parasite control in human and veterinary medicine. The avermectins are effective in the control of internal and external parasites in a wide range of host species, particularly

mammals (Fisher and Mrozik, 1989). Two avermectin compounds have been used to treat sea lice infestations, IVM and EMB. In invertebrates, the mechanism of action is binding and opening of glutamate-gated chloride channels at inhibitory synapses resulting in an increase in chloride concentrations, hyperpolarization of muscle and nerve tissue, and inhibition of neural transmission leading to erratic movement, convulsions and eventually paralysis and death (Grant, 2002; Roy et al., 2000). Avermectins are substantially more toxic to invertebrate taxa than to vertebrates. This is due to the high binding affinity of avermectins to the invertebrate-specific glutamate-gated chloride channels, and the lack of a blood brain barrier (BBB) to exclude avermectins from the central nervous system (CNS).

In vertebrates, avermectins can exhibit CNS toxicity through reactions at the receptor for the inhibitory neurotransmitter γ-amino butyric acid (GABA) (Stevens and Breckenridge, 2001). The avermectins open the $GABA_A$ receptor chloride channel by binding to the receptor and acting as partial agonists (Abalis et al., 1986). Chloride ions then flow into the postsynaptic neuron leading to hyperpolarization of the neuron and a dampening effect on nerve impulse firing. Avermectin intoxication in vertebrates begins with tremors, and incoordination, which later develops into ataxia and coma-like sedation (Stevens and Charles, 2001). This is similar to the mode of action of ethanol, barbiturates (Eldefrawi and Eldefrawi, 1987), and benzodiazepine sedatives (Williams and Yarbrough, 1979). As one of the predominant inhibitory neurotransmitters in the vertebrate brainstem and spinal cord, GABA is critically important for the regulation of the circuitry underlying locomotor behaviours such as escape response, swimming, metabolic capacity and control, as well as vision and visual responses (Estrada-Mondragon and Lynch, 2015; Marc et al., 1978; Powrie et al., 2022; Saint-amant and Drapeau, 2000; Sajovic and Levinthal, 1983; van Ginneken et al., 1996; Yan et al., 2017; Zajic and Podrabsky, 2020). Excessive activation of $GABA_A$ receptors from the continued binding of avermectins can lead to toxic effects related to that inhibitory signalling including sedation and muscle relaxation (Estrada-Mondragon and Lynch, 2015).Vertebrate intoxication by avermectins is highly dependent on the presence of an intact P-glycoprotein (Pgp) BBB, especially for IVM which more readily crosses the fish BBB compared to EMB (Høy et al., 1990; Sevatdal et al., 2005; Stevens and Breckenridge, 2001). This has been demonstrated by increased CNS entry and toxicity observed in mammals with a knockout mutation leading to deficiency in Pgp, and for increased CNS entry and toxicity observed in teleost fish, which have a more permeable BBB (Brown et al., 2018; Stevens and Breckenridge, 2001).

1.5.1. Emamectin Benzoate (SLICE®)

Emamectin benzoate is used globally to treat sea lice infestations in open-net pen salmonid aquaculture as the active ingredient in the formulation SLICE® (0.2% w/w; Schering-Plough, 2000). EMB is comprised of two homologues of avermectin: ≥90% of 9:1 4'- epimethyamino-4'deoxyavermectin B1a benzoate and ≥10% of 4'epimethyamino-4'-deoxyavermectin B1b benzoate. There several are other inactive ingredients of the SLICE® formulation such as cornstarch, maltodextrin, butylated hydroxyanisole, and propylene glycol (2.5% w/w), which is the carrier molecule for EMB and may serve to increase bioavailability and binding to food pellets when applying to feed. EMB, like all avermectins, exhibits a broad spectrum of activity against nematodes and arthropods, with the B1a compound being the most efficacious for control of a variety of terrestrial and aquatic pest species (Bright and Dionne, 2005; Korystov et al., 1999). SLICE® has been approved for use in Atlantic Salmon aquaculture in Canada since 2009 (Chang et al., 2022). EMB is administered to farmed salmon orally using their feed at a targeted dose of 50 µg EMB per kg of salmon daily for 7 d repeated in three to five treatments over a 2-year fish growth period provides protection from sea lice infection for up to 69 d (Schering-Plough, 2000). EMB is taken up by sea lice from fish skin and mucous, and exert their antiparasitic actions through irreversible activation of glutamate-gated chloride channels at invertebrate inhibitory synapses (Cornejo et al., 2014; Grant, 2002; Roy et al., 2000). Activation of chloride channels in inhibitory neurons results in the hyperpolarization of muscle and nervous tissue, and inhibition of neural transmission leading to sea lice paralysis and death (Grant, 2002; Roy et al., 2000).

EMB enters the receiving marine environment surrounding the net pens by deposition of uneaten food pellets treated with EMB, or fecal matter containing EMB, to the sea floor below. Feed waste has been shown to be as high as 15-20% of total feed used and 162 g of feces are produced for each kg of salmon (Milewski, 2001). Given that most of the EMB and IVM applied to Atlantic Salmon feed is released as uneaten pellets or unmetabolized in feces, this could lead to a large amount released into the area around the net pens (Davies and Rodger, 2000; Høy et al., 1990; Olsvik et al., 2008; Shaikh et al., 2007; Wang et al., 2013). EMB has a low water solubility ranging from 5 to 24 mg L^{-1} depending on salinity and a high affinity for organic material with a higher octanol-water partition coefficient (log K_{ow}) of 5.0, which suggests that the major portion of environmental releases will partition to, or remain in, suspended and settled particles (Bright and Dionne,

2005). Based on the solubility limits once present in benthic substrates there is potential for leaching of up to 5% of EMB or its metabolites from medicated feed or faecal pellets into the water column or sediment interstitial water (Bright and Dionne, 2005). In marine sediments the half-life estimates of EMB have ranged from 225 d to >404 d (Benskin et al., 2016; Scottish Environment Protection Agency (SEPA), 2004). Given long sediment half-lives, as well as strategies of multiple applications within a single year, there is the potential for compounding of the environmental concentrations. This can lead to a prolonged exposure to non-target benthic fauna that may result in toxic effects (Benskin et al., 2016; Davies et al., 1998; Prasse et al., 2009). Reported sediment concentrations of EMB in the environment near open-net salmon pens ranges from non-detectable to as high as 35 to 167 μg kg^{-1} sediment, with the highest concentrations usually measured on the last day of 7 d treatment programs (DFO, 2012; Ikonomou and Surridge, 2013; Veldhoen et al., 2012). Modeled marine sediment concentrations of EMB have been reported as high as 366 μg kg^{-1} (SEPA, 1999). In freshwater sediments downstream of a land-based aquaculture facility in Atlantic Canada, EMB concentrations as high as 2500 μg kg^{-1} were found (Lalonde et al., 2012).

Bioaccumulation is a process whereby a substance is accumulated by organisms directly from the surrounding media and through consumption of food containing the substances. Bioconcentration is a process whereby there is a net accumulation of a substance directly from water into aquatic organisms to higher concentrations than in the source environment. In the absence of bioaccumulation factor (BAF) or bioconcentration factor (BCF) information, the octanol-water partition coefficient (log K_{ow}) may be used to predict bioaccumulation potential. A BAF or BCF of 5000 is typically used as a threshold value whereby a chemical with a lower value is not likely to accumulate in an organism during exposure. A log K_{ow} of 5 is also used as a similar threshold. EMB has not been shown to meaningfully bioaccumulate in exposures to several taxa from different trophic levels (Bright and Dionne, 2005; Chukwudebe et al., 1996). The reason EMB and other avermectins show limited tendency to bioconcentrate or bioaccumulate is potentially due to its large molecular size, and also can be due to the ability of many biota to metabolize them (Bright and Dionne, 2005; SEPA, 1999). Low bioaccumulation potential helps to reduce to risk to non-target marine taxa, however at the concentrations observed in the marine environment and with the persistence of EMB, there is potential for exposure and toxicity to benthic marine taxa, including teleost fish.

With the targeted organisms of EMB being invertebrates, many toxicity studies have focused on lethal and sublethal effects of EMB to invertebrate taxa (Bright and Dionne, 2005; SEPA, 2017). Fewer studies have been completed assessing the toxic effects of EMB on teleost fish, and even fewer yet using exposure conditions, duration and benthic taxa that would align with the physical and chemical properties of EMB that would lead to distribution and persistence in marine sediments. The only study that was found that exposed a benthic marine fish species to an environmentally realistic exposure scenario and concentration was completed in 2021, using Tidepool Sculpin (*Oligocottus maculosus*) to EMB in sediment for 10-d (Strachan and Kennedy, 2021). A summary of toxicity studies assessing the effects of EMB on fish in seawater can be seen in Table 1-1.

Table 1-1. Summary of toxic effect levels of emamectin benzoate and ivermectin to target and non-target teleost fish in seawater.

Spp.	Exp.	Time (d)	Effect	Endpoint	Conc.	Units	Source
Emamectin Benzoate							
S. salar	IP	52	LOEC	Growth	0.4	mg kg^{-1}bw	Skilbrei et al 2015
-	Food	52	LOEC	Growth	0.4	mg kg^{-1}bw	-
-	Food	4	LOEC	Accumulation	0.15	mg kg^{-1}bw	Igboeli et al 2013
C. variegatus	F-T	4	LC50	Mortality	1.43	mg kg^{-1}bw	USEPA 1992
-	F-T	4	NOEL	Mortality	0.86	mg L^{-1}	-
O. mykiss	Food	84	Zero	Mortality	0.05	mg kg^{-1}fd	Roy et al 2006
A. Affinis	S-R	4	IC25	Survival	0.35	mg L^{-1}	Strachan and Kennedy 2021
-	S-R	4	NOEL	Survival	0.3	mg L^{-1}	-
-	S-R	4	LOEL	Survival	0.6	mg L^{-1}	-
P. stellatus	Static	1	LC50	Survival	1208	µg L^{-1}	-
-	Static	1	NOEL	Survival	100	µg L^{-1}	-
-	Static	1	LOEL	Survival	500	µg L^{-1}	-
G. aculeatus	Static	1	LC50	Survival	1310	µg L^{-1}	-
-	Static	1	NOEL	Survival	100	µg L^{-1}	-
-	Static	1	LOEL	Survival	500	µg L^{-1}	-
O. maculosus	Static	1	LC50	Survival	1307	µg L^{-1}	-
-	Static	1	NOEL	Survival	100	µg L^{-1}	-
-	Static	1	LOEL	Survival	500	µg L^{-1}	-

-	Sed	10	LC50	Survival	1980	µg kg^{-1}sd	-
-	Sed	10	NOEL	Survival	50	µg kg^{-1}sd	-
-	Sed	10	LOEL	Survival	100	µg kg^{-1}sd	-
O. gorbuscha	Static	2	LC50	Survival	1090	µg L^{-1}	Sahota et al 2021
-	Static		NOEC	Survival	30	µg L^{-1}	-
-	Static		LOEC	Survival	100	µg L^{-1}	-
-	Sed	10	LC50	Survival	2065	µg kg^{-1}sd	-
-	Sed	10	NOEC	Survival	100	µg kg^{-1}sd	-
-	Sed	10	LOEC	Survival	300	µg kg^{-1}sd	-
-	Static	0.02	NOEC	Avoidance	300	µg L^{-1}	-
-	Static	0.02	LOEC	Avoidance	500	µg L^{-1}	-
-	Static	2	NOEC	Olfaction	750	µg L^{-1}	-
-	Static	7	NOEC	Olfaction	500	µg L^{-1}	-
-	Sed	10	NOEC	Olfaction	1000	µg kg^{-1}sd	-
-	Static	2	NOEC	Swimming	750	µg L^{-1}	-
Ivermectin							
S. aurata	S-R	0.05	LT50	Mortality	5.6	mg L^{-1}	Mladineo et al 2006
-	S-R	2.56	LT50	Mortality	0.1	mg L^{-1}	-
-	Food	10	NOEC	ApoA1 mRNA	0.2	mg kg^{-1}fd	Varo et al 2010
-	Food	10	NOEC	Hb_{β} mRNA	0.2	mg kg^{-1}fd	-
D. labrax	Gavage	40	LD50	Mortality	0.34	mg kg^{-1}bw	Athanassopoulou et al 2002
-	IP	40	LD50	Mortality	0.11	mg kg^{-1}bw	-
-	IP	40	Lethal	Mortality	0.3	mg kg^{-1}bw	-
-	Gavage	40	Lethal	Mortality	0.7	mg kg^{-1}bw	-
-	Food	113	Zero	Mortality	0.5	mg kg^{-1}bw	-
S. salar	Food	30	LD50	Mortality	0.17	mg kg^{-1}bw	Ucan-Marin et al 2012
-	Food	30	LOEC	Weight	0.25	mg kg^{-1}bw	- -
-	Food	30	LOEC	Vitellogenin	0.25	mg kg^{-1}bw	- -
-	Food	30	LOEC	AChE	0.05	mg kg^{-1}bw	-

-	Food	30	NOEC	HSI	0.25	mg kg^{-1}bw	- -
-	Food	30	NOEC	Weight	0.05	mg kg^{-1}bw	- -
-	Food	30	NOEC	Vitellogenin	0.25	mg kg^{-1}bw	
-	Food	30	NOEC	Weight	0.05	mg kg^{-1}bw	
-	Food	30	NOEC	Vitellogenin	0.05	mg kg^{-1}bw	
-	Food	30	NOEC	HSI	0.25	mg kg^{-1}bw	
-	Food	30	NOEC	AChE	0.25	mg kg^{-1}bw	
O. tshawytscha	Food	64	ZERO	Mortality	9.1	mg kg^{-1}bw	Johnson et al 1993
O. kisutch	Food	64	ZERO	Mortality	11	mg kg^{-1}bw	-
L. guttatus	Static	0.63	ZERO	Mortality	4.5	mg L^{-1}	Fajer-Avila et al 2007

Spp.=species, Exp.=exposure, Conc.=concentration, hyphen (-) = species and reference repeated, IP=intraperitoneal, F-T=flow-through, ApoA1=apolipoprotein A1, Hb_{β}= hemoglobin subunit beta, AChE=acetylcholinesterase, HSI=hepatosomatic index, mg L^{-1}= milligrams per Litre of seawater, mg kg $^{-1}$bw= milligrams per kilogram of bodyweight, mgkg^{-1} fd=milligrams per kilogram of food, S-R=static renewal, Sed=sediment, µg kg^{-1} sd=micrograms per kilogram of sediment

In vertebrates, avermectin intoxication begins with tremors and incoordination and later develops into ataxia and coma-like sedation; and it is thought that the effects are mediated by avermectin agonistic binding to $GABA_A$ receptors ($GABA_AR$) (Stevens and Charles, 2001). Common effects observed in fish exposed to EMB have included lethargy, loss of appetite, darkened skin colouration, reduced mobility, erratic swimming, loss of equilibrium, paralysis and in multiple fish species and life stages (Ananda Raja et al., 2020; Roy et al., 2000). In the environmentally realistic exposure completed by Strachan and Kennedy (2021), *O. maculosus* exposed to EMB in sediments for 10-d showed effects on survival observed as low as 300 µg EMB per kg of sediment and found an LC_{50} of 1980 µg kg^{-1} (95% CI 1249-3750). The lowest observed effect concentration (LOEC) was within the range modeled or measured sediment concentrations observed around Atlantic Salmon net pens (DFO, 2012; Ikonomou and Surridge, 2013; SEPA, 1999; Veldhoen et al., 2012). The LC_{50} was an order of magnitude higher than what has been observed in marine sediments around the net pens, but within the range observed downstream of a land-based aquaculture facility in freshwater sediment (Lalonde et al., 2012).

In fish, EMB has been shown to be less toxic and have a higher therapeutic index than IVM, though effects similar to those observed with other avermectin exposures are found at higher concentrations of EMB. This difference in toxicity can potentially be attributed to the difference in distribution into the CNS between EMB and IVM. Høy et al (1990) showed that tritium-labeled IVM readily distributed and accumulated in the brain of Atlantic Salmon, indicating that IVM can cross the blood-brain barrier (BBB). A similar study by Sevatdal et al (2005) assessed the distribution of EMB in tissues of *S. salar* and found relatively little accumulation in the brain with most residues found in the viscera. The findings of Sevatdal et al (2005) show that some EMB can cross the BBB but very little does relative to IVM. These differences in tissue distribution, with more IVM accumulation in the CNS, might explain the difference in toxicity observed for IVM compared to EMB teleost avermectin exposures. The increased accumulation of IVM in the CNS relative to EMB, along with sublethal effects on locomotion observed for IVM and not EMB, corroborates a neurotoxic mechanism of toxic action of IVM. One study that directly compared the safety and efficacy of EMB and IVM applied to medicated feed on *D. rerio* reach similar conclusions with EMB being well tolerated and showing no signs of behavioural effects, including on swimming, and IVM having a low margin of safety and having a concentration-dependent effect on swimming behaviour marked by lethargy, loss of equilibrium and body twitching (Collymore et al., 2014). Data gaps still exist on the long-term effects of EMB in sediments and/or feed to teleosts. Given the amount of EMB potentially released and present in sediments surrounding Atlantic Salmon farms, the persistence of EMB in marine sediments and sublethal effects observed in teleost fish exposed to environmentally relevant concentrations, there is the need for further studies assessing the effects of long-term sediment- or food-based EMB exposures to benthic marine teleosts.

1.5.2. Ivermectin

Ivermectin (22,23-dihydroavermectin B1) is the second avermectin-type chemotherapeutant used to treat sea lice infestations in open-net pen Atlantic Salmon farms in Canada. It available for use in this application as an “off-label” veterinary prescription (i.e., for use in pesticidal applications other than the control of sea-lice). It was formerly used in emergency situations, but recently is more commonly applied in Atlantic Canada, and has not been used in BC in this application since the year 2000 (Chang et

al., 2022; Hamoutene et al., 2022). IVM is a semisynthetic compound derived from avermectin fermentation by-products produced by the soil-dwelling bacterium *S. avermitilis*. IVM is administered to Atlantic Salmon feed as the active ingredient (0.5% w/v liquid) in a formulation called IVOMEC® (Hamoutene et al., 2022; Haya et al., 2005). IVM is a relatively large molecule with a molecular weight of 875.1 g mol^{-1}. IVM has a log K_{ow} of 3.2, indicating some hydrophobicity and a strong binding affinity to organic matter, where it is likely to partition out of the water column and into marine sediments below the net pens. In a study assessing the partitioning of IVM in the marine environment <5% of ivermectin leached off medicated feed for a 48-h period (Davies et al., 1997). This finding and IVM's physicochemical properties suggest that it would adsorb to marine sediments (Davies et al 1997).

IVM is released into the receiving marine environment around the net pens in the same way as EMB through uneaten food pellets and fecal matter containing IVM. In the benthic environment below net pens, IVM can persist for long periods of time with half-life estimates of >188 d (Davies et al., 1998). In instances where IVM was used to treat sea lice infestations, sediment concentrations below the net pens ranged from non-detectable to 6.8 μg kg^{-1} (Cannavan et al 2000), and in another study up to 17.3 μg kg^{-1} (DFO, 1996). Recent use of IVM in farms in Atlantic Canada have increased, so an updated assessment of IVM concentrations in marine sediments is warranted. Direct exposure through the consumption of feed, sediment contact and burrowing, as well as indirect exposures by consumption of contaminated prey organisms are pathways of uptake available to benthic marine fish. The aggregation of benthic fish species and their prey in areas around Atlantic Salmon farms, with the persistence of IVM leave the potential for wild benthic fish to be exposed (Brooks, 1994; Brooks et al., 2002; Costelloe et al., 1998).

In most experiments assessing uptake and accumulation with chronic exposures to IVM, the chemotherapeutants were not found to meaningfully bioaccumulate or biomagnify with increasing concentrations (Chukwudebe et al., 1996; Davies et al., 1997). These findings suggest that IVM is unlikely to bioaccumulate in the tissues of other benthic fauna or biomagnify with increasing trophic level. Limited bioaccumulation potential helps to reduce to risk to non-target marine taxa, however at the concentrations observed in the marine environment and with the persistence of IVM, there is potential for exposure and toxicity to benthic marine taxa, including teleost fish.

Ivermectin-induced toxicity in fish has been studied for several species and life stages and presents in a similar way to EMB with lethargy, loss of appetite, loss of

equilibrium and darkened skin, which are consistent with effects that would be observed from a CNS depressant activating GABAergic neurotransmission (Collymore et al., 2014; Domingues et al., 2016; Johnson et al., 1993; Katharios et al., 2001; Kennedy et al., 2014; Ogueji et al., 2019; Oliveira et al., 2016a; Palmer et al., 1996; Thiripurasundari et al., 2014; Ucán-Marín et al., 2012). Unlike EMB, which has recent studies with a more environmentally realistic exposure scenario, IVM exposures have all been to pelagic species, often in freshwater, with the route of exposure being intraperitoneal injection, or immersion in static, static-renewal or flow-through systems; and by application to feed. Often, they are lethal exposures, when concentrations observed in the environment would be sublethal, and for a short duration relative to the persistence of IVM (See Table 1-1). In fish, IVM has been shown to have a lower therapeutic index than EMB (Collymore et al., 2014). Data gaps still exist on the effects of IVM in sediments and/or feed long-term to benthic teleosts. Given the increased toxicity compared to EMB and lower therapeutic index in fish, recent use of IVM in Atlantic Salmon mariculture on Canada's east coast, and the persistence of IVM in marine sediments, there is the need for further studies assessing the effects of long-term sediment- or food-based to benthic marine teleosts exposed to IVM.

1.5.3. Emamectin Benzoate and Ivermectin Combined Toxicity

Ivermectin and emamectin benzoate are both substrates to P-glycoprotein (Pgp) transporters which are efflux transporters found on the BBB, key in reducing xenobiotic toxicity in the CNS (Kennedy et al., 2014; Stevens and Charles, 2001). Both chemotherapeutants also can lead to toxicity in the vertebrate CNS by their agonistic action on $GABA_A$Rs. Teleost BBBs tend to be more permissive than mammalian barriers, though the cellular makeup and level of protection varies across taxa of teleost fish (Brown et al., 2018). The implications of an incomplete BBB observed in fish would be a potential increase in susceptibility to the effects of an exposure to IVM and EMB due to a reduced ability to exclude their entry across the BBB. IVM has been shown to more readily cross the teleost BBB and accumulate in the CNS compared to EMB (Høy et al., 1990; Sevatdal et al., 2005). Additionally, IVM and to some extent EMB, have been shown to inhibit Pgp, with IVM being a more potent inhibitor, up to 4-fold more potent than the known Pgp inhibitor, cyclosporin-A (CsA; Griffin et al., 2005; Kennedy et al., 2014; Pouliot et al., 1997). Inhibition of Pgp by IVM could lead to reduced efflux of IVM and EMB, more accumulation

in the CNS and additional toxic effects, whereby the combination of the two and their inhibitory action on Pgp would increase the toxicity. This is of particular concern for farms in Atlantic Canada where IVM and EMB have been co-applied or applied within the same year, often with other chemotherapeutants as well (Chang et al., 2022; Hamoutene et al., 2022). With the long half-life of EMB and IVM in marine sediments, and the expected partitioning into marine sediments based on the physicochemical properties of both, there is a potential risk for sublethal effects to benthic teleosts for either IVM without EMB, and especially so for the combination of the two at one farm site given their action on Pgp (Bright and Dionne, 2005; Davies et al., 1997; Griffin et al., 2005; Hamoutene et al., 2022; Kennedy et al., 2014; Pouliot et al., 1997).

1.6. Non-target benthic teleosts

Due to the physicochemical properties of IVM and EMB and their persistence in marine sediments, with a half-life >188 d for both, there is potential for non-target teleost species with a demersal life-history, many of which are relatively sedentary, to be exposed to these chemotherapeutants below the open-net pen Atlantic Salmon farms. Salmon farms can act as fish aggregating structures where numerous fish species, including benthic fish such as sole species (*Pleuronichthys spp.*) have been found in abundance within 30 m of a farm (Brooks, 1994; Brooks et al., 2002; Costelloe et al., 1998). Fish are attracted to these facilities due to the availability of pelleted food below the cages, food organisms growing on facility structures, and other habitat attributes including cover (Carss, 1990; Gowen, 1991; Weston, 1986). Christensen et al (1991) reported a high biomass of mainly benthic-dwelling fish species (e.g., flatfish) near marine farm sites compared to control areas, and that 25% of flounder caught near the farms had eaten feed pellets. Increases in the abundance of sediment-dwelling prey species have also been shown to increase near farms. The aggregation of benthic fish species and their prey in areas around Atlantic Salmon farms, leaves the potential for benthic fish exposure by feed consumption, incidental sediment and feed consumption and indirect consumption of prey which has eaten treated feed.

There is a broad range of taxa of benthic-dwelling marine fish, occupying different ecological niches that could potentially be exposed to IVM and EMB in sediments below the farms. The Pacific Sand Lance (*Ammodytes personatus*) are small short-lived forage fish that range from northern California to Alaska in nearshore intertidal and subtidal

benthic environments of the northeastern Pacific Ocean (Sisson and Baker, 2017; Staudinger et al., 2020). Sand Lance play a key role in coastal marine ecosystems and support trophic structures of several culturally and ecologically important species such as salmon, seabirds, and whales and are known prey for more than 100 predators (Robards et al., 1999). Their life history characteristics including sediment-dwelling and spawning, sedentary with a small home range, limited habitat availability makes them especially susceptible to exposure to EMB and IVM, given their persistence and partitioning into marine sediments. There are also benthopelagic taxa which are in contact with or occupy the benthic marine environment for at least part of their life, that are at risk of exposure to avermectins from Atlantic Salmon aquaculture. Lumpfish (*Cyclopterus lumpus*), a species of fish that has been tested for an alternative to chemotherapeutants for the removal of sea lice from farmed Atlantic salmon, was recently listed as Threatened by the Committee on the Status of Endangered Wildlife in Canada and met the criteria to be listed as endangered (COSEWIC, 2017; Imsland et al., 2018). This is a semi-demersal species that is seasonally and diurnally located on the sea floor, that spawns in the benthic substrates where the embryonic and larval life stages rear. This species has some commercial importance in Canada and globally and a distribution that overlaps with Atlantic Salmon aquaculture across the North Atlantic. Other commercially important species such as cods and related species including Lingcod (*Ophiodon elongatus*), Sablefish (*Anoplopoma fimbria*), Pacific Cod (*Gadus macrocephalus*), Kelp Greenling (*Hexagrammos decagrammus*) and Atlantic Pollock (*Pollachius pollachius*) which was recently listed by COSEWIC, each have varying levels of contact with sediment and the benthic marine environment that could leave them vulnerable to avermectin exposure. Commercially, culturally and recreationally important to indigenous and non-indigenous communities are the 38 species of Pacific Rockfish of the genus *Sebastes* in BC (DFO, 2022). Several are listed as species of conservation concern, and are all share the relatively slow growth, late age of maturity and territorial behaviour which leave the populations slow to recover once depleted (DFO, 2022). The taxa within the genus *Sebastes* range in their distribution, depth, substrate preferences and contact which leave for the potential to exposure to EMB and IVM in marine sediments from Atlantic Salmon farms. Due to prior overharvest of this group of teleosts and their slow recovery, additional pressures such as contaminant exposure could be concerning.

Lastly, the order of flatfish (Pleuronectiformes), present in marine environments globally, are of particular among benthic teleost species. Flatfish, similar to rockfish, serve

as economically and culturally significant indigenous and non-indigenous communities related to commercial, recreational and ceremonial harvest. Several species of Pacific flatfish at various life stages including Starry Flounder (*Platichthys stellatus*), Pacific Halibut (*Hippoglossus stenolepsis*), Yellowfin Sole (*Pleuronectes asper*), Rock Sole (*Pleuronectes bilineatus*) have been shown to prefer finer-grained sediments, which allow the flatfish to bury themselves (Moles and Norcross, 1995). Fine-grained sediments are often observed below net pen aquaculture operations due to the locations of the farms and increased sedimentation from the farms in the surrounding marine benthos. The primary nurseries for many species of juvenile flatfishes on the Pacific coast of North America are located in sediments of inshore coastal areas (Kramer, 1991; Krygier and Pearcy, 1986; Rooper et al., 2003). Flatfish are thought to settle onto fine-grained sediments where the quality and quantity of suitable nursery habitat, as well as other factors such as water quality and predation, affect recruitment of flatfishes (Gibson, 1994). Taxa within the order of flatfish (Pleuronectidae) have evolved to dwell almost exclusively in the benthic marine environment in near-constant contact with marine substrates. Metamorphosis of all flatfish occurs early in life from pelagic to benthic-dwelling anatomical features (i.e., eyed on one side). With their flat slender body shape, advanced chromatic biology allowing for camouflage, physiology suited to sedentary sit-and-wait behaviour, and anatomy that allows for rapid burrowing in sediments; flatfish are well adapted to the benthic environment (Burton, 2010; Moles and Norcross, 1995; Orcutt, 1950).

With most of their life spent in direct contact, and often completely buried, in marine sediments, flatfish are especially susceptible to extended exposure to persistent organic contaminants often found associated with marine sediments through dermal or gill contact, respiration of sediment pore water, ingestion of contaminated sediments or ingestion of benthic prey species containing contaminants within their tissues. This also applies to exposure to chemotherapeutants used in open-net pen Atlantic Salmon aquaculture, which has attracted flatfish which have been shown to eat the salmon feed to which EMB and IVM are applied (Brooks, 1994; Brooks et al., 2002; Carss, 1990; Christensen, K.D, Hoffman, E., Horsted, 1991; Costelloe et al., 1998). The life history behaviours and presence of flatfish around net pen facilities demonstrates that they are especially susceptible to exposure and potential effects of chemotherapeutants released by the farms into the surrounding marine environment, including IVM and EMB. For these reasons, juvenile Starry Flounder were used as the subject organism of the current study exposing a flatfish species to EMB, IVM and a combination of both.

1.7. Study Goal and Objectives

Protection of coastal ecosystems in BC is a priority as evidenced by recent increases in protection offered by marine protected areas and rockfish conservation areas; fisheries closures or improved management to maintain or recover key commercial species such as rockfish and salmon; protection of marine mammals such as the southern resident killer whale through regulation of boat traffic, noise and harvest of their prey species; and even by the recent decision of the DFO to remove open-net pen Atlantic Salmon farms from the Discovery Islands region. Atlantic Salmon aquaculture and the associated environmental impacts, which are much improved in recent decades, are somewhat at odds with this trend of coastal protection. However, Atlantic Salmon aquaculture is a critical industry in Canada for both food and economic security, including for many coastal indigenous and non-indigenous communities. For this industry to sustainably continue in BC, efforts need to be made to mitigate and eliminate impacts associated with open-net pen aquaculture, to coastal ecosystems and the fauna therein.

This study aims to provide knowledge on the sublethal effects of SLICE®, ivermectin, and a combination of both on a Pacific flatfish species, juvenile Starry Flounder. The information collected will aid in closing gaps in knowledge of the effects of avermectin sea-lice chemotherapeutants to non-target benthic marine teleost fish. The findings will provide guidance on the use of avermectins in sea lice management strategies, as it relates to non-target fish impacts, in BC and other jurisdictions. The specific objectives of the study were:

1. To determine the sub-chronic (30-d) sublethal toxicity of SLICE®, ivermectin and a combination of both chemicals in sediments to the physiology of Starry Flounder using a variety of endpoints including:
    - Burst swimming performance;
    - Oxygen consumption;
    - Growth and condition;
    - Effects on metabolism and stress using biochemical indicators and;
    - Burying and skin colouration.
2. To determine the sub-chronic (30-d) sublethal toxicity of SLICE®, ivermectin and a combination of both chemicals in sediments to the behaviour of Starry Flounder using:
    - Avoidance behaviours of starry flounder towards the avermectin pesticides;

- Burying behaviour and;
- Camouflage behaviour.

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Chapter 2. Biochemical, physiological and behavioural effects of SLICE® and ivermectin on juvenile starry flounder (*Platichthys stellatus*)

2.1. Introduction

Marine aquaculture is a rapidly growing industry, with the farmed supply of seafood exceeding wild-caught seafood as of 2013 (Engle, 2016). The world's human population is estimated to grow to between 9.2 to 9.5 billion by 2050 and aquaculture represents a sustainable and efficient means to meet global meat and protein needs (Engle, 2016). Atlantic Salmon (*Salmo salar*) is an important species in marine aquaculture globally and are predominantly farmed using open-net pen aquaculture. With this increasing contributions in both Canada and globally, marine aquaculture and its associated waste and effluents have the potential to be a significant source of near-shore coastal pollution (Vikas and Dwarakish, 2015).

Contamination associated with Atlantic Salmon open-net pen aquaculture is a concern due to the open exchange of water and nutrients between the pens and the surrounding ocean environment. One of the impacts associated with open-net pen systems come from uneaten feed, feces, pharmaceuticals applied to the feed, and chemicals excreted into feces, all of which end up in the surrounding environment (Brooks et al., 2002). The density of fish rearing in a relatively small area in the open-net pens can lead to diseases and parasitic infestations that can require pharmaceutical intervention to prevent loss of fish and maintain fish health (Bateman et al., 2022; Mordecai et al., 2021; Wootten et al., 1982), making these compounds of concern in the surrounding marine environment.

Central to preventing and mitigating the impacts of sea lice infestations on farmed and wild fish are good husbandry practices that may include pharmaceutical interventions (Haya et al., 2005). Two avermectin-derived compounds have been used to treat sea lice infestations in farmed Atlantic Salmon in Canada; emamectin benzoate (EMB), which is used as the active ingredient (0.2% w/w dry powder) of a formulation in Canada called SLICE®, and ivermectin (IVM), which is used as the active ingredient (0.5% w/v liquid) in a formulation called IVOMEC® (Hamoutene et al., 2022; Haya et al., 2005) . EMB is comprised of two homologues of avermectin: 9:1 4'-epimethyamino-4'deoxyavermectin B1a and 4'epimethyamino-4'-deoxyavermectin B1b benzoate (Schering-Plough, 2000)

and ivermectin is a mixture consisting of ≥90% 22,23-dihydroavermectin B1a and ≤10% 22,23-dihydroavermectin B1b (National Center for Biotechnology Information, 2023). SLICE® is the most commonly used chemotherapeutant approved for use to prevent and treat farmed Atlantic Salmon for sea lice in Canada, and IVM has been used off-label in experimental or emergency situations where resistance to SLICE® was observed (E. Horsberg, 2012; Hamoutene et al., 2022). Both EMB and IVM are administered to Atlantic Salmon in-feed (Davies and Rodger, 2000; Hamoutene et al., 2022); these compounds are taken up by the parasite from fish skin and mucous, and exert their antiparasitic actions through irreversible activation of glutamate-gated chloride channels at invertebrate inhibitory synapses (Cornejo et al., 2014; Grant, 2002; Roy et al., 2000). Activation of chloride channels in inhibitory neurons results in the hyperpolarization of muscle and nervous tissue, and inhibition of neural transmission leading to paralysis and death (Grant, 2002; Roy et al., 2000).

A substantial amount of the SLICE® and IVM used in Atlantic Salmon farms is released into the environment surrounding net-pens (Davies and Rodger, 2000; Høy et al., 1990; Olsvik et al., 2008; Shaikh et al., 2007; Wang et al., 2013). Feed waste has been shown to be as high as 15-20% of total feed used and 162 g of feces are produced for each kg of salmon (Milewski, 2001). Given that most of the EMB and IVM applied to Atlantic Salmon feed is released as uneaten pellets or unmetabolized in feces, this could lead to a large amount released into the area around the net pens (Davies and Rodger, 2000; Høy et al., 1990; Olsvik et al., 2008; Shaikh et al., 2007; Wang et al., 2013). Both IVM and EMB have low water solubilities, with log K_{ow} values of 3.2 and 5, respectively (Bright and Dionne, 2005; Davies and Rodger, 2000), and therefore accumulate in the sediments and associated with organic matter and partition out of the water column (Davies and Rodger, 2000; Strachan and Kennedy, 2021). In the benthic environment below net pens, EMB and IVM can persist for long periods of time with half-life estimates of 225 d to >404 d for EMB and > 188 d for IVM (Benskin et al., 2016; Davies et al., 1998; Prasse et al., 2009; SEPA, 2004). Given long sediment half-lives, as well as strategies of multiple applications within a single year, there is the potential for compounding of the environmental concentrations. This can lead to chronic exposure situations at higher concentrations, exposure conditions that may result in toxic effects to non-target benthic species (Benskin et al., 2016; Davies et al., 1998; Prasse et al., 2009).

Reported sediment concentrations of EMB in the environment near open-net salmon pens ranges from non-detectable to as high as 35 to 167 μg kg^{-1} sediment, with

the highest concentrations usually measured on the last day of 7 d treatment programs (DFO, 2012; Ikonomou and Surridge, 2013; Veldhoen et al., 2012). Modeled marine sediment concentrations of EMB have been reported as high as 366 µg kg^{-1} (SEPA, 1999). In freshwater sediments downstream of a land-based aquaculture facility in Atlantic Canada, EMB concentrations as high as 2500 µg kg^{-1} were found (Lalonde et al., 2012). In instances where IVM was used to treat sea lice infestations, sediment concentrations below the net pens ranged from non-detectable to 6.8 µg kg^{-1} (Cannavan et al 2000), and in another study up to 17.3 µg kg^{-1} (DFO, 1996). In most experiments assessing uptake and accumulation with chronic exposures to EMB and IVM, the chemotherapeutants were not found to meaningfully bioaccumulate or biomagnify with increasing trophic level (Bright and Dionne, 2005; Chukwudebe et al., 1996; Davies et al., 1997). Slootweg et al (2010), however, demonstrated the bioaccumulation of IVM in a sediment exposed worm, *Lumbriculus variegatus*, though the bioaccumulation factors ranged substantially from 0.2 to 11.0 depending on organic carbon content and feeding behaviours of the worms, and the study was over a relatively short time period of 28-d. Brooks et al (2019) suggested that due to a longer elimination half-life of EMB in Blue Mussel (*Mytilus edulis*), there is the potential to bioaccumulate in mussel populations, though the bioconcentration factor was low at 49. To date, most findings suggest that avermectins are unlikely to bioaccumulate in the tissues of marine fauna, though there is still uncertainty associated with this assertion based on the findings of (Brooks et al., 2019; Slootweg et al., 2010), which indicates the need for further studies assessing the bioaccumulation potential of these chemotherapeutants in more marina taxa, including fish.

Direct exposure through the consumption of feed, sediment contact and burying, as well as indirect exposures by consumption of contaminated prey organisms are pathways of uptake available to benthic marine fish. Salmon farms can act as fish aggregating structures, where numerous fish species, including benthic fish such as sole species (*Pleuronichthys spp.*) have been found in abundance within 30 m of a farm (Brooks, 1994; Brooks et al., 2002; Costelloe et al., 1998). Fish are attracted to these facilities due to the availability of pelleted food below the cages, food organisms growing on facility structures, and other habitat attributes including cover (Carss, 1990; Gowen, 1991; Weston, 1986). Wild marine fish caught in the vicinity of farms eat pelleted feed and can be significantly larger than those captured at control sites (Carss, 1990). Christensen et al (1991) reported a high biomass of mainly benthic-dwelling fish species (e.g., flatfish) near marine farm sites compared to control areas, and that 25% of flounder caught near

the farms had eaten feed pellets. Increases in the abundance of sediment-dwelling prey species have also been shown to increase near farms. The aggregation of benthic fish species and their prey in areas around Atlantic Salmon farms, with the persistence and potential for bioaccumulation, leave the potential for wild benthic fish to be exposed to EMB and IVM.

Several studies report the effects of EMB and IVM exposure in fish, including several salmonids and other fish species (Athanassopoulou et al., 2002; Berg and Horsberg, 2009; Bowker et al., 2013; Bright and Dionne, 2005; Cárcamo et al., 2014, 2011; Davies and Rodger, 2000; Haya et al., 2005; Igboeli et al., 2013; Johnson et al., 1993; Kennedy et al., 2014; Lu et al., 2022; Oliveira et al., 2016b; Olsvik et al., 2008; Sahota et al., 2022; Strachan and Kennedy, 2021; Ucán-Marín et al., 2012; Varó et al., 2010). However, these studies exposed fish *via* water, intraperitoneal, or treated-food exposures, and most used species with a pelagic life-history. Additionally, most of the exposures were short-term (e.g., <10 d) and at concentrations not typically observed in the marine benthic environment. Data gaps exist on the sublethal effects of EMB and IVM in sediment at environmentally observed concentrations, on benthic-dwelling fish species endemic to the areas where Atlantic Salmon aquaculture exists, and with long-term exposures. Elucidating the potential effects under these conditions is necessary to determine the potential ecological impacts of EMB and IVM use to benthic marine ecosystems.

The objective of this study was to evaluate the sublethal effects of the anti-sea lice chemotherapeutants EMB (using the formulation SLICE® 0.2% premix) and IVM on starry flounder following 30 d treated-sediment exposures using growth, relative condition, burst swimming performance, aerobic scope, skin coloration, burying behaviour and tissue biochemical endpoints as metrics of toxicity. *P. stellatus* were chronically exposed to EMB, IVM or a combination of both, at or near environmentally relevant concentrations.

2.2. Methods

2.2.1. Fish

Juvenile Starry Flounder, *P. stellatus* were captured from Boundary Bay, Surrey, British Columbia (BC), Canada. This area is a low-gradient intertidal zone with mixed tides of up to a 3.5-m range. Fish were collected during daytime hours at low tide ±4-h

depending on the elevation of the tide, between June 21 and August 23, 2019. Fish were captured using a 10 m long and 1.2 m high beach seine with 3.18 mm mesh size (stretched). The number of individuals captured in a sampling day ranged from 11 to 103. *P. stellatus* weighed on average 4.00 ± 2.11 g (range 0.76 – 12.35 g) and had an average total length of 69.6 ± 12.0 mm (range 45.0 – 104.7 mm). Fish were transported from Boundary Bay to Simon Fraser University (SFU) on the same day as collection. The fish were transported in 45 L coolers filled with seawater collected at the sampling location, with aeration, and topped with dechlorinated ice to maintain a temperature of approximately 8-10°C. This method of transport was tolerated well by the fish with no transport-related mortalities occurring. Upon arrival, fish were transferred to 180 L (152 cm length x 58 cm width x 20 cm depth) long shallow fiberglass tanks, containing seawater (29.37 ± 3.19 ‰), maintained at 11 ± 1°C under a 12:12-h photoperiod. Water quality was maintained using Hagen® Fluval® FX6 mechanical and biological filters, Coralife® hang-on-back protein skimmers, and Coralife® ultraviolet sterilizers. Water temperature was maintained by a temperature-controlled room maintained at 10-12 °C and directly using a Coralife 1/4 HP flow-through aquarium chiller. Holding density did not exceed 1 fish per 1 L (0.025 ± 0.013 g L^{-1}) of seawater. Seawater changes were conducted 3-4 times per week with 50-75% of the water changed each time. *P. stellatus* were fed with Cargill® EWOS 1.2 mm farmed fish salmon pellets 3-4 times weekly *ad libitum* and any excess food was removed by vacuum suction during each water change. Fish were acclimated for at least 2 weeks prior to use in an exposure or experiment. Individuals were not size selected for experiments. All sampling was approved by the Simon Fraser University Animal Care Committee (protocol # 1245B-17) which abides by regulations set by the Canadian Council for Animal Care.

2.2.2. Sediment and water

Seawater was obtained from the Vancouver Aquarium (VA, Vancouver, BC) and was sourced directly from Burrard Inlet. At the VA, the water was slow sand filtered and disinfected with ultraviolet (UV) radiation. Treated water was transported to SFU by truck and the seawater was pumped into a large temperature-controlled storage tank for use.

Sediment was collected from Centennial beach (Tsawwassen, BC). Sediment was collected from the upper 10 cm, sieved during collection using 1 mm metal sieves to remove debris and dried prior to experimental use. Sediment was air dried and then stored

in 19 L food grade plastic buckets with a lid in a dark room at 4°C. Boundary Bay is considered an acceptable uncontaminated reference site based on results from the Boundary Bay Assessment and Monitoring Program (BBAMP) (2009 – 2015), completed by Hemmera (2017). Sediment from this region has an organic carbon content of 0.02 – 0.2 % (Hemmera, 2014).

2.2.3. Chemicals

SLICE® 0.2% Premix (Merck Animal Health, Itervet Canada Corp., Kirkland, QC), which contained 0.2% EMB w/w, was obtained from Fisheries and Oceans Canada (DFO). IVM (22,23-Dihydroavermectin B1, CAS Number 70299-86-7 95% purity) was obtained from Sigma-Aldrich (Oakville, ON). Target concentrations of EMB- and IVM-treated sediment were prepared by creating a stock sediment with a nominal concentration of 12,000 µg EMB kg^{-1} dry sediment or 10,000 µg IVM kg^{-1} dry sediment, and serially diluting the stock with clean, dry sediment to achieve the target concentrations. The stock and other concentrations were created by hand mixing the SLICE® and IVM with a small amount of clean, dry sediment in a 17 L stainless steel bucket with a stainless-steel garden trowel, and then by adding the remaining sediment and hand mixing. The hand-mixed sediment was then stirred at low speed vigorously for 2 min using a ½" 2-speed hammer drill with a 67 mm Jiffy Mixer HS-1 stainless steel mixing head. Preparations were stored in the stainless-steel bucket at 4°C in the dark for a maximum of 2-weeks before use in an exposure or disposal.

The sediment concentrations used in the current study are nominal, however in a recent study where sediments were treated in a similar manner chemical analyses for emamectin benzoate and ivermectin were performed on representative samples (range 1–1000 µg kg^{-1}; [Strachan and Kennedy, 2021; Xie et al., 2011]). Sediments were air-dried and extracted three times with dichloromethane, followed by centrifugation at 1500 *xg*. Supernatants were combined and cleaned by elution through anhydrous sodium sulfate and dried by rotary film evaporation. The evaporated residues were reacted with derivatization reagents according to Xie et al., (2011). The derivatization solutions were dried down under N_2, and the residue was taken up in 1 mL of high-performance liquid chromatography (HPLC) grade acetonitrile (Xie et al., 2011). Samples were analyzed by HPLC on a Hewlett-Packard model 1050 with an HP model 1046A programmable fluorescence detector and HP 3396 Series II Integrator (Hewlett-Packard). The detection

limit for emamectin benzoate and ivermectin was between 10 and 25 ng kg^{-1}. Measured concentrations were 86%–95% of all target concentrations. Recoveries were determined by comparing five spiked samples in sediment with one another. Recoveries from sediment were 87%–96%, with a between-day variability of 5%.

2.2.4. Exposures

Range-finding trials were performed for EMB and IVM to determine the range of concentrations of IVM and EMB that would result in mortality of <20%; these mortality data were used to select the appropriate concentration ranges for subsequent sublethal exposures. The range of concentrations for the exposures were selected based on a lack of mortality in addition to using concentrations that are environmentally relevant, such that concentrations of EMB and IVM in sediment had been previously observed within an order of magnitude. Three 30-d exposures were completed for each of EMB, IVM or a combination of EMB and IVM. The lowest EMB exposure concentrations were derived from the proposed "near-field" environmental quality standard (EQS) maximum acceptable concentration in sediment of 0.12 µg EMB per kg of sediment (dry weight) from the Scottish Environment Protection Agency, at which there would be no expected ecological effects (SEPA, 2017). The lowest concentration of EMB used in the current study was a 10-fold increase to the EQS (1.2 µg kg^{-1}), because the EQS value was based on the LC_{50} of a benthic marine invertebrate, which would be more sensitive to EMB than a teleost species.

Spiked sediment (1 kg) was added to 10 L glass aquaria (31 cm length x 16 cm width x 21 cm height) which were filled with aerated seawater (~9 L). The sediment and seawater in the aquaria were then left to settle for 12- to 24-h prior to the addition of *P. stellatus*. Fish (n=6) were randomly selected from their holding tank and lightly anaesthetized using 0.1 g L^{-1} buffered MS222. During anaesthesia fish were measured for total length (mm), weight (g), and then tagged with a Visible Implant Elastomer (VIE) tag according to the manufacturers protocol (Northwest Marine Technology Inc., Anacortes, WA) on the ventral surface immediately under the dorsal fin to allow for individual fish identification. Fish were recovered in aerated seawater and gently added to aquaria and exposed to contaminated sediments for 30 d. For each exposure there were five concentrations and a negative control that contained uncontaminated sediment. For each concentration there were 4 replicate tanks each with 6 fish in each tank. Nominal

treatment concentrations in sediments were 1.2, 12, 120, 600 and 1200 µg kg^{-1} EMB; 1.0, 10, 100, 500 and 1000 µg kg^{-1} IVM, and 1.2/1.0, 12/10, 120/100, 600/500, 1200/1000 µg kg^{-1} EMB/IVM for the combination exposures.

Animals were fed Cargill® EWOS 1.2 mm farmed fish salmon pellets 3-4 times weekly *ad libitum* and any excess food present during the subsequent feeding was removed by vacuum suction during each water change. The density of the food was lower than the treated sediment which allowed for the removal of excess food while avoiding the removal of any treated sediment. Water quality (salinity, dissolved oxygen, pH, ammonia [Ammonia Alert®, Seachem Laboratories, Madison, GA] and temperature) were measured prior to each water change of 75% of seawater 3-4 times each week. All exposure tanks were kept in temperature-controlled water baths and held at 11 ± 1°C under a 12:12 h photoperiod.

Following the exposure, 3 randomly selected fish were removed and used immediately for respirometry, and swim performance measurements, and the remaining 3 fish were euthanized, and target tissues removed for biochemical analysis. The experimental design and summary of the results can be seen in Figure 2-1.

2.2.5. Swimming performance and respirometry

Oxygen consumption

Following exposure, fish were gently transferred to one of 3 randomly selected respirometry chambers for measurements of oxygen consumption. Oxygen consumption rate (MO_2; mg O_2 kg^{-1} h^{-1}) was recorded for 12 h using an automated intermittent-flow respirometry system (Loligo® Systems, Denmark). Cylindrical respirometry chambers (~200 mL, ID = 100 mm, depth = 25 mm) were submerged in a 50 L temperature-controlled, aerated and UV-filtered seawater bath maintained at 11 ± 1°C. The oxygen concentration within each chamber was measured each second using a flow-through oxygen sensor with a fiber-optic probe connected to a Witrox 4 minisensor oxygen meter, and real-time oxygen levels were recorded using AutoResp™ 2 software (Loligo® Systems). The 12 h standard metabolic rate (SMR) measurements were started between 15:00 and 20:00 under the 12:12 h photoperiod. The results of a pilot experiment indicated that a 5.5 h habituation period was sufficient for recovery from handling stress. Oxygen consumption from the start of the intermittent flow cycle closest to 5.5 h, through 12 h (approx. 6 h) was used to determine SMR. A cardboard barrier was used to surround the assay chambers to prevent any disturbances to the fish. Fish were fasted 24 to 48 h prior

to the SMR assay depending on the date of their last feeding. Oxygen consumption measurements were made using a 120 s flushing, 240 s mixing, and 1020 s measuring cycle. All components of the respirometry chambers and flow-through seawater bath were cleaned and disinfected with a 5% bleach solution weekly, and the seawater was completely replaced weekly. Background oxygen consumption rates within the chamber were negligible and therefore excluded from determining oxygen consumption. Plastic ball valves with ½" barbed hose fittings were used within the intermittent flow system to reduce the flow created by the pumps to a rate that allowed for sufficient mixing but did not require fish swimming activity to stay in place. The barbed hose fittings were disassembled and made air-tight using silicone caulking and Teflon tape. Trials were completed with and without the barbed hose fittings to ensure that they had no effect on oxygen exchange.

Following the burst swim performance test described in the burst swim performance (U_{burst}) section below, fish were transferred back to the respirometry chamber to determine maximum metabolic rate (MMR). For MMR oxygen consumption measurements, the intermittent flow respirometry cycles were left on "flush" until the fish were added to the chambers. Chambers were then sealed, and oxygen consumption was measured for 600 s. MMR was determined from the initial negative linear trend in oxygen consumption ($R^2 \geq 0.9$). After oxygen consumption measures were recorded, fish were euthanized using buffered MS222 (1 g L^{-1}), then measured and weighed.

Burst swim performance (U_{burst})

After the first 12-h oxygen consumption measurements to determine SMR, fish were transferred to a swim tunnel previously described (Goulding et al., 2013; Mackinnon and Farrell, 1992; Osachoff et al., 2014) for swimming performance trials. Briefly, the swim tunnel consisted of a 2470 L ovoid raceway, containing filtered, disinfected, and aerated seawater maintained at 11 ± 1°C, and fitted with two electric motors to create current, laminar flow vanes in the corners and a condensing cone to concentrate water velocity into the single, cylindrical swimming chamber where the fish were contained. Fish density in the swim tunnel never exceeded 0.01 g L^{-1} and solid blocking was <10% so no correction factor to adjust for flow disruption from the fish's body was required (Bell and Terhune 1970). Water in the swim tunnel was maintained at a constant level and flow standard curves (relating propeller motor voltages to water velocities cm s^{-1}) were prepared daily using a current meter (Forestry Suppliers Inc.; www.forestry-suppliers.com). All swim trials began with a 20-min acclimation period in the swim tunnel

at water velocity of 5 cm s^{-1}, followed by the burst-swimming speed test (Farrell, 2008; Nendick et al., 2009; Reidy et al., 1995). In this test, water velocity was increased in 5 cm s^{-1} increments at 1 min intervals (Farrell, 2008). The burst acceleration swimming speed test was initiated at 5 cm s^{-1} in the first min after the 20 min acclimation period. Due to the burst-and-coast swimming behaviour and a lesser prolonged swimming ability compared to salmonids (the organisms originally used in this method [Farrell, 2008]), the test was initiated at this velocity rather than 20 cm s^{-1}. The test was terminated after all fish were exhausted, as exhibited by inactivity and laying on the rear net of the swim chamber for several seconds; fish were at this point unable to resume swimming after mechanical prodding. Fish were then immediately removed *via* a rear access panel of the swim tunnel, which had no effect on the remaining upstream swimmers, and returned to the respirometer chambers to measure MMR.

2.2.6. Skin pigmentation and burying behaviour

Following the exposure, alterations in the burying behaviour and skin pigmentation of *P. stellatus* in response to chemicals in sediment were assessed. This was determined by counting the number of fish completely or partially buried in the exposure chamber, which was the expected behaviour under the exposure conditions. The number of fish that were noticeably darker and did not exhibit a normal camouflage condition were also counted. An example of the difference between the body colour and burying behaviour of *P. stellatus* from the highest combination concentration (1200+1000 µg kg^{-1} EMB+IVM) compared to fish from a negative control treatment are seen in Fig. 2-2.

2.2.7. Biochemical analysis

At the end of the 30-d exposure, 3 randomly selected fish were removed from each exposure tank and euthanized using buffered MS222 (1 g L^{-1}). Fish were measured, weighed, and then dissected for the dorsal white muscle section on the top of each fish, and the liver. The white muscle, liver and remaining whole body fish were then snap-frozen using liquid nitrogen and stored on dry ice temporarily until long-term storage at -80 °C for tissue biochemical analysis. Tissue concentrations of glucose and L-lactate (Glucose Assay Kit I and L-Lactate Assay Kit I, Eton Bioscience, San Diego, CA) were determined spectrophotometrically using commercial colorimetric assay kits according to the respective protocols provided by the manufacturers. Sub-samples of the whole-liver and

upper dorsal white muscle filets were reduced to a mass which resulted in a 1:8 tissue/Ethanol (80%) ratio up to 1.5 mL.

Whole body homogenization and steroid isolations were performed according to Arukwe et al., (2008). Briefly, remaining whole body samples of *P. stellatus* stored at -80 °C were thawed on ice, weighed, and homogenized in 0.2 M sodium citrate buffer at pH = 5 (EMD Chemicals Inc., Gibbstown, NJ) using a Tissue Tearor homogenizer (#985370-07, Fisher Scientific, Houston, TX) and centrifuged at 14,000 *xg* for 15 min at 4 °C. The supernatant was purified by mixing with 4 mL diethyl ether using a vortex mixer. After phase separation, the aqueous portion was frozen in an ethanol/dry ice bath. The lipophilic phase was decanted into a clean tube, and the ether phase was evaporated at 25 °C. The dry extract was reconstituted in 300 µL of EIA buffer (Cayman Chemical Company, Ann Arbor, MI, USA) by vortexing. Cortisol concentrations in each individual sample were measured in duplicate using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA) performed according to the manufacturer's protocol. Data were quantified against a standard curve that was linearized using a logit transformation of B/B0 (bound sample/maximum bound). All colorimetric assays were performed in duplicate using an Epoch™ 2 microplate spectrophotometer (Bio-Tek, Winooski, VT) and Corning® 96-well microplate (Greiner Bio-One International, Monroe, NC).

2.2.8. Calculations and statistics

The weight-normalized oxygen consumption rate for each fish was calculated using the slope values for oxygen concentration (mg L^{-1}) over time collected using the AutoResp™ 2 software according to the manufacturer's instructions (Loligo® Systems). Water temperature was recorded automatically with a temperature probe in the seawater bath; the estimated barometric pressure for the elevation (365 m) and the salinity were entered into the AutoResp™ 2 software. Oxygen consumption rates (MO_2, mg O_2 kg^{-1} h^{-1}) were calculated as $MO_2 = bV_cM$ where b = slope (mg L^{-1} O_2 h^{-1}), V_c = chamber volume, and M = fish wet mass in g. The negative slopes of oxygen concentration over time during the measurement period of each intermittent flow cycle were used to determine oxygen consumption. To provide a more accurate measurement of metabolism, slope values with a minimum R^2 of 0.9 were included and those with an R^2 of less than 0.9 were excluded from the analysis (Svendsen et al., 2016). The standard metabolic rate (SMR) value was

the 20% quantile ($q_{0.2}$) of all MO_2 values from the intermittent flow cycles recorded over the 12 h as described by Chabot et al (2016). The MMR value for each fish was calculated in the same way as MO_2 using oxygen consumption measurements during the highest rate of respiration immediately after swimming to exhaustion. MMR was determined from a single measurement of oxygen consumption, which was different from the SMR value which was the 20th percentile of multiple MO_2 values collected over a 12-h period. Aerobic scope (AS) was calculated as the difference between MMR and RMR for each fish (Fry, 1971, 1947). U_{burst} was calculated by normalizing water velocity to fish length by converting the velocity in cm s^{-1} to body lengths s^{-1} ($L_B\,s^{-1}$) by $U_{burst} = V\,L_T^{-1}$, where V = water velocity and L_T = fish total length.

For body condition, Le Cren's Relative condition (K_n; Le cren, 1951) was used to determine body condition over Fulton's Condition (K, Fulton, 1904) because it is preferred when evaluating within-species variation in weight-length relationships and estimating the relative condition of individuals within a sample, which was the goal for body condition in this study (Froese, 2006). Relative condition of individuals was calculated by the equation $K_n = W_0\,W^{-1}$ where K_n is relative condition, W_0 is the observed weight and W is the expected weight. Expected weight was calculated using the fish total length in the linear regression equation from the length-weight relationship of initial lengths and weights for the wild-caught population used in these exposures. Expected weight was calculated according to the equation W = log a + b log L where W is the body weight (g), L is the total length of the fish (mm), a is the intercept of the regression and b is the growth coefficient related to body form, determined from the slope off the regression curve. Percent growth was calculated according to the equation $G_{\%} = (W_F - W_0)\,W_0^{-1}\,100$ where $G_{\%}$ = percent growth, W_F = final weight (g) and W_0 = initial weight (g).

A restricted maximum likelihood (REML) estimation treating exposure tank as a random factor was used to test for tank effects. If tank effect was determined to be statistically significant, the statistical analysis was completed using exposure tank as the experimental unit with individual fish values within that exposure tank averaged. If tank effect was not statistically significant between replicate tanks for a given endpoint, then statistical analysis was completed using individual fish within an exposure tank as the experimental unit. For all endpoints, whether at the tank- or fish-level, a quantile outliers test was completed (0.1 and 3 times the interquartile range (IQR)) and if an outlier was detected it was removed. Endpoint values were then tested for a normality using an Anderson-Darling test, and the Levene test was used to determine homogeneity of

variance. If the data exhibited a normal distribution and equal variances then a one-way analysis of variance (ANOVA) was completed using exposure concentration as the factor, and Tukey's post hoc multiple comparison test. If the data were normally distributed and did not exhibit equal variances, then the Welch's ANOVA test was used with the Games-Howell nonparametric post-hoc test. If the data were not normally distributed and exhibited unequal variances, then the Kruskal-Wallis test was used with the Steel-Dwass test for multiple comparisons. For evaluating a concentration-response effect and calculation of EC_{50} values for endpoints that demonstrated this effect, a log-logistic 3-parameter regression was used where for continuous data the percent response relative to the mean of the negative control response was plotted as a function of concentration (i.e., U_{burst}, AS). For burying and camouflage analyses, binary data of buried or unborrowed and camouflaged or not were plotted as proportion with an effect out of total observations as a function of concentration using a log-logistic 3-parameter regression to determine EC_{xx} values, and significant differences in these counts were assessed using Pearson's χ^2 test for independence. For all statistical tests, significance was determined using $p<0.05$. All data analyses using hypothesis testing were performed using JMP 15 software (SAS Institute, Cary, NC) and all figures and concentration-response analyses were prepared using R (R Core Team, 2021), the package ggplot2 (Wickham, 2016) and the package drc (Ritz et al., 2015).

2.3. Results

2.3.1. Water quality

During the 30 d exposures, oxygen consumption and swim performance assays, the seawater was consistently measured at 11 ± 1°C, pH of 7.6 ± 0.3, dissolved oxygen 8.5 ± 0.9 mg L^{-1}, ammonia <0.02 ppm, and salinity 27 ± 3 ‰. These ranges of water quality parameters were well within normal ranges for this species were expected to support the health of *P. stellatus.*

2.3.2. Mortality

The exposure concentration ranges were chosen from range-finding trials previously performed with *P. stellatus* exposed to sediment treated with EMB, IVM and a combination of the two to reduce potential mortality to <20% at the highest concentration

used. Mortality was monitored for the duration of the 30 d exposures, and did not exceed 10% for EMB, IVM and 12.5% for the EMB/IVM exposure (Table 2-1). These results confirmed that the ranges selected were sublethal to *P. stellatus*.

2.3.3. Body condition metrics

Growth

All treatment groups for EMB, IVM and combination exposures (including negative controls) lost weight, and therefore showed negative growth over the 30-d exposure period. In negative controls, the change in fish mass was -12.5 ± 1.5 %. None of the mean percent change in mass values for the EMB treatments were significantly different from controls. Fish in the 600 µg kg^{-1} EMB treatment group lost less mass compared to fish in the other EMB treatment groups ($p = 0.001$, $\chi^2 = 19.95$). The percent change in mass in the other EMB treatment groups (i.e., 1.2, 12, 120 and 1200 µg kg^{-1}) were not significantly different from each other (see Fig. 2-3). In IVM-exposed fish, the IVM 1 µg kg^{-1} treatment lost significantly more mean percent mass (-20.4 ± 2.3 %) compared to controls ($Z = 3.10$, $p=0.02$). The 500 µg kg^{-1} IVM treatment group lost significantly less mass (-5.6 ± 1.9%) than all other treatment groups except for the control ($p < 0.0001$, $\chi^2 = 40.42$, Fig. 2-3, Table 2-1). For the combination EMB/IVM exposures the mean percent change in mass was not significantly different between controls and treatment groups, or between treatments ($p = 0.20$, $\chi^2 = 7.29$, Fig. 2-3, Table 2-1).

Relative condition

The mean relative condition (K_n) values decreased over the 30-d exposure for all treatment groups including the negative controls, which is consistent with the negative percent change in mass. The mean K_n for the negative control group was 0.83 ± 0.02. For the EMB, IVM and combination EMB/IVM exposures; the mean K_n values for all treatments were not significantly different from the negative control, or between treatments (Fig. 2-4, Table 2-1).

Hepatosomatic index

The mean hepatosomatic index (HSI) of the negative control group at the end of the 30-d exposure was 6.02 ± 0.70, which was the highest HSI value for all groups except for the 1 µg kg^{-1} IVM treatment (6.64 ± 0.92). For the EMB, IVM and combination EMB/IVM

exposures; the mean HSI values for all treatments were not significantly different from the negative control, or between treatment groups (Fig. 2-5, Table 2-1).

2.3.4. Burst swim performance

The mean burst swimming performance (U_{burst}) value for fish in the control group was 4.85 ± 0.21 body lengths (BL) s^{-1}. There was no significant difference between fish in the negative control group, and those in any EMB treatment group (Fig. 2-6, Table 2-1). The mean U_{burst} value for the highest concentration IVM 1000 µg kg^{-1} treatment (U_{burst} = 2.83 ± 0.3 BL s^{-1}) was significantly lower than the negative control (U_{burst} = 4.85 ± 0.21 BL s^{-1}) and all other treatment groups (range 4.14 - 4.73 ± 0.2 BL s^{-1}, Fig. 2-4). A concentration-response effect was assessed when U_{burst} values were normalized to the mean value of the negative control as a function of IVM concentration and then fit to a log-logistic 3-parameter curve (Fig. 2-7, Table 2-1), effective concentration (EC) values for reductions in burst swimming, EC_{50} and EC_{25} values of 1184.3 µg kg^{-1} ± 149.6 and 800.0 ± 93.1 µg kg^{-1}, respectively, were calculated.

The mean U_{burst} values of the three highest combined concentrations (120+100, 600/500, and 1200/1000 µg kg^{-1} EMB/IVM) were significantly lower than the negative control (F = 17.9, p<0.0001). The U_{burst} of the highest concentration group of EMB/IVM 1200/1000 (U_{burst} = 2.48 ± 0.2 BL s^{-1}) was significantly lower than all other treatment groups and the second highest treatment group of EMB/IVM 600/500 (U_{burst} = 3.53 ± 0.2 BL s^{-1}) was significantly less than the two lowest concentration groups of EMB/IVM 1.2/1 (U_{burst} = 4.64 ± 0.2 BL s^{-1}) and 12/10 µg kg^{-1} (U_{burst} = 4.71 ± 0.2 BL s^{-1}, Fig. 2-7). There was evidence of a concentration-dependent effect on burst swimming performance for the EMB/IVM exposure group with a decreasing mean U_{burst} as the combination EMB/IVM concentration increased. A concentration-response curve of U_{burst} normalized to the mean value of the negative control as a function of concentration with the EMB and IVM values added together (see Fig. 2-8 and Table 2-1). This curve was used to calculate the EC50 and EC25 values for burst swimming: 3108.2 ± 905.6 µg kg^{-1} and 629.18 ± 245.26 µg kg^{-1}, respectively.

2.3.5. Oxygen Consumption

Standard Metabolic Rate

The mean SMR value for negative control fish was 128.05 ± 13.63 mg O_2 kg^{-1} h^{-1}. Mean SMR values for all concentrations of the EMB, IVM and combination EMB/IVM

exposures were not significantly different from the negative control (Fig. 2-9, Table 2-1). For the IVM and combination EMB/IVM exposures, no significant differences in SMR from between treatments was observed.

Maximum Metabolic Rate

The mean MMR value for the negative control fish was 367.92 ± 15.86 mg O_2 kg^{-1} h^{-1}, and significant reductions in MMR were seen for the IVM and EMB/IVM combination exposures. For the IVM exposure, the mean MMR values for all but the lowest concentration (IVM 1 µg kg^{-1}) were significantly lower than the negative control (χ^2 = 33.22, p<0.0001). Between treatments, the two highest concentrations (500 and 1000 µg kg^-1IVM) had a mean MMR significantly lower than the lowest concentrations. The magnitude of the decrease in mean MMR generally increased with concentration. For the combination EMB/IVM exposure, the mean MMR values for the 600/500 µg kg^{-1} EMB/IVM (248.64 ± 22.15 mg O_2 kg^{-1} h^{-1}) and 1200/1000 µg kg1EMB/IVM (193.44 ± 22.15 mg O_2 kg^{-1} h^{-1}) were significantly lower than the control and 12/10 µg kg^{-1} EMB/IVM treatment group (301.56 ± 22.15 mg O_2 kg^{-1} h^{-1}, Fig. 2-10, Table 2-1).

Aerobic Scope

The mean aerobic scope (AS) value for the negative control was 247.06 ± 17.20 mg O_2 kg^{-1} h^{-1}), and significant reductions in AS for the EMB, IVM and EMB/IVM combination exposures were found. The mean AS for the EMB 1200 µg kg^{-1} treatment was significantly lower than the control (165.30 ± 17.90 mg O_2 kg^{-1} h^{-1}). No other significant differences were found between treatments and a concentration-dependent effect was not observed (see Fig. 2-11, Table 2-1).

For the IVM exposure, the two highest concentrations of 500 and 1000 µg kg^{-1} IVM had mean AS values significantly lower than the negative control (142.42 ± 18.78 mg O_2 kg^{-1} h^{-1} and 115.79 ± 18.78 mg O_2 kg^{-1} h^{-1}, respectively [see Fig. 2-11]). A concentration-response curve of AS normalized to the mean value of the negative control as a function of concentration, fit to a log-logistic 3-parameter curve, (see Fig. 2-12). This curve was used to calculate the EC50 and EC25 values for reductions in were 1581.30 ± 1345.40 µg kg^{-1} and 12.27 ± 17.95 µg kg^{-1}, respectively.

For the combination EMB+IVM exposure, mean AS values for the three highest concentrations of 120/100, 600/500 and 1200/1000 µg kg^{-1} EMB/IVM were significantly lower than the negative control (174.67 ± 15.84 mg O_2 kg^{-1} h^{-1}, 167.15 ± 16.54 mg O_2 kg^{-1}

h^{-1} and 101.10 ± 16.54 mg O_2 kg^{-1} h^{-1}, respectively [see Fig. 2-9]). There were also significant differences in mean AS between treatments. The mean AS values of the two highest treatments were significantly lower than the two lowest treatments of 1.2/1.0 µg kg^{-1} EMB/IVM (184.17 ± 16.54 mg O_2 kg^{-1} h^{-1}) and 12/10 µg kg^{-1} EMB/IVM (198.35 ± 16.54 mg O_2 kg^{-1} h^{-1}); and the EMB/IVM 1200/1000 mean AS was significantly lower than the EMB/IVM 120/100 treatment (see Fig. 2-11, Table 2-1). There was a concentration-dependent reduction in AS with increasing concentration of EMB/IVM (see Fig. 2-13). The concentration-response curve was used to calculate the EC50 and EC25 for a reduction in AS (3528.73 ± 2978.01 µg kg^{-1} and 35.11 ± 66.77 µg kg^{-1}, respectively).

2.3.6. Skin pigmentation and burying behaviour

The effect of EMB, IVM and EMB/IVM exposure on the behavioural endpoints of burying in sediment and darkened skin pigmentation were measured. Darkened pigmentation is a commonly observed effect of IVM and EMB on teleosts. At the end of the 30-d exposure, fish in all EMB treatment concentrations were completely or partially buried in the sediment and demonstrated normal colouration and camouflage. For the IVM exposure, the counts of individuals not buried and with darkened skin colouration were equal and showed a significant increase with concentration of IVM ($p < 0.0001$, $\chi^2 = 168.5$; see Table 2-1) with an EC_{50} of 267 ± 1068 µg kg^{-1} where all fish in the 500 and 1000 µg kg^{-1} IVM treatments presented with dark skin pigmentation and were not buried in the sediment. In the current study darkened skin pigmentation was always accompanied by *P. stellatus* not burying. Similar results were observed in the two highest concentrations in the combination exposure (600+500 and 1200+1000 µg kg^{-1} EMB+IVM), with the exception of 25% of fish presenting with darkened pigmentation and decreased burying in the next lowest treatment concentration (120+100 µg kg^{-1} EMB+IVM). The counts of individuals not buried and with darkened skin colouration were equal and showed a significant increase with concentration of EMB and IVM ($p < 0.0001$, $\chi^2 = 146.1$; see Table 2-1) with an EC_{25} and EC_{50} of 220 ± 14 µg kg^{-1} and 255 ± 151 µg kg^{-1}, respectively.

2.3.7. Biochemical analysis

Cortisol

Mean whole-body cortisol concentration in the negative control group was 1489.39 ± 246.27 pg g^{-1}. There were no significant differences in mean tissue cortisol concentration between the negative control and EMB ($p = 0.40$, $\chi^2 = 5.13$), IVM ($p = 0.10$, $\chi^2 = 9.17$) and

combination ($p = 0.01$, $\chi^2 = 14.76$) treatment groups (see Fig. 2-14, Table 2-1). The Kruskall-Wallis test for the combination exposure indicated a significant difference between treatments, however, the Steel-Dwass post hoc multiple comparison test indicated no significant differences between treatments, including the negative control.

Glucose

Mean liver and white muscle glucose concentrations in the negative control group was 37.70 ± 6.62 μg g^{-1} and 15.69 ± 10.07 μg g^{-1}, respectively. There were no significant differences in mean liver and white muscle glucose concentrations between the negative control and treatment groups or between treatments for EMB, IVM and the combination exposures (See Fig. 2-15, Fig. 2-16 and Table 2-1).

Lactate

Mean liver and white muscle lactate concentrations in the negative control group were 0.09 ± 0.04 μg g^{-1} and 0.02 ± 0.01 μg g^{-1}, respectively. No significant differences were seen between the negative control and treatment groups or between treatments for white muscle lactate concentrations in the EMB exposure (See Fig. 2-18). Mean liver lactate concentrations in the EMB exposure group were not significantly different from the negative control, however, the EMB 600 μg kg^{-1} group had the lowest mean liver lactate concentration (0.01 ± 0.003 μg g^{-1}), and EMB 1200 μg kg^{-1} EMB had the highest (0.10 ± 0.05 μg g^{-1}). No other significant differences in mean liver lactate were found (see Fig. 2-17, Table 2-1). For the IVM exposure group, mean liver lactate concentrations were not significantly different from the negative control or between treatments ($p = 0.36$, $\chi^2 = 5.49$; see Fig. 2-17, Table 2-1). White muscle lactate concentrations were significantly different between treatment groups ($p = 0.0037$, $F = 4.91$; see Fig. 2-18). The mean white muscle lactate concentration for the IVM 500 μg kg^{-1} group (0.02 ± 0.007 μg g^{-1}) was significantly higher than the IVM 1 μg kg^{-1} treatment group (0.01 ± 0.01 μg g^{-1}) treatment. No other significant differences in mean liver lactate concentration for the IVM exposure was found. In the combination EMB/IVM exposure group, liver ($p = 0.16$, $F = 1.81$; see Fig. 2-18, Table 2-1) and white muscle ($p = 0.51$, $\chi^2 = 2.85$; see Fig. 2-18, Table 2-1) lactate concentrations were not significantly different from the negative control or between treatment groups.

2.4. Discussion

The aim of the present study was to determine the sublethal effects of the anti-sea lice chemotherapeutants SLICE® (AI EMB), IVM and a combination of both, on the physiology and behaviour of *P. stellatus*. The assays provided the first evidence of sublethal effects of avermectins on a benthic teleost (e.g., flatfish order Pleuronectiformes) exposed to contaminated sediment at environmentally relevant concentrations found at Atlantic Salmon farm operations.

Cumulative mortality rates for all concentrations of EMB (1.2 - 1200 µg kg^{-1}), IVM (1.0 - 1000 µg kg^{-1}) and the combination of both (1.0+1.2 – 1000+1200 EMB+IVM µg kg^{-1}) did not exceed 10%, indicating that these sediment concentrations are not lethal to *P. stellatus*. A recent study by Strachan and Kennedy (2021) exposed adult Tidepool Sculpin (*Oligocottus maculosus*) to sediment treated with EMB for 10 d, and an LC_{50} (95% CI) of 1980 (1249-3750) µg EMB kg^{-1} sediment. Sahota et al. (2022) exposed Pink Salmon fry, a pelagic fish species, to EMB in sediment for 10-d and found an LC_{50} (95% CI) of 2065 (1384-3720) µg EMB kg^{-1} sediment. These results are consistent with the findings of this study with the maximum treated used (1200 µg kg^{-1}) being below the LC_{50} found by Strachan and Kennedy (2021). All other experiments found which exposed teleosts to IVM and EMB were by other exposure methods, so direct comparison to the conditions used in the current study cannot be made.

2.4.1. Body condition metrics

Growth

Relative change in mass was assessed over the 30-d exposure and for all treatments, including the negative control, a reduction in mass was observed. One difference in growth observed was significantly less of a reduction in mass for the IVM 500 µg kg^{-1} treatment group compared to the control, and though not significantly different, the second highest concentrations for EMB (600 µg kg^{-1}) was significantly lower reduction in mass compared to all treatments except for the control, and the combination (600+500 EMB+IVM µg kg^{-1}) also showed the lowest reductions in mass among treatments though not significant. A non-monotonic trend with the lowest reduction in mass observed at intermediary concentrations was seen here but has not been reported in other studies (Bowker et al., 2013; Domingues et al., 2016; Du et al., 2023; Lu et al., 2022; Roy et al.,

2000; Skilbrei et al., 2015; Ucán-Marín et al., 2012). In fish exposed to IVM (Domingues et al., 2016; Ucán-Marín et al., 2012), EMB (Skilbrei et al., 2015) and avermectin (Du et al., 2023) negative growth in a concentration-dependent manner was observed, as well as no effect on growth in a study exposing fish to EMB (Ananda Raja et al., 2020; Bowker et al., 2013; Kent et al., 2019) and IVM (Katharios et al., 2001). One study which exposed the European Eel (*Anguilla anguilla*) to IVM by a bath treatment showed an increase in growth at one concentration, but only two concentrations were tested (Buchmann and Bjerregaard, 1990). In several fish species, IVM and EMB caused significant reductions in appetite and feeding response, which may explain the general trend of reduced growth observed (Azevedo and Kennedy, 2022; Bowker et al., 2013; Domingues et al., 2016; Johnson et al., 1993; Roy et al., 2000; Ucán-Marín et al., 2012). The few effects on change in mass observed in this study are consistent with previous findings showing no growth-related effects following abamectin-type chemotherapeutant exposure (Bowker et al., 2013; Katharios et al., 2001; Kent et al., 2019; Kilercioglu et al., 2020). Given the novelty of sediment-based exposures to EMB and IVM in fish, the uptake and tissue concentrations accrued over the 30 d exposure period are unknown. Perhaps at the sublethal concentrations used, or the duration of the exposure (30 d) effects on growth in a concentration-dependent manner could not be observed, where growth was not a sensitive enough endpoint to demonstrate a sublethal effect in *P. stellatus*. A range-finding exposure, more limited in scope to the current study was completed prior to this study using the same experimental conditions and feed, where growth and feeding were observed. With previously observed feeding and growth, and the suitable water quality parameters recorded throughout the 30 d exposure, it is unknown why negative growth was observed in control fish. Given the variable results in change in mass observed in the current study, including in the negative control group, it was clear that fish were not eating enough which precluded the ability to elucidate any effects on growth related to the avermectin exposure.

Condition and hepatosomatic index

Relative condition and HSI were not significantly different between treatments and the negative control for EMB, IVM and the combination of both. The K_n values in all individuals but 13 were reduced from their value at the beginning of the 30-d exposure meaning that body condition declined, even in the control group, which is consistent with

the findings for percent change in mass. These results match the findings of Lozano et al (2021) which found sub-optimal condition values with no significant difference in HSI between treatments for *Prochilodus lineatus* exposed to IVM. In another study exposing *S. salar* to IVM applied to feed the opposite was found where weight, condition factor and HSI were significantly reduced in exposed fish (Ucán-Marín et al., 2012). Hepatosomatic index is a metric of body condition that can indicate overall health, toxicant exposure, hepatotoxicity and energy utilization and storage.

Though no effect was seen, other sublethal biochemical effects on liver health have been observed following avermectin exposures. In African Catfish (*Clarias gariepinus*) exposed to IVM in water, HSI was significantly reduced in a concentration dependent manner and biomarkers of hepatotoxicity were significantly elevated including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP; Odo et al., 2020). Antioxidant enzyme levels including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were also significantly lowered in a concentration dependent manner, which can indicate oxidative stress and injury in the liver following use and removal of these enzymes following reactions with reactive oxygen species (ROS), which was corroborated by elevated levels of lipid peroxidation from which ROS are created and cellular toxicity can be inferred (Odo et al., 2020). Similar results of elevated liver enzymes and depressed antioxidant enzymes were found in the liver of *L. rohita* (Choudhary et al., 2022) and *Oreochromis niloticus* (Fırat and Tutus, 2020), exposed to EMB. Olsvik et al (2008) assessed the effect of EMB exposure on liver gene expression in *S. salar* and found significant increases in the expression of genes related to oxidative stress, protein stability and an inflammatory response. Proteomic analyses from liver tissue of Gilthead Seabream (*Sparus aurata*) exposed to IVM showed changes in protein and protein subunit levels relative to control fish that indicated an oxidative stress response and alterations in energy generation (Varó et al., 2010). Fish exposed to IVM and EMB have also presented without changes in metrics that indicate hepatotoxicity. Palmer et al (1996) found no histopathological changes in the liver tissues of *S. salar* exposed to IVM, and Kilercioglu et al (2020) found no significant changes in serum liver enzymes in *O. mykiss* exposed to EMB, with the exception of AST only on the 21st day of sampling. EMB and IVM can be hepatotoxic and affect HSI depending on concentration, species and exposure medium, where some studies have, and some have not, demonstrated significant effects on these metrics following fish exposure to EMB and IVM.

The conditions used in the current study of the treated sediment exposure to a flatfish species using environmentally relevant concentrations might delay the uptake and distribution in tissues to the levels that cause hepatotoxicity when compared to most studies exposing fish to IVM and EMB by water, applied to feed, oral gavage or intraperitoneal injection. To account for the different exposure route and simulate the long half life (>100 d) in the benthic marine environment for EMB and IVM, a longer exposure duration might be necessary. Additionally, to pair with HSI as a metric for liver health, tissue or serum biochemical endpoints such as liver enzyme and antioxidant enzyme levels, as well as an *omics* approach would be informative.

2.4.2. Swimming

Behavioural endpoints have seen an increase in use in animal toxicity testing for several reasons. Animal welfare as behavioural metrics are normally sublethal effects that avoid lethal concentrations and undue harm and suffering to the animal, behavioural metrics can be more sensitive than conventional endpoints (Gerhardt, 2007; Kennedy et al., 2014; Little and Finger, 1990) and they often have more ecological relevance demonstrating exactly how a toxicant might impact animal behaviour in the wild (Sandheinrich and Atchison, 1990; Scott and Sloman, 2004). In toxicity testing using teleosts, swim performance has a been used as a behavioural endpoint for decades, given the importance of the behaviour to the survival and fitness of all fish (Beamish, 1978). Swimming performance is an important component of viability as it relates to a fish's capacity to maintain station against current, avoid predators and acquire food (Beamish, 1978). In this study burst swimming (U_{burst}) was chosen as the swim performance endpoint because of its applicability to the life history characteristics of *P. stellatus*, as well as flatfish and other demersal fish in general, being burst-and-coast swimmers and ambush predators (Orcutt, 1950). Though the available studies on flatfish swim performance are limited, the relative swim performance of *P. stellatus* (2.48-4.85 BL s^{-1}) is within the range of values found for the swim performance of the European Flounder (3.8 BL s^{-1}; *Pleuronectes flesus*), Lemon Sole (1.9 BL s^{-1}; *Pleuronectes microcephalus*), Winter Flounder (0.65-6.6 BL s^{-1}; *Pseudopleuronectes americanus*) and European Plaice (0.6-16 BL s^{-1}; *Pleuronectes platessa*) in other studies between (Beamish, 1978, 1966; Blaxter and Dickson, 1959; Ryland, 1963; Wagner and Gamperl, 2004).

In the current study significant effects on U_{burst} in *P. stellatus* were observed in a concentration-dependent manner for the IVM and combination exposures, but not for the EMB exposure. Given that no effect on swim performance was observed in the EMB exposure, these results suggest that IVM was causing most of the toxic effect on U_{burst} observed in the combination exposure. However significant reductions were also seen at the 100+120 and 500+600 μg kg^{-1} IVM+EMB treatments but not the 100 or 500 μg kg^{-1} IVM treatments, which suggests a potential additive effect of the combined treatments. Previous studies exposing teleosts to EMB matched the findings of the current study and showed no significant effects on swimming behaviour and swimming performance in fingerling *O. mykiss* (Bowker et al., 2013) and juvenile *O. gorbuscha* (Sahota et al., 2022). Two studies, however, showed an effect of lethargy and loss of equilibrium at the highest concentration of 500 μg kg^{-1} body weight in Asian Seabass (*Lates calcarifer*; Ananda Raja et al., 2020)) as well as *O. mykiss* and *S. salar* (Roy et al., 2000) exposed to EMB. Studies investigating the effects of IVM on fish swimming behaviour and performance demonstrated more effects at lower concentrations, matching the findings of the current study. Common effects on swimming behaviour included lethargy, reduced mobility, erratic swimming, loss of equilibrium, paralysis and reduced swimming performance (e.g., U_{CRIT}) in multiple fish species and life stages included *S. salar, D. rerio, C. catla*, *O. tshawytscha*, *O. kisutch*, *O. mykiss*, Sea Bream (*Sparus aurata*), and *Clarias gariepinus* (Azevedo and Kennedy, 2022; Domingues et al., 2016; Ezenwaji et al., 2017; Johnson et al., 1993; Katharios et al., 2001; Kennedy et al., 2014; Ogueji et al., 2019; Oliveira et al., 2016b; Palmer et al., 1996; Thiripurasundari et al., 2014; Ucán-Marín et al., 2012). One study that directly compared the safety and efficacy of EMB and IVM applied to medicated feed on *D. rerio* reach similar conclusions with EMB being well tolerated and showing no signs of behavioural effects, including on swimming, and IVM having a low margin of safety and having a concentration-dependent effect on swimming behaviour marked by lethargy, loss of equilibrium and body twitching (Collymore et al., 2014). To date, no studies testing the swim performance of a benthic (or any) teleost species exposed to sediment treated with IVM and EMB were found, so direct comparison of concentration and effects was not possible.

The trend observed across all studies exposing fish to avermectins seems to be that EMB has little effect on swimming behaviour and performance except at the highest concentrations and IVM a more substantial effect on swimming at lower concentrations. The effects of both EMB and IVM present as lethargy, loss of appetite, loss of equilibrium

and darkened skin similar to what was observed in the current study; most of which match the effect that would be observed from a central nervous system (CNS) (Ananda Raja et al., 2020; Collymore et al., 2014; Domingues et al., 2016; Johnson et al., 1993; Katharios et al., 2001; Kennedy et al., 2014; Ogueji et al., 2019; Oliveira et al., 2016b; Palmer et al., 1996; Roy et al., 2000; Thiripurasundari et al., 2014; Ucán-Marín et al., 2012). This difference in toxicity can potentially be attributed to the difference in distribution into the CNS between EMB and IVM. Høy et al (1990) showed that tritium-labeled IVM readily distributed and accumulated in the brain of Atlantic Salmon, indicating that IVM can cross the blood-brain barrier (BBB). A similar study by Sevatdal et al (2005) assessed the distribution of EMB in tissues of *S. salar* and found relatively little accumulation in the brain with most residues found in the viscera. The findings of Sevatdal et al (2005) show that some EMB can cross the BBB but very little does relative to IVM. These differences in tissue distribution, with more IVM accumulation in the CNS, might explain the difference in toxicity observed for IVM compared to EMB in fish in the current study, and in previous teleost avermectin exposures. The increased accumulation of IVM in the CNS relative to EMB, along with sublethal effects on locomotion observed for IVM and not EMB, suggests a neurotoxic mechanism of toxic action of IVM.

In vertebrates, avermectin intoxication begins with tremors and incoordination and later develops into ataxia and coma-like sedation (Stevens and Charles, 2001). IVM and EMB are also substrates to P-glycoprotein (Pgp) transporters which are efflux transporters found on the BBB, key in reducing xenobiotic toxicity in the CNS (Kennedy et al., 2014; Stevens and Charles, 2001). Teleost BBBs tend to be more permissive than mammalian barriers, though the cellular makeup and level of protection varies across taxa of teleost fish (Brown et al., 2018). The implications of an incomplete BBB observed in fish would be a potential increase in susceptibility to the effects of an exposure to IVM and EMB due to a reduced ability to exclude their entry across the BBB.

It is thought that the mechanism by which IVM exerts its toxic effect in the CNS once accumulated in the brain in teleost fish is by its agonistic action on $GABA_AR$, opening or potentiating ligand-gated chloride channels (Estrada-Mondragon and Lynch, 2015; Stevens and Charles, 2001). Xu et al (2016) assessed and reviewed EMB and IVM binding to two cation-selective nicotinic acetylcholine receptors (NAChR) and two GABA-gated chloride channels and found that EMB could directly activate both NAChRs as well as $GABA_AR$ and $GABA_CR$ all with similar potency, whereas IVM only activated the $GABA_ARs$. As one of the predominant inhibitory neurotransmitters in the vertebrate brainstem and

spinal cord, GABA is critically important for the regulation of the circuitry underlying locomotor behaviours such as escape response and rhythmic swimming (Saint-amant and Drapeau, 2000; Yan et al., 2017). In zebrafish it has been shown that $GABA_AR$ plays a vital role in mediating the majority of fast inhibitory synaptic transmission in the CNS, including neurotransmission related to movement (Sajovic and Levinthal, 1983). Given that toxic effects on swim performance observed in the current study were likely due to IVM exposure and not EMB exposure, IVMs tendency to cross the BBB compared to EMB, and the exclusive binding to $GABA_ARs$ of IVM, it is possible that the effect on locomotion caused by IVM is through activation of GABA-gated chloride channels and the subsequent inhibitory neurotransmission which can produce a sedative-like effect which can directly impact swimming and muscle contraction (Høy et al., 1990; Xu et al., 2016). It is worth considering the previously observed effect of IVM and EMB on fish appetites as an additional indirect effect on swim performance, as fish weight and condition can influence swimming performance and capacity (Beamish, 1978; Bowker et al., 2013; Domingues et al., 2016; Johnson et al., 1993; Roy et al., 2000; Ucán-Marín et al., 2012). However, since no significant differences in K_n or percent change in mass was observed between treatments and the control, the concentration-dependent effects observed on burst swimming performance swimming are likely due to the CNS action of IVM and potentially EMB on $GABA_ARs$, not due to changes in body condition or energy utilization and storage due to reductions in appetite.

Significant effects on swim performance compared to the negative control group were observed at the highest concentration for the IVM exposure (1000 μg kg^{-1} IVM) and the highest three concentrations in the combination exposure (120+100, 600+500, 1200+1000 μg kg^{-1} EMB+IVM) with U_{burst} decreasing in a concentration-dependent manner; with no effects observed in the EMB exposure. These findings indicate that IVM is more toxic to *P. stellatus* swim performance than EMB, and that a combination exposure can increase the effect on swim-performance compared to an exposure to either, individually. The additive effect observed may be due to the binding and inhibition of Pgp by IVM and EMB. In a study by Kennedy et al (2014), *O. mykiss* exposed to IVM and EMB by intraperitoneal injection yielded similar results to those found in the current study on critical swim speed and other swimming behaviours with IVM affecting swimming at significantly lower concentrations than EMB. Each of EMB and IVM were also co-administered with a known Pgp inhibitor (i.e., chemosensitizer), Cyclosporin-A (CsA; Kennedy et al., 2014). When co-administered, significant increases in the effects on swim

performance caused by IVM and EMB were observed, with a larger change in effect seen with EMB (Kennedy et al., 2014). These findings suggest that Pgp inhibition can increase IVM and EMB movement into the CNS across the BBB and therefore increase the concentration acting on $GABA_AR$ and potentially other ion receptors (Xu et al., 2016). The was corroborated in a study by Pouliot et al (1997) showing IVM accumulation in a drug-resistant cancer cell line when co-administered with CsA. The increase in effect observed for the EMB exposure following Pgp inhibition also suggests that EMB is more readily bound to and excluded by Pgp, which likely explains the decrease in toxicity seen for teleost EMB exposures. It has also been previously shown that IVM and EMB can inhibit Pgp, with IVM being a more potent inhibitor up to 4-fold more potent than CsA (Griffin et al., 2005; Kennedy et al., 2014; Pouliot et al., 1997). Given that Pgp inhibition has been shown to increase CNS concentrations of IVM and EMB, and that IVM is a potent Pgp inhibitor, it is suggested that the increased sensitivity and effect on U_{burst} seen in the combination exposure is due to Pgp inhibition and increased CNS concentrations of EMB and IVM.

A significant reduction in burst swimming ability seen from exposure to IVM- and EMB-treated sediments at environmentally observed concentrations, for a teleost species with a life history that keeps it in near-constant contact with marine sediments, leads to potentially concerning ecological implications for demersal teleost species with distributions that overlap with active open-net pen Atlantic Salmon farms (Cannavan et al., 2000; DFO, 2012, 1996; Ikonomou and Surridge, 2013; Orcutt, 1950; Veldhoen et al., 2012; Wen et al., 1999). With the exposure conditions used in the current study (30-d with treated sediment), the effects observed could understate the effects that might be observed in an environmental exposure. In marine sediments the persistence of EMB and IVM are much longer than 30 d at >225 d and >188 d, respectively, and there would be an additional route of exposure to benthic fish in the form of uneaten feed with EMB or IVM applied, instead of just a sediment-based exposure. The current study only demonstrates the effects of contact exposure via respiration in sediment pore water, skin-to-sediment contact and direct sediment ingestion, without being exposed to treated feed (Benskin et al., 2016; Davies et al., 1998; Prasse et al., 2009; SEPA, 2004). Given the persistence, additional route of exposure (i.e., feed) and that the concentrations used in this study are within or near those observed in the environment surrounding the net pens, there is the potential for a longer exposure to higher exposure concentrations than those used in this study, and thus the potential for even greater reductions in burst swimming

performance in *P. stellatus*. This is especially concerning given that fish have not shown a tendency to avoid avermectins when given the opportunity (Sahota et al., 2022).

Swim speed and performance can be directly related with the capacity to escape and avoid predators, capture food and access preferred habitats with a velocity barrier (Beamish, 1978; Weis and Candelmo, 2012). Teleosts exposed to contaminants in sediments in the wild compared to fish from uncontaminated reference sites have been shown to take more risks leaving refuge to find food, have a poor fast-start performance, reduced stamina, altered schooling behaviour, altered activity and increased conspicuousness, all leading to increased predator vulnerability (Scott and Sloman, 2004). Given that IVM and potentially EMB can cause reductions in burst swimming ability at environmentally relevant concentrations, and can potentially reduce appetite, exposure to these chemotherapeutants could lead to reduced caloric intake and prey capture which could further potentiate the effects of IVM and EMB by altering energy utilization and storage (Díaz-Rúa et al., 2021; Ginneken et al., 1996). This in turn could lead to increased predation and reduced survival. The $GABA_AR$ has also been shown as a key component of neuroendocrine and CNS signalling involved in growth, reproduction, early development, learning and memory and metabolic processes in teleost species (Basu et al., 2009; Collinson et al., 2002; Martyniuk et al., 2007; Orger et al., 2000; Song et al., 2017; Yan et al., 2017). Sublethal effects on mediated by IVM and EMB $GABA_AR$ binding could lead to population-level effects on key processes that influence the survival and reproduction.

2.4.3. Oxygen consumption

The decrease in U_{burst} may be associated with the reduced aerobic scope (AS) observed in *P. stellatus* exposed to EMB, IVM and the combination of EMB and IVM. AS represents the oxygen available to for activities excluding basal metabolism (Fry, 1947). Reduced AS my affect an organisms burst swimming ability such that the mobilization of energy resources for repeated bursts and therefore the frequency of rapid swimming may be restricted by moderate oxygen deficiency (Beamish, 1978). The AS was significantly reduced compared to the negative control in the highest concentration of EMB (1200 μg kg^{-1}), the two highest IVM treatments (500 and 1000 μg kg^{-1}) and the three highest in the combination exposure (120+100, 600+500 and 1200+1000 μg kg^{-1} EMB+IVM). AS is the difference between MMR and SMR which can be used as a metric to estimate the overall

metabolic budget that an animal can allocate for oxygen consuming processes including growth, digestion, reproduction, movement such as swimming, as well as coping with stressors or disease (Killen et al., 2016). The depression of an organisms AS can be in response to environmental stressors such as toxicant exposures, where xenobiotic processing and response can remove available aerobic capacity from what would normally be available to processes such as movement. A reduced AS can be attributed to elevated standard metabolic rate (SMR), and/or a reduction in maximum metabolic rate (MMR; Killen et al., 2016).

There was no significant difference in *P. stellatus* SMR from the control for any of the EMB, IVM and combination exposures, whereas the MMR showed a concentration-dependent reduction for IVM and the combination exposures, matching the reductions seen in AS. The effect in aerobic capacity was therefore caused by a limiting stressor leading to reduction in the MMR. Limiting stress (reduced MMR) indicates the IVM and the combination of IVM and EMB impair oxygen uptake and/or oxygen transport, thereby reducing oxygen delivery to tissues and limiting the overall metabolic capacity of the fish (Stieglitz et al., 2016). When compared to previously determined SMR values (34.99-75 mg O_2 kg^{-1} h^{-1}) for other flatfish species including *P. stellatus* (Milligan and McDonald, 1988), the range of mean of SMR values found in this study were slightly higher (99.83 ± 4.92 mg O_2 kg^{-1} h^{-1}) (Duthie, 1982; Frame, 1973; Lefrançois and Claireaux, 2003; Priede and Holliday, 1980). The mean of MMR values found in the current study (277.30 ± 11.47 mg O_2 kg^{-1} h^{-1}) was similar to those previously recorded for flatfish (149.77-202.83 mg O_2 kg^{-1} h^{-1}; Duthie, 1982; Frame, 1973; Lefrançois and Claireaux, 2003; Milligan and McDonald, 1988; Priede and Holliday, 1980). The methodologies for determining MMR, as they related to how the fish were swam and exhausted varied across studies which makes direct comparison difficult. Only one study by Al-Kahtani (2011) was found where the effects of exposure to an avermectin pesticide on a teleost species was assessed. Nile Tilapia (*Oreochromis niloticus*) exposed to sublethal concentrations of another avermectin-type chemotherapeutant (abamectin) saw significant reductions in oxygen consumption compared to the control. However, the units of oxygen consumption (mL g^{-1} h^{-1}) and concentration of abamectin (µg L^{-1}) were not directly comparable to those used in the current study. Clearly additional efforts should be made to assess the effects of avermectin-type sea lice chemotherapeutants on the metabolic capacity of wild teleost that possess a range of life history characteristics (e.g., benthic and pelagic, resident and migratory) and ranges that overlap with Atlantic Salmon farming operations.

There are several potential mechanisms by which IVM and EMB exhibit the reduction in MMR and AS in *P. stellatus.* Direct damage to the gills and gill epithelium could affect the uptake and transport of oxygen thereby reducing aerobic capacity. Toovey et al (1999) found that IVM caused a significant, concentration-dependent reduction in oxygen consumption by isolated *O. mykiss* gill tissue and Athanassopoulou et al. (2002) using histopathological examination of the gill tissues of Sea Bass (*Dicentrarchus labrax*) exposed to IVM by oral intubation and intraperitoneal injection showed marked signs of gill pathology including edema, necrosis of the epithelial cells and sloughing of the secondary lamellae. A similar effect of pathologies associated with gill morphology and altered gas exchange on teleost exposure to a multitude of organic contaminants (Evans, 1987). This effect on gill tissues is not necessarily due to a specific mechanism, but can be attributed to general signs of oxidative stress and toxicity such as reactive oxygen species (ROS) generation (Evans, 1987). Teleost species exposed to IVM and EMB have shown significant increases in signs of oxidative stress and injury, and a reduction antioxidant enzymes, which could potentially demonstrate a mechanism of toxicity to gills from IVM and EMB (Choudhary et al., 2022; Fırat and Tutus, 2020; Huang et al., 2019; Kumar et al., 2022; Odo et al., 2020; Olsvik et al., 2008; Varó et al., 2010). Given the potential for direct toxicity to gill tissues through oxidative injury, additional investigation into the potential effects on gill tissue and AS following and environmentally realistic exposure to IVM and EMB is pertinent.

Cardiotoxicity and haematological effects on O_2 binding from IVM and EMB could also explain the observed reduction in AS. Avermectin has previously been shown to be cardiotoxic to zebrafish where cardiac rhythm and vascular smooth muscle contraction was affects as well as cardiac development in embryos (Du et al., 2023; Lu et al., 2022). Similar reductions in AS have also been observed in fish with demonstrated cardiotoxic effects leading to arrhythmias and reduced cardiac output from crude oil exposure (Brette et al., 2014). The O_2 binding capacity in fish exposed to both EMB and IVM has also been reduced by significant decreases in hemoglobin and/or red blood cells (Kumar et al., 2022; Ogueji et al., 2019). Overall, cardiotoxic effects and reduced O_2 binding capacity potentially caused by EMB and IVM at sublethal concentrations could cause reduced oxygen uptake, and consequently a reduced MMR and AS.

The reduction in AS and MMR from IVM and the combination of EMB and IVM can also be potentially explained by their binding and activation of $GABA_AR$ similar to what was suggested for the effect on U_{burst}. $GABA_ARs$ are responsible for much of the inhibitory

neurotransmission in the brain and excessive activation of these receptors from the continued and irreversible binding from ivermectin can lead to toxic effects related to that inhibitory signalling including sedation and muscle relaxation (Estrada-Mondragon and Lynch, 2015). In fact, increased GABAergic neurotransmission to cause metabolic depression by supressing oxygen uptake and transport is used as a survival mechanism for fish tolerant of anoxic conditions (Ginneken et al., 1996; Zajic and Podrabsky, 2020). This survival mechanism using GABA in anoxic-tolerant teleost species demonstrates that binding and activation of $GABA_AR$ by EMB and IVM can cause metabolic depression, and therefore reduced oxygen availability for MMR and AS (Ginneken et al., 1996; Xu et al., 2016; Zajic and Podrabsky, 2020). Alterations to respiration rates is an early indication of potential fitness ramifications. There is evidence that metabolic rate alterations affect the survival, growth and reproductive output of organisms (Auer et al., 2015; Burton et al., 2011) (Burton et al. 2011, Cooke et al. 2013, Auer et al. 2015). Similar to the reduction in U_{burst} observed in *P. stellatus,* following environmentally relevant exposures to EMB, IVM and a combination of both, reductions in metabolic capacity (i.e., AS) can have population-level implications for demersal fish species around open-net pen Atlantic Salmon farms. Long-term exposures to concentrations of IVM or EMB and IVM in sediment around the farms could lead to sublethal effects on swimming and metabolic capacity which can then impact growth, feeding, predator avoidance and survival.

2.4.4. Skin pigmentation and burying behaviour

Darkened skin pigmentation

Reduction in an organism's ability to camouflage resulting in an increase in conspicuousness can affect feeding, growth, predator avoidance and survival; similar to depressed swimming ability and AS (Sumner, 1935, 1934). There is substantial diversity in teleost chromatic biology and flatfish (Pleuronectidae) have relatively complex chromatic and patterning ability (Burton, 1998). Flatfish are exceptional among animals in their responses to differences in background patterns, an important adaptation for flat-bodied fish with demersal life histories (Parker and Brown Jr., 1948). Overall, regulation of the movement of pigment containing cells (chromatophores) and organelles within cells to accomplish cryptic colouration and patterns in flatfish is a complex process (Burton, 2010). The cell type responsible for creating the darkened pigment and colouration are melanophores, which are regulated by several processes. Two peptide hormones

released by the pituitary gland can cause darkening (α-melanophore stimulating hormone [α-MSH]) and lightening (melanin concentrating hormone [MCH]) have an antagonistic effect and modulate the effect of the other, with MCH shown to be more potent than α-MSH (Burton, 2010; Burton and Vokey, 2000). To further complicate the process of neurohormone control by α-MSH and MCH, neurons that contain and release both were recently shown to be inhibited by $GABA_A$ receptors and GABA inhibitory neurotransmission (Molagoda et al., 2021; Toossi et al., 2016). In addition to circulating pituitary hormones there are localized signalling factors stored and released from the integument surrounding chromatophores called melanization inhibiting factor (MIF) and melanization stimulating factor (MSF) which work together or independently in establishing pigmentation patterns (Burton, 2010). Teleost chromatophore responses to background patterns may be “physiological”, rapid or short-term responses (seconds to hours) or longer term (days to weeks) “morphological” increases or decreases in pigments and chromatophores (Burton, 2010). The abundance and distribution of chromatophores and the mechanism by which they are regulated can even vary within tissues, with for example, more melanophores observed in the epidermis and iridophores in the dermis of *Pseudopleuronectes americanus* (Burton, 2010, 1980). Chromatophores are also controlled by direct sympathetic innervation by adrenergic axons where norepinephrine and epinephrine as well as α- and β-adrenergic receptors and their subclasses can have opposing effects on aggregation or dispersal of pigments (Burton, 2010). The effects of adrenergic neurotransmission of pigment-containing cells has also shown to be influenced by concentration of the neurotransmitter with a threshold-based effect which have been shown to have more control over the neuroendocrine control of α-MSH and MCH (Burton, 2010). Several environmental stimuli can influence the hue and pattern of flatfish bodies including visual influence from fish retinal stimulation by background substrates, diurnal influences from night and daylight regulated by melatonin released from the pineal gland; as well as localized influences from UV light on pigment-containing cells (Burton, 2010). Excitation, stress and rearing conditions can also influence colour with localized darkening observed in excited fish and hypomelanosis in aquacultured flatfish (Burton, 2010).

One common effect observed across studies and taxa exposing teleosts to IVM and EMB at high concentrations was fish presented with darkened skin pigmentation, which was also observed in the current study (Domingues et al., 2016; Johnson et al., 1993; Katharios et al., 2001; Ogueji et al., 2019; Palmer et al., 1996; Roy et al., 2000; Ucán-Marín et al., 2012). At the end of the 30-d exposure, *P. stellatus* exposed to the two

highest concentrations of IVM and the three highest concentrations of the combination of IVM and EMB showed significantly more fish with substantially darkened bodies with a reduced ability to match their substrate background and were completely unborrowed within the substrate. An EC_{50} value of 255 (95% CI = -41 - 552) for the combination EMB and IVM exposure and 267 (-1953 to 2488) for IVM were found; there was clearly an effect at a certain concentration of IVM and IVM+EMB on *P. stellatus* body colour. Given the complexity of the chromatic biology of flatfish, elucidating a specific mechanism of toxicity for the effect observed of darkened colour and lack of an ability to camouflage for *P. stellatus* following exposure to IVM and a combination of EMB and IVM is beyond the scope of the current study. The chromatic biology of flatfish is a field still being actively studies where gaps in understanding still exist, which also increases the complexity of the discussion of mechanisms of toxicity from IVM and EMB. However, several potential mechanisms are discussed.

External visual stimuli influence the camouflage of flatfish skin where chromatophores will aggregate or concentrate to create a colour and pattern that closely matches their environment, normally the substrate (Burton, 2010). When vision is deprived in flatfish, although pigment changes can still occur, fish do not adaptively camouflage to their substrate background (Burton, 2010; Sumner, 1911). In juvenile plaice (*Pleuronectes platessa*; Healey, 1999; Kelman et al., 2006) as well as *P. stellatus* (Iwanicki, 2016) there is a significant relationship between visual background and expressed pattern. Given the influence on camouflage, an effect on vision and eyesight from IVM or EMB exposure could potentially effect *P. stellatus* ability to camouflage. Zebrafish exposed to IVM have shown an altered response to light-dark transition stimulation, which could be related to effects on vision (Powrie et al., 2022). Other vertebrate species have shown optical effects when exposed to IVM. Ivermectin-induced blindness and retinopathy has been shown for certain breeds of *Canis familiaris* (i.e., domestic dog; Epstein and Hollingsworth, 2013; Kenny et al., 2008) as well as the African Lion (*Panthera leo*; Saqib et al., 2015). In dog breeds known to be deficient in P-glycoprotein, IVM caused mydriasis and other signs of IVM toxicosis (i.e., abnormal pupillary responses), which reinforces the potential visual effect on teleosts known to have a more permissive BBB than mammals (Gupta and Milatovic, 2014; Pulliam, 1985). The action of IVM and EMB on GABAergic neurotransmission could also have an indirect effect on teleost visual stimuli and the subsequent response on camouflage and skin pigment. It has been shown that GABA-mediated feedback in the retina has an important role in converting trichromatic cone

response into colour opponency in horizontal cells (Marc et al., 1978). Exposure of fish to darkness induced depolarization of cone horizontal cells and increased release of GABA (Marc et al., 1978). If GABA is the neurotransmitter associated with the response to dark visual stimuli in fish, then there is potential that uncontrolled or excessive GABA activation and release by IVM and EMB binding to $GABA_A$Rs could lead to continuous “dark” visual stimulus response and therefore darkened body pigment and pattern.

Another potential mechanism of toxicity causing the effect of darkened colouration is the inhibition or alteration of neuroendocrine processes that regulate the release of α-MSH and MCH through inhibitory GABAergic neurotransmission caused by the action of IVM and EMB as $GABA_A$ agonists. GABA receptors and neurons have been found in the brain regions where MCH (Eggermann et al., 2003; Toossi et al., 2016; van den Pol et al., 2004; Xie et al., 2006) is released and have been shown to inhibit MCH release. MCH is responsible for reducing dark melanophore pigments and modulates and inhibits the melanophore dispersal effect of α-MSH, thereby lightening skin tone, and inhibition of MCH release and signalling could lead to unbalanced release and binding of α-MSH which could potentially cause skin darkening. However, $GABA_A$-mediated neurotransmission has also been shown to inhibit α-MSH release (Molagoda et al., 2021). Given that MCH and α-MSH is a process influenced and regulated by several factors, perhaps the GABAergic neurotransmission caused by IVM and EMB exposure potentially inhibiting the release of both neuropeptides alters the balanced release and modulation of both allowing the signal transduction of α-MSH to dominate over the lightening effect of MCH (Burton, 2010; Burton and Vokey, 2000).

Given the myriad of stimuli and neuroendocrine process involved in regulating crypsis in flatfish, presumably the mechanism of avermectins causing darkened colouration in teleosts could be multifaceted. Overall, the obvious concentration-dependent effects causing dark colouration and a potentially reduced camouflage ability in multiple teleost species, including *P. stellatus* at environmentally relevant concentrations, is cause for concern. Camouflage is a key process for predation and feeding as well as predator avoidance in demersal fish species and alterations to this process would increase conspicuousness and potentially reduce survival; which in turn could lead to population-level implications (Burton, 2010; Sumner, 1935, 1934).

Burying behaviour

Another observed effect of IVM and EMB exposure to *P. stellatus* which always accompanied the darkened skin pigmentation was a reduction in burying behaviour. Several flatfish species, including *P. stellatus*, have been shown to select and exhibit burying behaviour in finer grain sediments (e.g., sand and mud) and completely avoid coarser substrates in which they are unable to bury themselves (Moles and Norcross, 1995). The preferred behaviour observed in the current study, was partial or complete body coverage with sediments. The observed behavioural response of burying, or not, was used as the endpoint to indicate an effect of avermectin exposure on *P. stellatus* ability to bury in sediments. The results of burying behaviour mirrored the darkened colouration response exactly, where the two were co-morbid effects of IVM and the combination exposure found at the same treatment concentrations. At the end of the 30-d exposure, *P. stellatus* exposed to the two highest concentrations of IVM (500 and 1000 μg kg^{-1}) and the three highest concentrations of the combination of IVM and EMB (120+100, 600+500 and 1200+1000 μg kg^{-1} EMB+IVM) showed significantly more completely unburied fish. *P. stellatus* in all other treatment concentrations were completely or partially buried at the end of the 30-d exposure. However, there was clearly an effect at a certain concentration of IVM and IVM+EMB on *P. stellatus* burying behaviour. Additional exposures using intermediary concentrations between the treatment where no effect and the treatment where 100% effects were observed would be warranted to refine the estimate of effective concentration values.

Information on whether, or not, teleost can detect and avoid toxicants in the aquatic environment can have profound implications on the level of exposure and toxicity to an organism. Teleosts have been shown to avoid certain toxicants (e.g., Cl^-, and copper), not avoid or detect others and in some cases be attracted by others (Tierney, 2016). Detection of contaminants and other stimuli by fish can be through gustation, olfaction, other chemosensory cells or nociception at the gills; with most avoidance or attraction response being driven by olfaction (Tierney, 2016). Avoidance of a contaminant by fish tends to have a concentration-dependent response between avoidance and the stimulus concentration, with a threshold point below which there is no detectable avoidance and above which there will be partial or complete avoidance (Tierney, 2016). This threshold response of reduced burying was observed in the current study and might indicate *P. stellatus* avoidance of the highest avermectin concentrations, with a complete response

observed above a certain concentration of IVM or the combination of EMB and IVM. Only one study was found which assessed fish avoidance response to an avermectin insecticide (EMB). Sahota et al., (2022) exposed juvenile Pink Salmon (*O. gorbuscha*) to EMB by treated sediment and in in water and found no attraction to EMB, no effect on olfaction and food response following the treated sediment and water exposure but did observe a significant avoidance effect at concentrations above 300 µg L^{-1} EMB. These findings only demonstrated reduced burying at an EMB concentration unlikely to be observed in the marine environment given the mode of application by feed, the low water solubility of EMB and the propensity to accumulate in sediments. Given the limited information on fish avoidance of avermectin-type insecticides, it is difficult to confirm that the effect of reduced burying behaviour was due to intentional avoidance behaviour by *P. stellatus* or due to lethargy and reduced swimming ability caused by inhibitory GABAergic neurotransmission from IVM and the combination of EMB and IVM binding to $GABA_A$ receptors (Estrada-Mondragon and Lynch, 2015; Stevens and Charles, 2001; Xu et al., 2016)

2.4.5. Biochemical analysis

Cortisol

During xenobiotic toxicokinetic responses, resources normally devoted to physiological process (e.g., respiration, digestion, muscle contraction) are diverted to metabolize, eliminate the toxicant and control and repair cellular or physiological damage caused by the xenobiotic (e.g., necrosis or oxidative stress). This response is partially influenced by the teleost stress response, mediated by serum concentrations of the stress hormone, cortisol. Cortisol, a principal corticosteroid that plays a role in intermediary metabolism, ion regulation and osmoregulation, and immune function (Mommsen et al., 1999; Norris and Hobbs, 2006). In response to a stressor causing serum cortisol concentrations increase, elevations in other haematological components such as glucose, lactate, sodium and potassium concentrations will also occur to provide the resources needed to respond to the stressor (Mommsen et al., 1999; Norris and Hobbs, 2006; Rotllant et al., 2001; Skjervold et al., 1999). In the current study, significant differences in whole-body cortisol concentrations in *P. stellatus* between the negative control and all treatments of EMB, IVM and the combination of both, were observed. Previous studies have assessed the effect of avermectin exposure on serum cortisol concentration in

teleosts. In *L. rohita* exposed to EMB (Kumar et al., 2022) and *O. niloticus* exposed to abamectin and EMB (Fırat and Tutus, 2020), significant alterations in hematological stress parameters including significantly elevated cortisol. Kilercioglu et al., (2020) found elevated expression of genes related to stress response in liver tissue of Rainbow Trout exposed to EMB. In sea-lice infected *S. salar* treated with EMB applied to feed, no significant elevation in serum cortisol was observed (Poley et al., 2013). Unfortunately, all studies found which measured cortisol values following an avermectin exposure used serum cortisol, rather than whole body. In the current study using *P. stellatus* from the pilot study, blood draws from the caudal vein were attempted using a capillary tube, with little success at drawing a sufficient sample even with larger individuals. This was potentially related to the anatomy of flatfish and caused the current study to focus on tissue instead of hematological, biochemical parameters. When comparing the whole body values from the current study to other fish whole-body cortisol values for *D. rerio* (Barcellos et al., 2007; Ramsay et al., 2006), *Jenynsia multidentate*, Rainbowfish (*Melanoteania duboulayi*; (Zuberi et al., 2014), and the juvenile flatfish *P. americanus* (Breves and Specker, 2005), the values of the current study (542.34 – 1589.21 pg g^{-1}) were within the ranges of values observed (100-45000 pg g^{-1}). The lower whole body cortisol values observed in the current study are likely due to the white muscle filet section and liver removal for metabolic parameters, and therefore the direct comparison is not possible.

The stress response of *P. stellatus* as indicated by whole-body cortisol concentration did not show an effect of avermectin exposure. It is possible that an effect on cortisol levels as an endpoint is less sensitive than the behavioural endpoints such as U_{burst}, AS, skin pigmentation and burying behaviour, where higher concentrations not yet observed in the environment could elicit a change in cortisol levels. The duration of the exposure might also explain no observed effect, where a longer exposure to avermectins might elicit a cortisol response. Additionally, there is potential that environmentally relevant concentrations of IVM and EMB in sediments do not induce a stress response in *P. stellatus* that would lead to elevated whole-body cortisol concentrations. Future studies should assess the statistical power and sample size required to confidently detect a significant alteration in cortisol levels, and attempt complete rather than partial whole-body sampling or blood sampling.

Glucose and Lactate

Liver and white muscle glucose levels were analyzed to elucidate whether alterations in U_{burst} were related to changes in energy utilization from aerobic to anaerobic respiration. Burst swimming relies heavily on the anaerobic mobilization of metabolites form carbohydrate sources (Beamish, 1978). Alterations in aerobic respiration is commonly observed toxicant exposure in animals, normally due to the diversion of metabolic resources normally used for normal bodily and behavioral process to responding to the toxicant. When the capacity for aerobic is exceeded, anaerobic respiration to create glucose is used, which can lead to elevated liver and white muscle glucose and lactate levels. Increases in anaerobic respiration due to xenobiotic defense mechanisms following exposure to IVM or EMB could reduce *P. stellatus* metabolic capacity (AS), which could have implications on growth, swimming and survival. Previous studies assessing the effect avermectin-type insecticides on teleost biochemical parameters have provided mixed results. Serum glucose concentrations in teleost fish were significantly elevated following exposure to IVM (Katharios et al., 2001; Ogueji et al., 2019) and EMB (Das et al., 2022; Julinta et al., 2020) but have also shown no significant changes when exposed to IVM (Kolarova et al., 2022; Lozano et al., 2021) and EMB (Kilercioglu et al., 2020). Tissue concentrations of glucose and lactate in animals exposed to avermectins also showed somewhat mixed results. In mice significant increases in liver and muscle lactate levels following administration of EMB in drinking water was observed (Naik et al., 2021). Elevations in liver lactate and glucose were also observed in *S. auratus* exposed to avermectin (Li et al., 2015), as well as increases to muscle glucose and liver lactate dehydrogenase (LDH) levels following abamectin exposure (Rohmah et al., 2022; Singha et al., 2022); all of which would indicate anaerobic metabolism in the liver. The findings of the current study did not corroborate the previous findings of significantly elevated liver and muscle lactate and glucose levels in teleosts exposed to avermectins as no significant differences from the control were found for liver and white muscle glucose and lactate for all exposures. Overall, the significant and concentration-dependent reduction in *P. stellatus* AS and burst swimming ability in the current study could not be attributed to altered tissue energy metabolism in liver and white muscle tissues.

2.5. Conclusions

The avermectin-type insecticides EMB (in the SLICE® formulation) and IVM are currently in use in Canada to treat sea lice infestations in open-net pen Atlantic Salmon aquaculture operations in both the Pacific and Atlantic regions, with sequential applications of SLICE® and IVM sometimes used when resistance is suspected or encountered (Hamoutene et al., 2022). Additionally, some recent avermectin application data reported by the aquaculture operations showed substantially more SLICE® applied to feed than was therapeutically indicated, which could increase the amount in the surrounding marine environment (Hamoutene et al., 2022). The tendency of EMB and IVM to persist in the benthic marine sediments for long periods of time (half-life >188 d), the potential combined or subsequent applications of EMB and IVM as well as excessive dosing of feed is cause for concern of the potential for the environmental concentrations and potential sublethal toxic effects to non-target benthic fauna of both EMB and IVM individually and in combination with EMB (Benskin et al., 2016; Davies et al., 1998; Hamoutene et al., 2022; Prasse et al., 2009; SEPA, 2004). In the current study, significant reductions were observed in burst swimming performance (U_{burst}) and metabolic capacity (AS), as well as significantly darkened skin pigmentation potentially affecting camouflage ability, and reduced sediment burying in *P. stellatus* exposed to IVM and the combination of EMB and IVM, but not to those treated with only EMB. These findings suggest that IVM was likely the cause of the observed toxic effects in the combination exposure. The physiological and behavioral effects observed herein for a benthic marine teleost exposed to IVM and EMB in sediment, at or near previously reported sediment concentrations, are a cause for concern for the fitness and survival individually and at the population level of non-target demersal teleost species present below the *S. salar* aquaculture operations. Reductions in IVM use in Atlantic Salmon aquaculture has declined in recent years (Hamoutene et al., 2022). However, given that EMB in the SLICE® formulation caused no significant effects, whereas IVM and the combination of IVM and EMB did, consideration should be given to eliminating Ivermectin use in open-net pen Atlantic Salmon aquaculture operations as a proactive measure to protect non-target teleost species from potential exposures that would induce sublethal effects that could affect fitness and survival.

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2.7. Tables and Figures

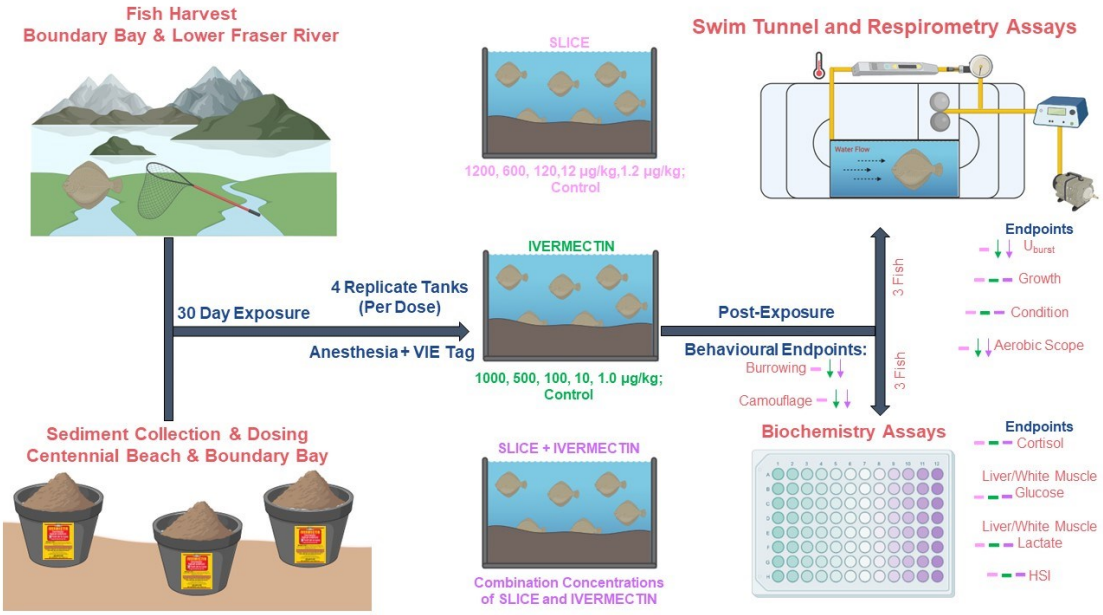

Figure 2-1. Exposure design and results summary for behavioural, physiological and biochemical effects in Platichthys stellatus exposed to IVM, EMB and the combination of both in marine sediments. The observed effects from EMB, IVM and the combination are denoted by pink, green and purple arrows and dashes, respectively.

Figure 2-2. Example image of the camouflage and burying behaviour of control fish (left) compared to fish from the highest combination concentration (right) of EMB and IVM (1200+1000 µg kg^{-1} EMB+IVM) at the end of the 30-d exposure period.

Table 2-1. **Behavioural, physiological and biochemical endpoints of Platichthys stellatus measured following a 30-d exposure to sediment treated with emamectin benzoate (EMB) prepared from SLICE® 0.2% premix, ivermectin (IVM) or a combination of both (EMB+IVM). Endpoints measured were mortality, growth (% change in mass), relative condition (K_n), hepatosomatic index (HSI), Burst swim speed (U_{burst}), resting metabolic rate (RMR), maximum metabolic rate (MMR), aerobic scope (AS), burying behaviour, darkened skin, liver glucose and lactate, white muscle glucose and lactate and remaining whole-body cortisol. Statistical tests selected based on satisfying required assumptions (e.g., normality for ANOVA) and statistical significance was denoted by $p<0.05$. SE=standard error; µg kg^{-1}=µg chemotherapeutant for each kg of sediment; NOEC=no observed effect concentration; LOEC=lowest observed effect concentration;*=significance between one or more treatments with no clear concentration-dependent trend; EC_x=effective concentration to elicit an x% response. NOEC and LOEC are based on no effects or observed effects significantly different from the negative control. EMB+IVM EC_x values are combined concentrations with 55%EMB and 45%IVM.**

Chemical	Endpoint	Test	Statistic (p-value)	NOEC/LOEC (µg kg^{-1})	EC_{25}/EC_{50} ±SE (µg kg^{-1})
EMB	Mortality	-	<10%	-	-
IVM		-	<10%	-	-
EMB+IVM		-	12.5%	-	-
EMB*	Growth (% Δmass)	Kruskal-Wallis	χ^2 =19.9 (p=0.001)	1200/-	-
IVM*		Kruskal-Wallis	χ^2 =40.42 (p<0.001)	-	-
EMB+IVM		Kruskal-Wallis	χ^2 =7.29 (p=0.20)	1200+1000/-	-
EMB	K_n	ANOVA	F=0.76 (p=0.58)	1200/-	-
IVM		ANOVA	F=1.89 (p=0.15)	1000/-	-
EMB+IVM		Kruskal-Wallis	χ^2 =10.3 (p=0.07)	1200+1000/-	-
EMB	HSI	Kruskal-Wallis	χ^2 =2.09 (p=0.84)	1200/-	-
IVM		Kruskal-Wallis	χ^2 =8.21 (p=0.15)	1000/-	-
EMB+IVM		Kruskal-Wallis	χ^2 =1.59 (p=0.90)	1200+1000/-	-
EMB	U_{burst}	ANOVA	F=1.75 (p=0.14)	1200/-	-
IVM		ANOVA	F=8.06 (p<0.0001)	500/1000	-/ 262±2398
EMB+IVM		ANOVA	F=17.89 (p<0.0001)	12+10/ 120+100	630±245/ 3096±895
EMB*	RMR	Kruskal-Wallis	χ^2 =13.0 (p=0.02)	1200/-	-
IVM		Kruskal-Wallis	χ^2 =10.3 (p=0.07)	1000/-	-
EMB+IVM		Welch's ANOVA	F=0.74 (p=0.62)	1200+1000/-	-
EMB	MMR	Kruskal-Wallis	χ^2 =10.0 (p=0.08)	1200/-	-
IVM		Kruskal-Wallis	χ^2 =33.2 (p<0.0001)	1/10	-

EMB+IVM		Kruskal-Wallis	χ^2 =23.2 (p=0.0003)	120+100/ 600+500	-
EMB	AS	ANOVA	F=2.40 (p=0.05)	600/1200	-
IVM		ANOVA	F=5.05 (p=0.005)	100/500	12.27±180/ 1581±1345
EMB+IVM		ANOVA	F=8.84 (p<0.0001)	12+10/ 120+100	35±67/ 3529±2978
EMB	Burying	-	-	1200/-	-
IVM		Likelihood Ratio	χ^2 =168.6 (p<0.0001)	100/500	- /267±1068
EMB+IVM		Likelihood Ratio	χ^2 =168.6 (p<0.0001)	100/500	211±67/ 3529±2978
EMB	Skin Darkening	-	-	1200/-	-
IVM		Likelihood Ratio	χ^2 =168.6 (p<0.0001)	100/500	- /267±1068
EMB+IVM		Likelihood Ratio	χ^2 =146.1 (p<0.0001)	12+10/ 120+100	220±14/ 255±151
EMB	Liver Glucose	Kruskal-Wallis	χ^2 =5.81 (p=0.33)	1200/-	-
IVM		Kruskal-Wallis	χ^2 =2.10 (p=0.84)	1000/-	-
EMB+IVM		Kruskal-Wallis	χ^2 =6.89 (p=0.23)	1200+1000/-	-
EMB	White Muscle Glucose	Kruskal-Wallis	χ^2 =8.93 (p=0.11)	1200/-	-
IVM		Welch's ANOVA	F=1.04 (p=0.42)	1000/-	-
EMB+IVM		Welch's ANOVA	F=1.18 (p=0.35)	1200+1000/-	-
EMB*	Liver Lactate	Kruskal-Wallis	χ^2 =12.7 (p=0.03)	1200/-	-
IVM		Kruskal-Wallis	χ^2 =2.10 (p=0.84)	1000/-	-
EMB+IVM		Welch's ANOVA	F=1.80 (p=0.16)	1200+1000/-	-
EMB	White Muscle Lactate	Kruskal-Wallis	χ^2 =8.47 (p=0.13)	1200/-	-
IVM*		Kruskal-Wallis	χ^2 =13.9 (p=0.02)	1000/-	-
EMB+IVM		Welch's ANOVA	F=0.26 (p=0.92)	1200+1000/-	-
EMB	Cortisol	Kruskal-Wallis	χ^2 =5.13 (p=0.40)	1200/-	-
IVM		Kruskal-Wallis	χ^2 =9.17 (p=0.10)	1000/-	-
EMB+IVM*		Kruskal-Wallis	χ^2 =14.8 (p=0.01)	1200+1000/-	-

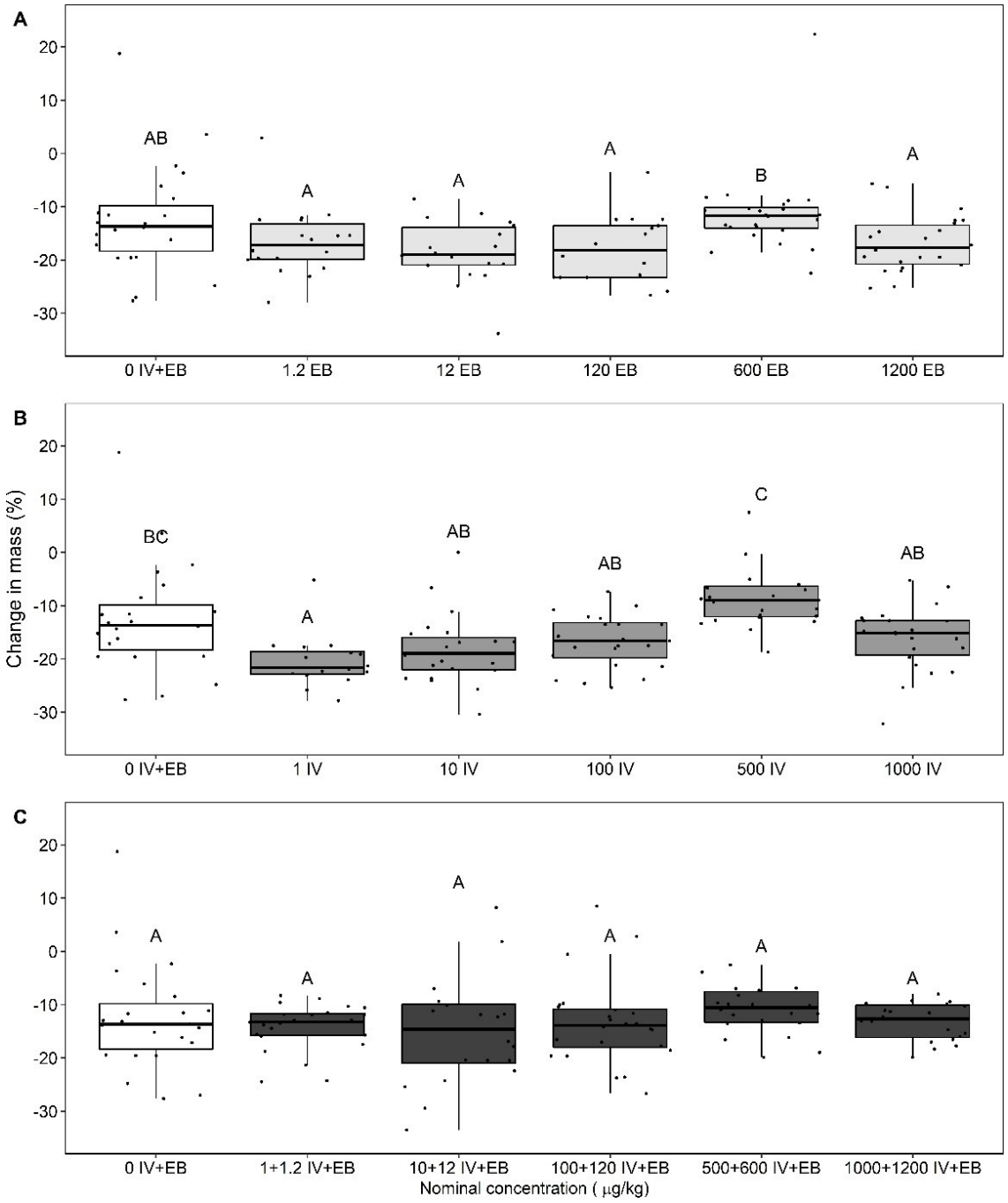

Figure 2-3. Percent change in mass of *P. stellatus* exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (n = 12, boxes = interquartile range (IQR), bolded line = median, whiskers = 1.5*IQR, dots = individual fish). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05).

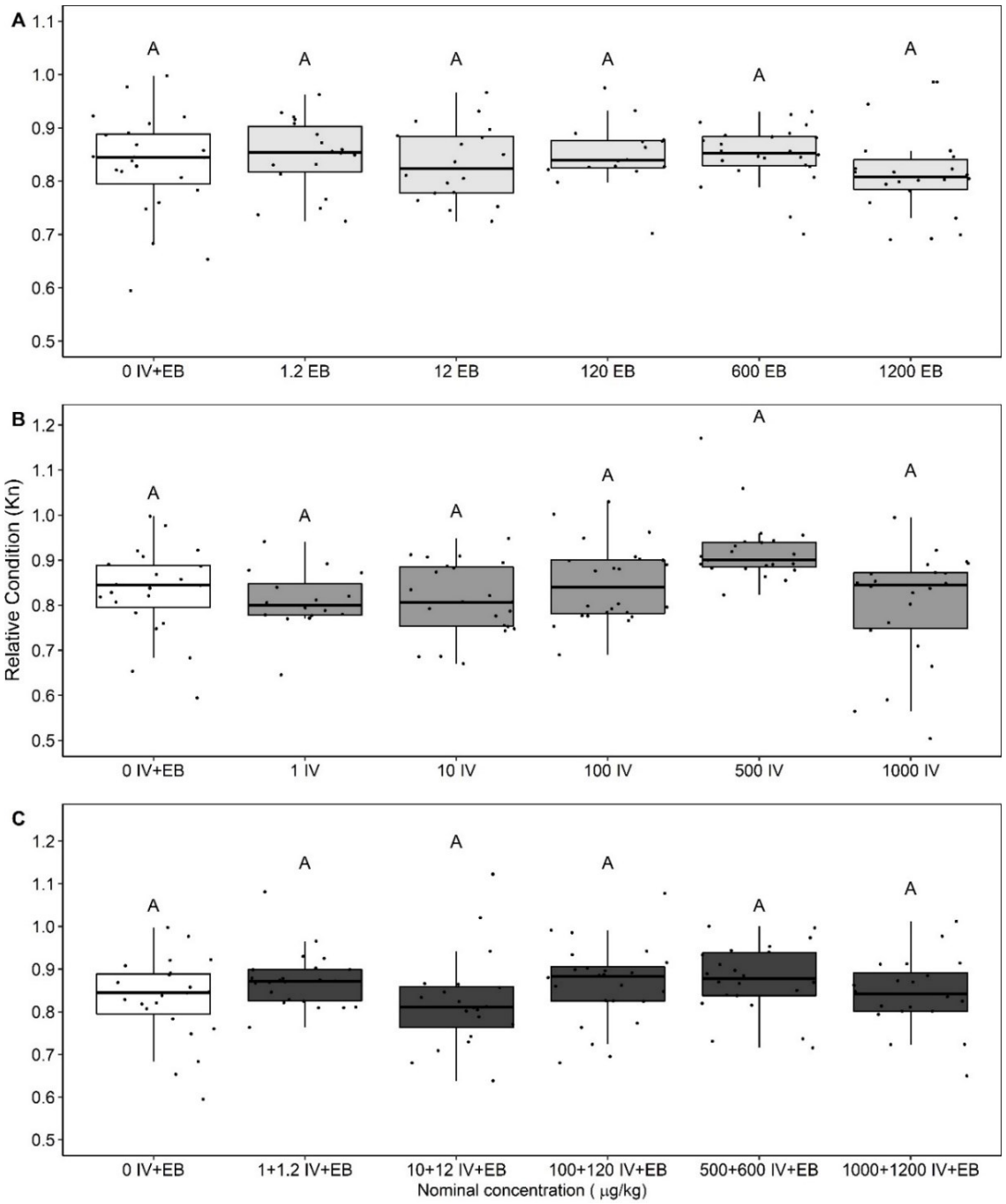

Figure 2-4. **Relative condition (K_n) of *P. stellatus* exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (n = 12, boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05).**

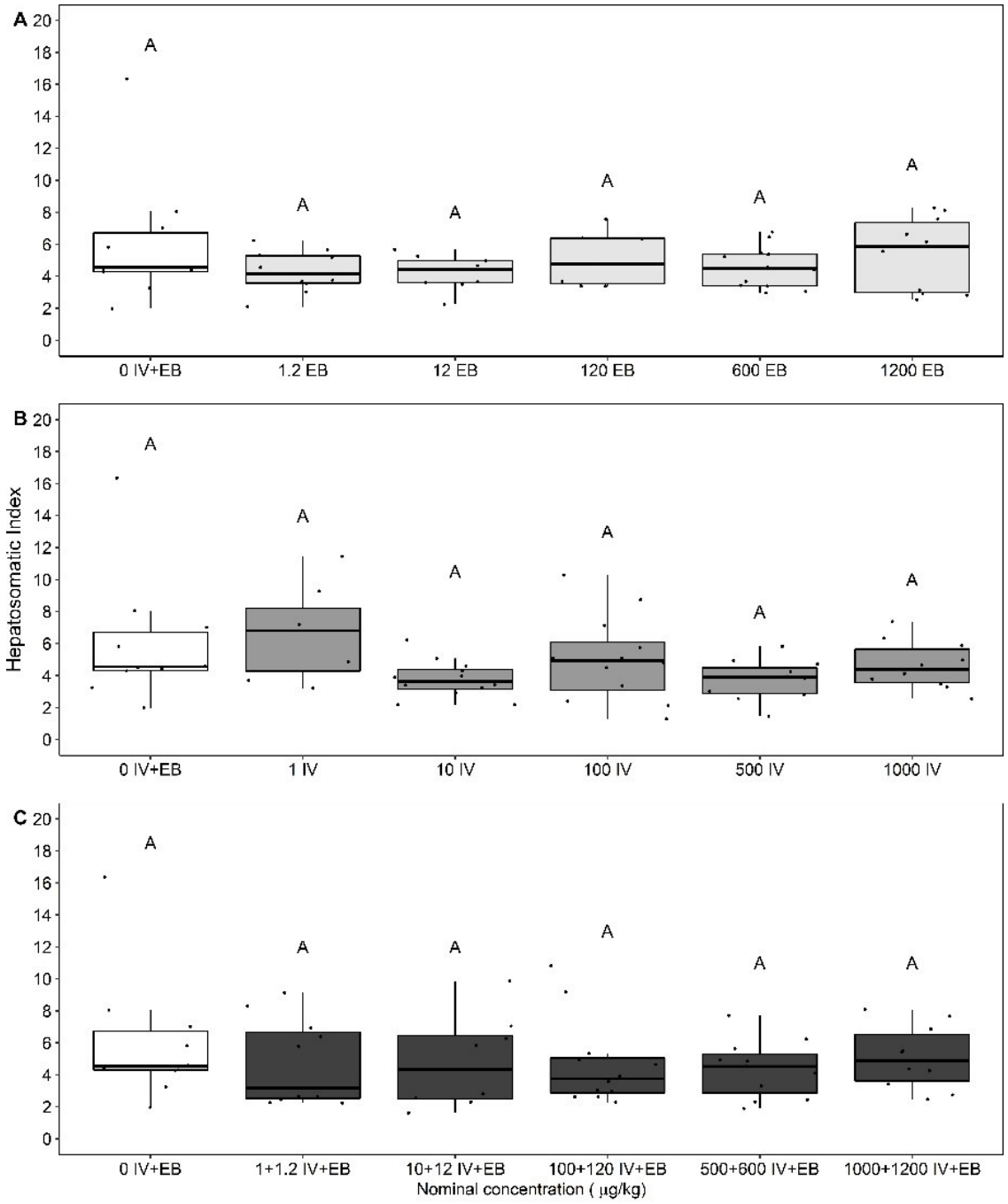

Figure 2-5. Hepatosomatic Index *(HSI),* of Platichthys stellatus exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM. (n = 12, boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish) Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control . Upper case letters represent statistically different groups (p < 0.05).

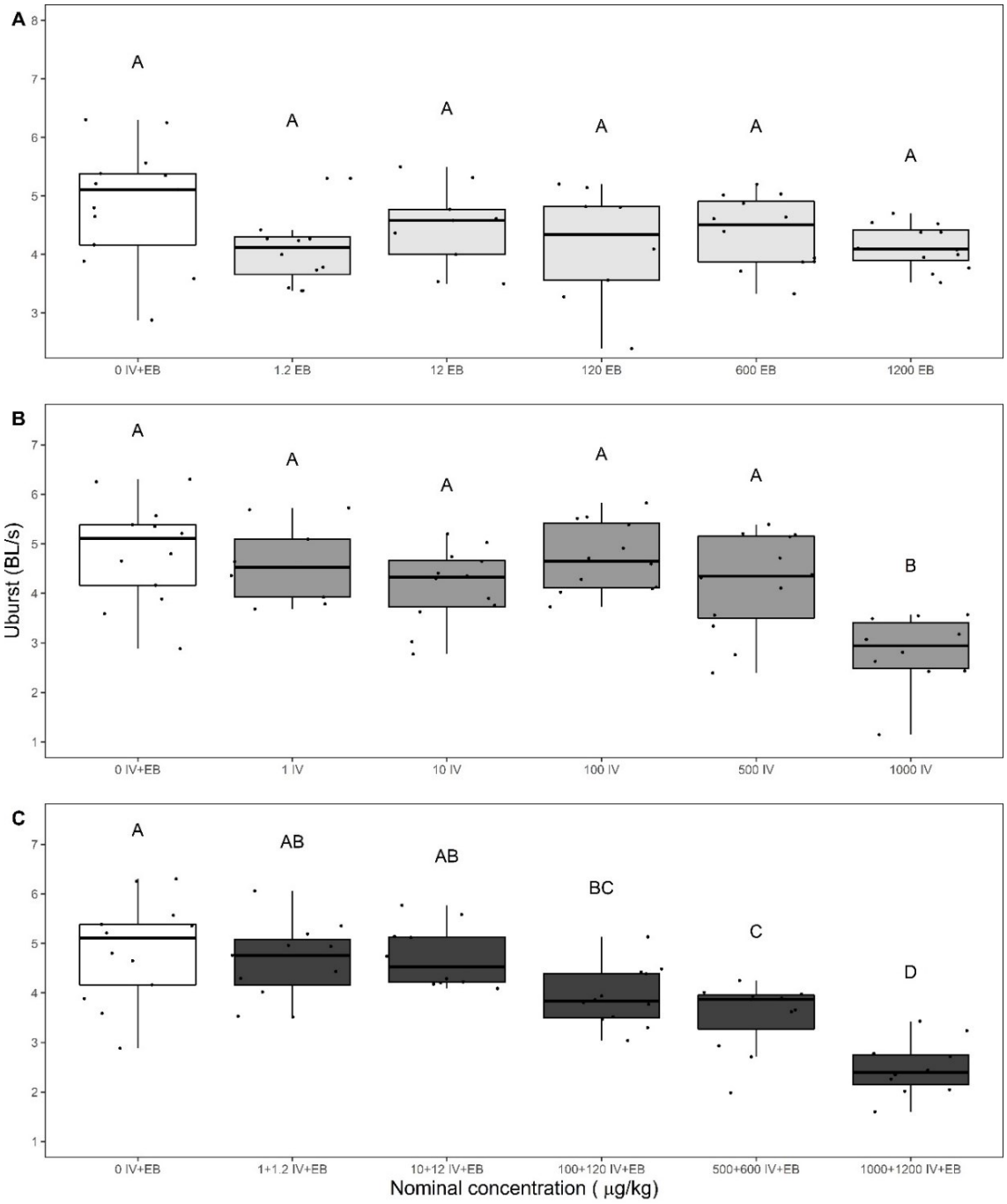

Figure 2-6. Burst swimming performance (U_{burst}) of *P. stellatus* exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (n = 12, boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05).

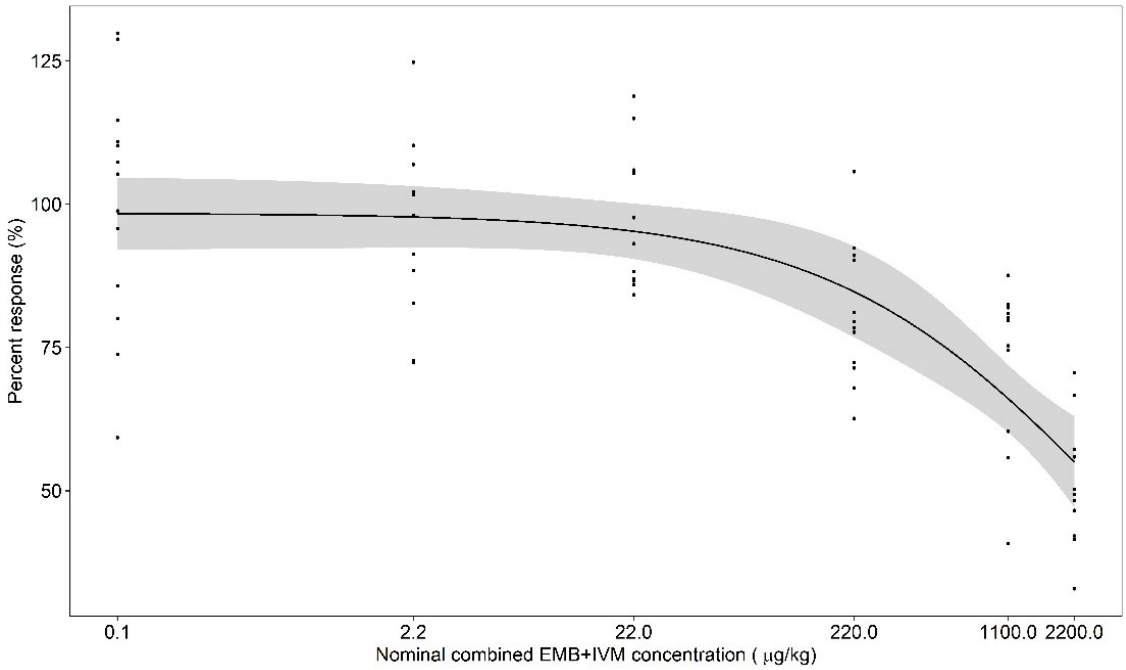

Figure 2-7. Percent response relative to the negative control of burst swimming performance (U_{burst}) of *P. stellatus* exposed for 30-d to sediment spiked with ivermectin (IVM, n = 12, black line = mean, dots = individual fish, grey shaded area = 95% confidence interval. Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05). Concentration-response curve created using 'drc' package in R using a 3-parameter log logistic model.

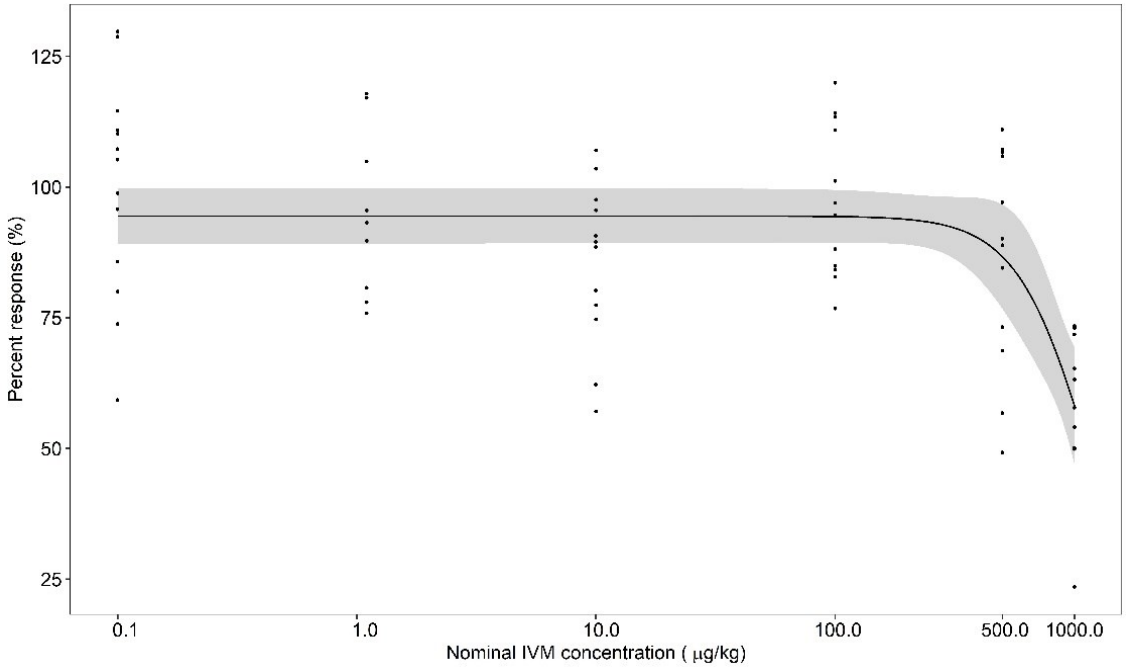

Figure 2-8. **Percent response relative to the negative control of burst swimming performance (U_{burst}) of P. stellatus exposed for 30-d to sediment spiked with a combination of both emamectin benzoate (EMB) and ivermectin (IVM, n = 12, black line = mean, dots = individual fish, grey shaded area = 95% confidence interval). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05). Concentration-response curve created using 'drc' package in R using a 3-parameter log logistic model.**

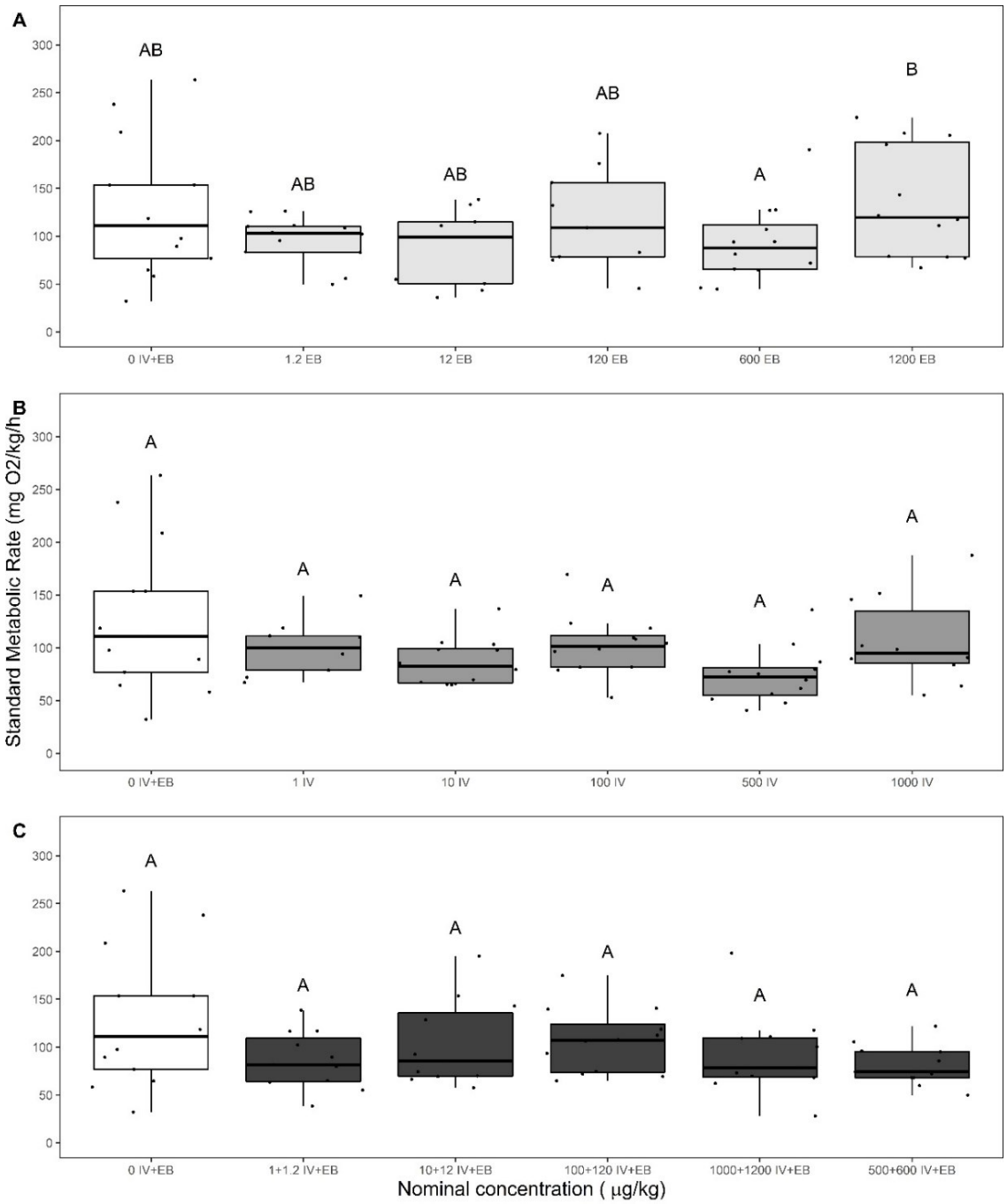

Figure 2-9. Standard metabolic rate (SMR) of *P. stellatus* exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (n = 12*, boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish*). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05).

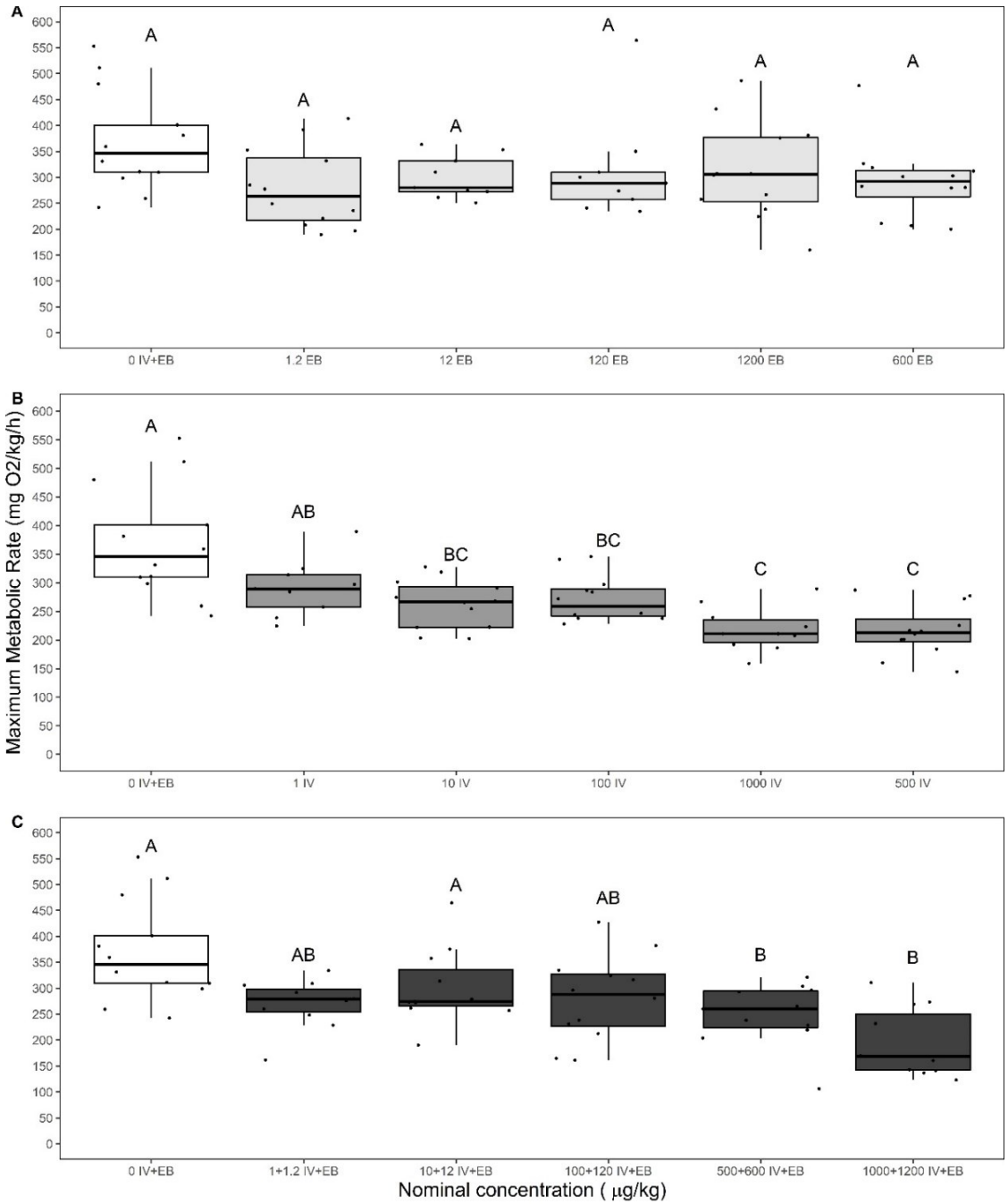

Figure 2-10. Maximum metabolic rate (MMR) of *P. stellatus* exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (n = 12*, boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish*). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control . Upper case letters represent statistically different groups (p < 0.05).

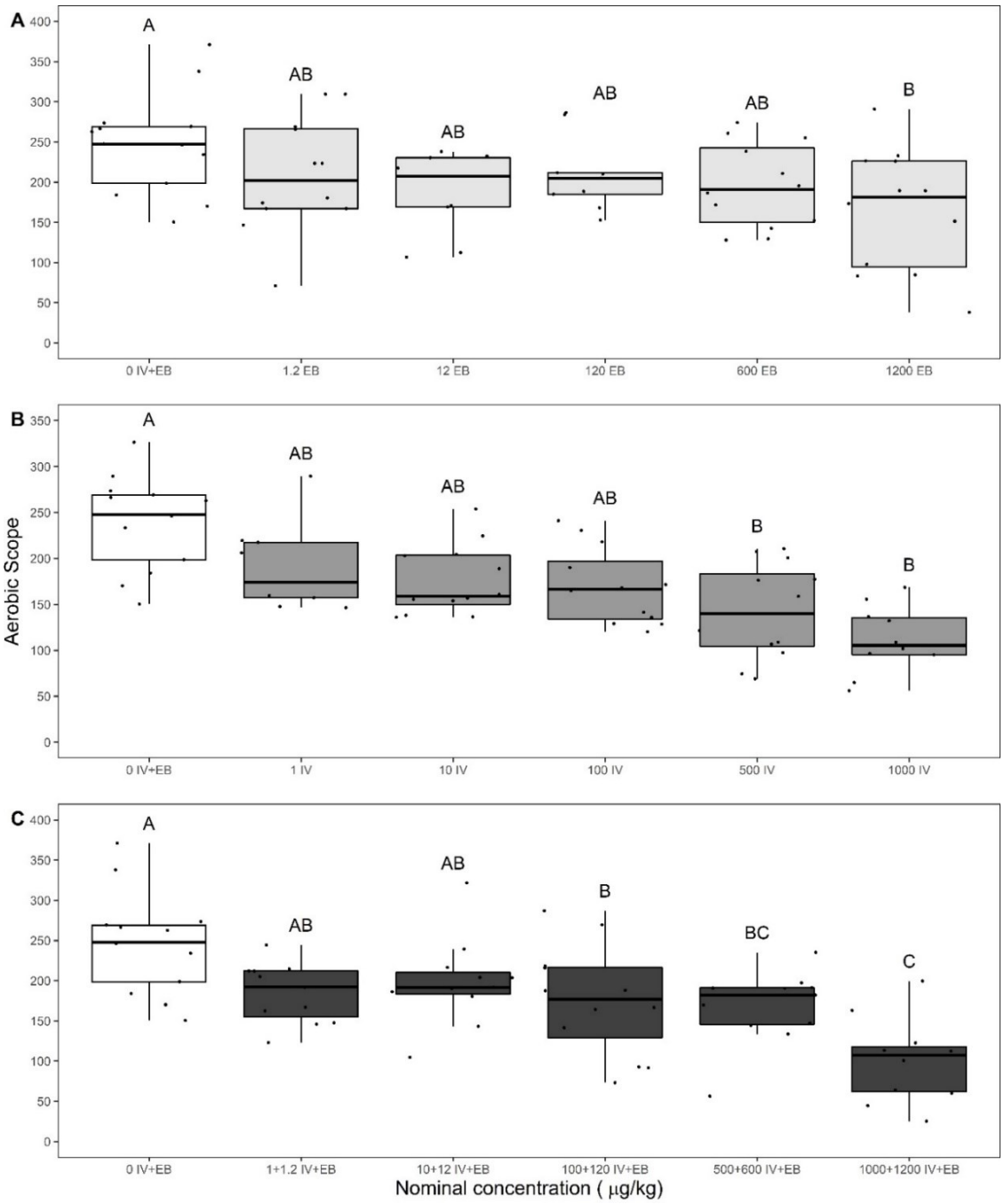

Figure 2-11. Aerobic scope (AS) of *P. stellatus* exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (n = 12*, boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish*). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05).

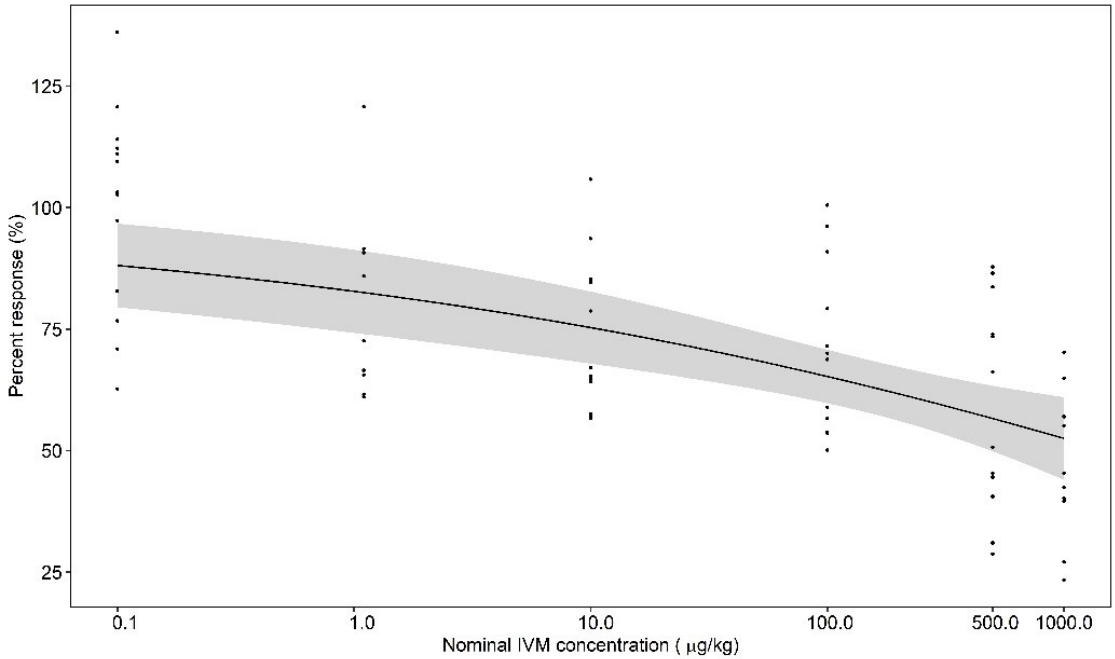

Figure 2-12. Percent response relative to the negative control of aerobic scope (AS) of P. stellatus exposed for 30-d to sediment spiked with ivermectin (IVM, n = 12, black line = mean, dots = individual fish, grey shaded area = 95% confidence interval Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05). Concentration-response curve created using 'drc' package in R using a 3-parameter log logistic model.

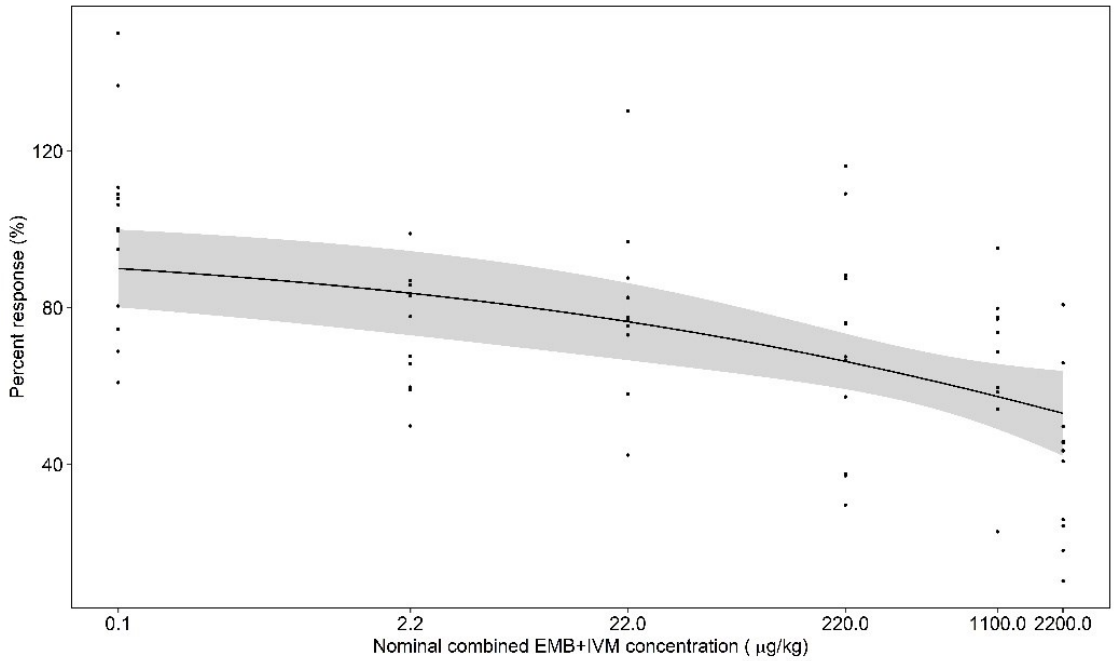

Figure 2-13. Percent response relative to the negative control of aerobic scope (AS) of Platichthys stellatus exposed for 30-d to sediment spiked with a combination of both emamectin benzoate (EMB) and ivermectin (IVM, n = 12, black line = mean, dots = individual fish, grey shaded area = 95% confidence interval. Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05). Concentration-response curve created using 'drc' package in R using a 3-parameter log logistic model.

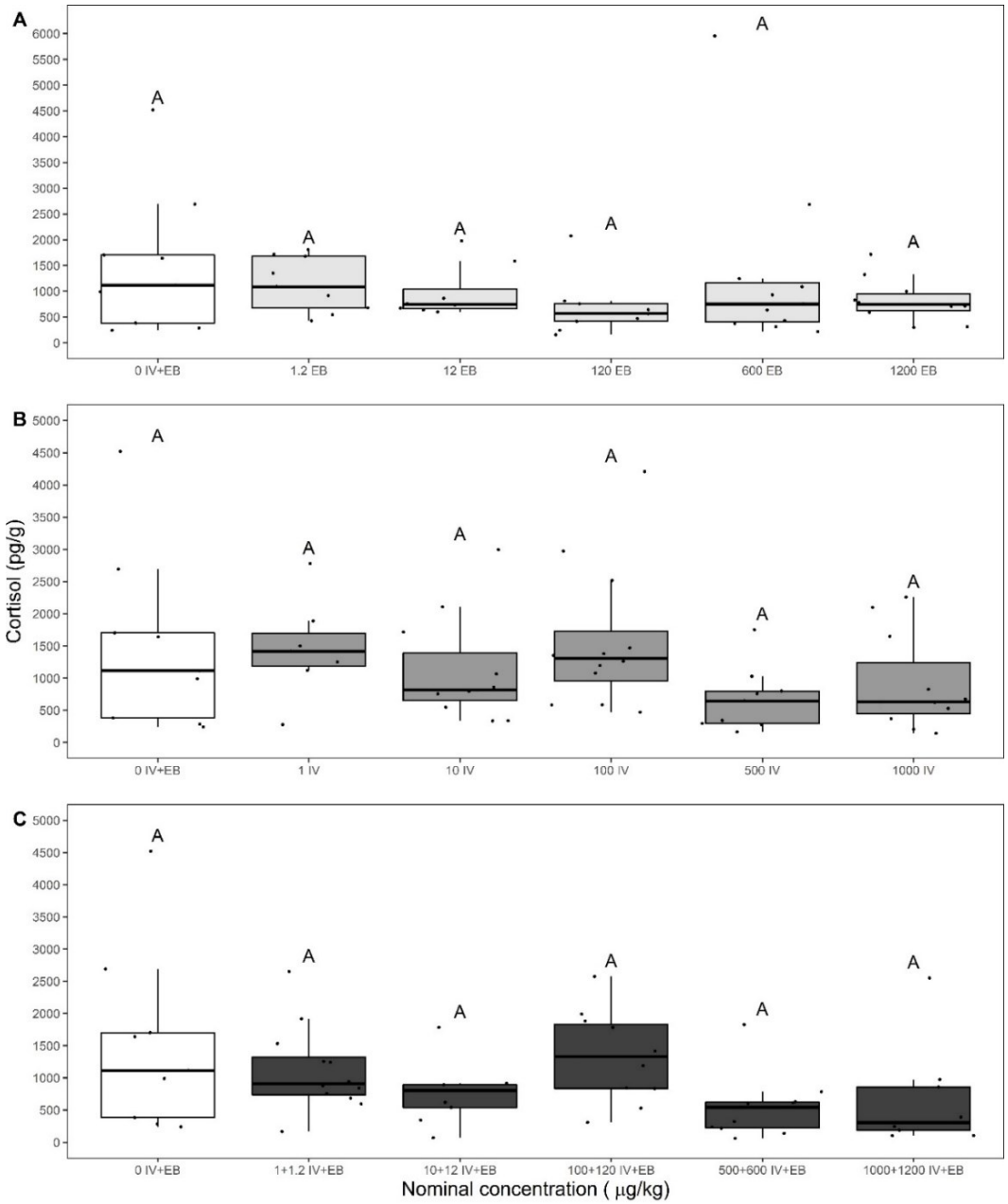

Figure 2-14. Remaining whole body cortisol concentration of *P. stellatus* exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (n = 12*, boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish*). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Whole body cortisol was quantified using the remaining tissue following the removal of the liver and left dorsal white muscle section of each fish. Upper case letters represent statistically different groups (p < 0.05).

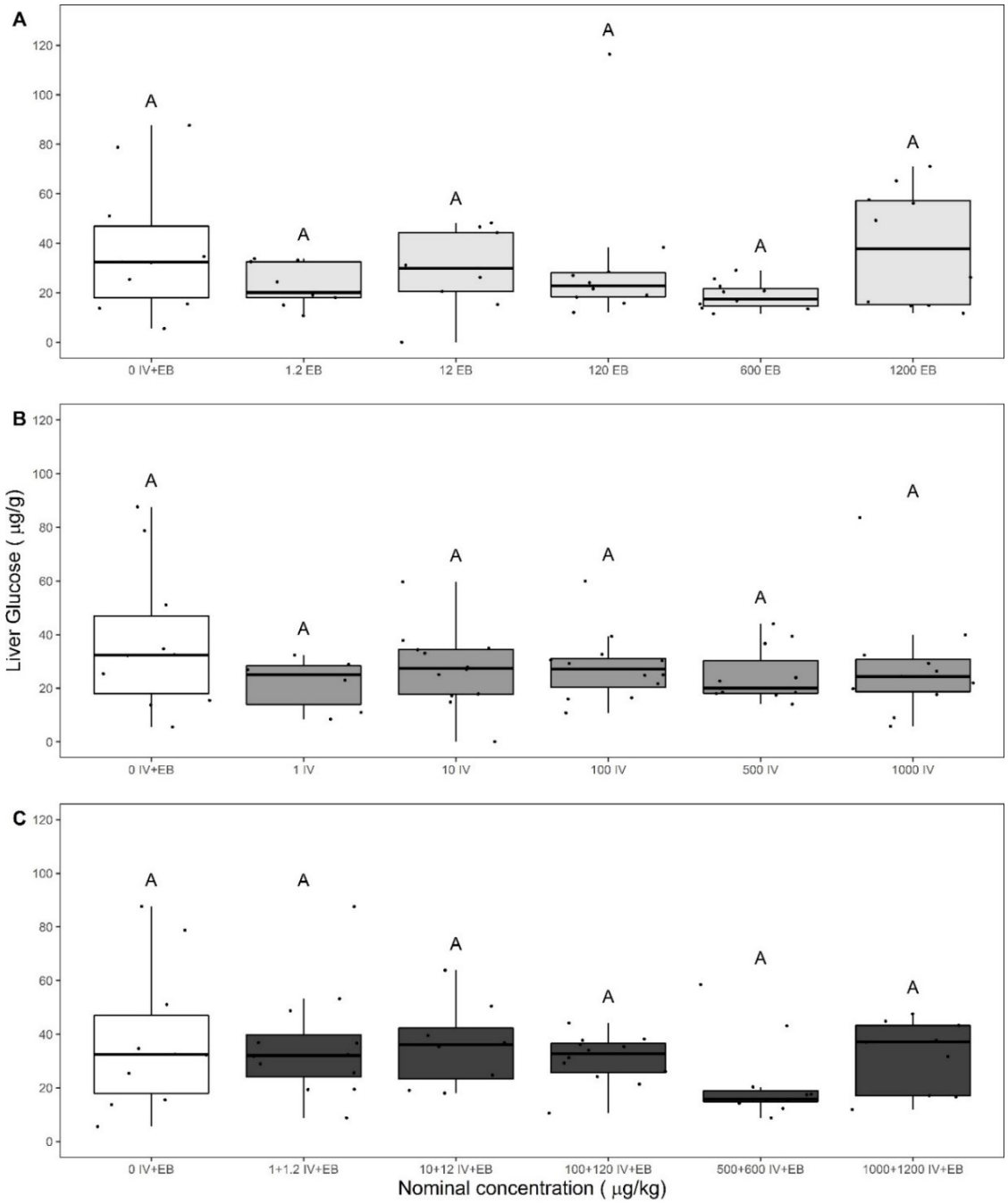

Figure 2-15. Liver glucose concentration of *P. stellatus* exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (n = 12*, boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish*). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control . Upper case letters represent statistically different groups (p < 0.05).

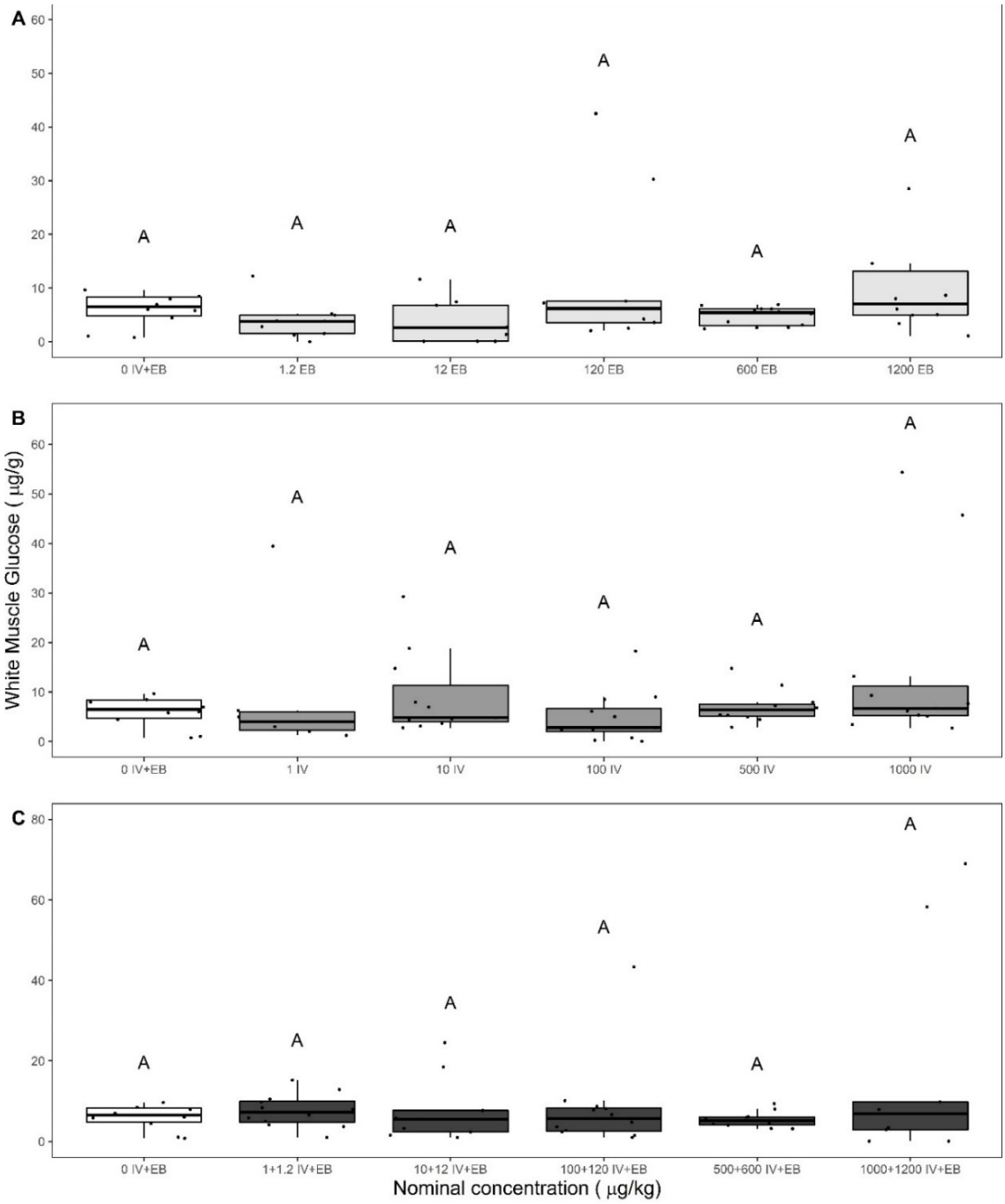

Figure 2-16. White muscle glucose concentration of *P. stellatus* exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (n = 12*, boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish*). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05).

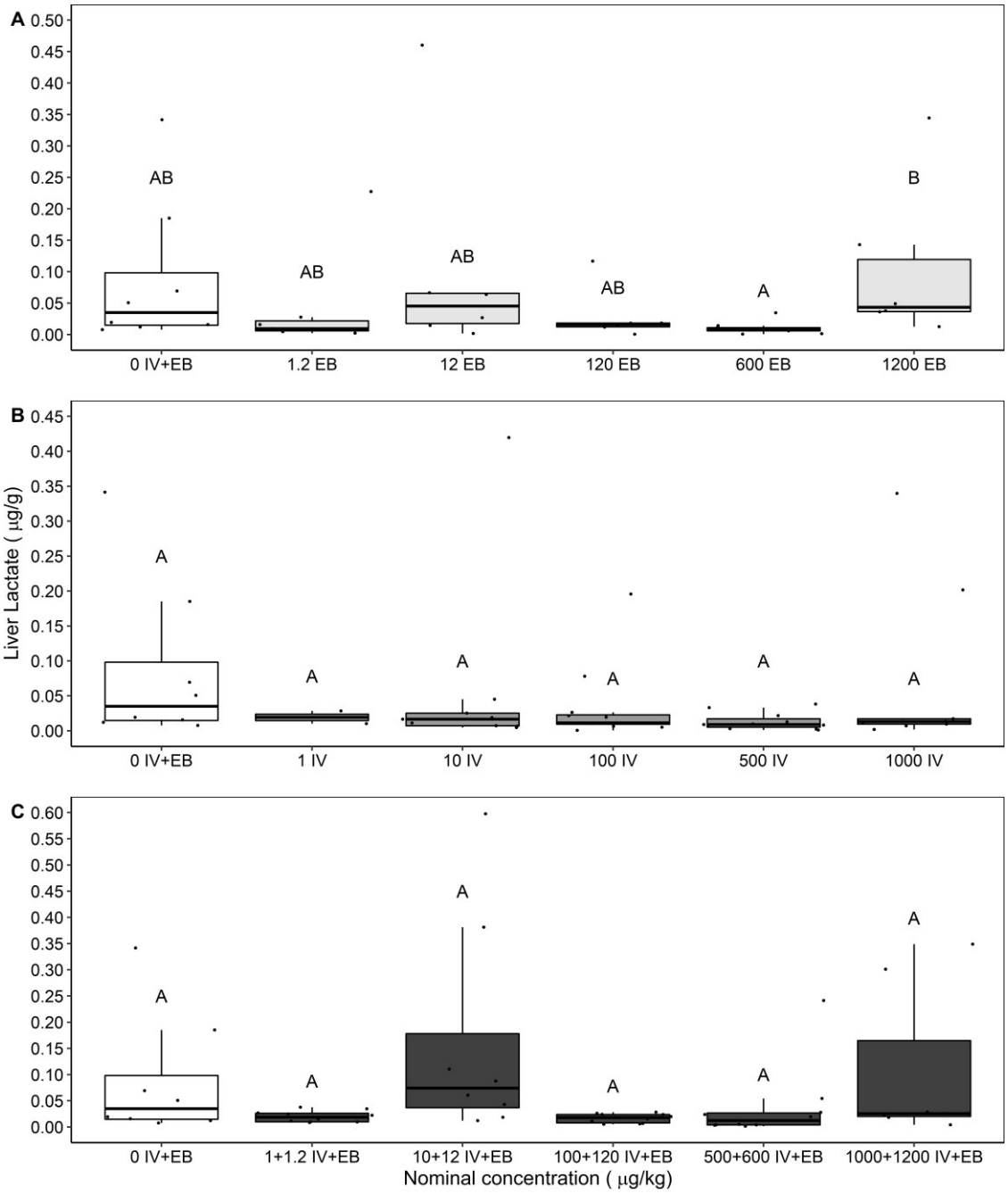

Figure 2-17. Liver lactate concentration of *P. stellatus* exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (n = 12*, boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish*). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control . Upper case letters represent statistically different groups (p < 0.05).

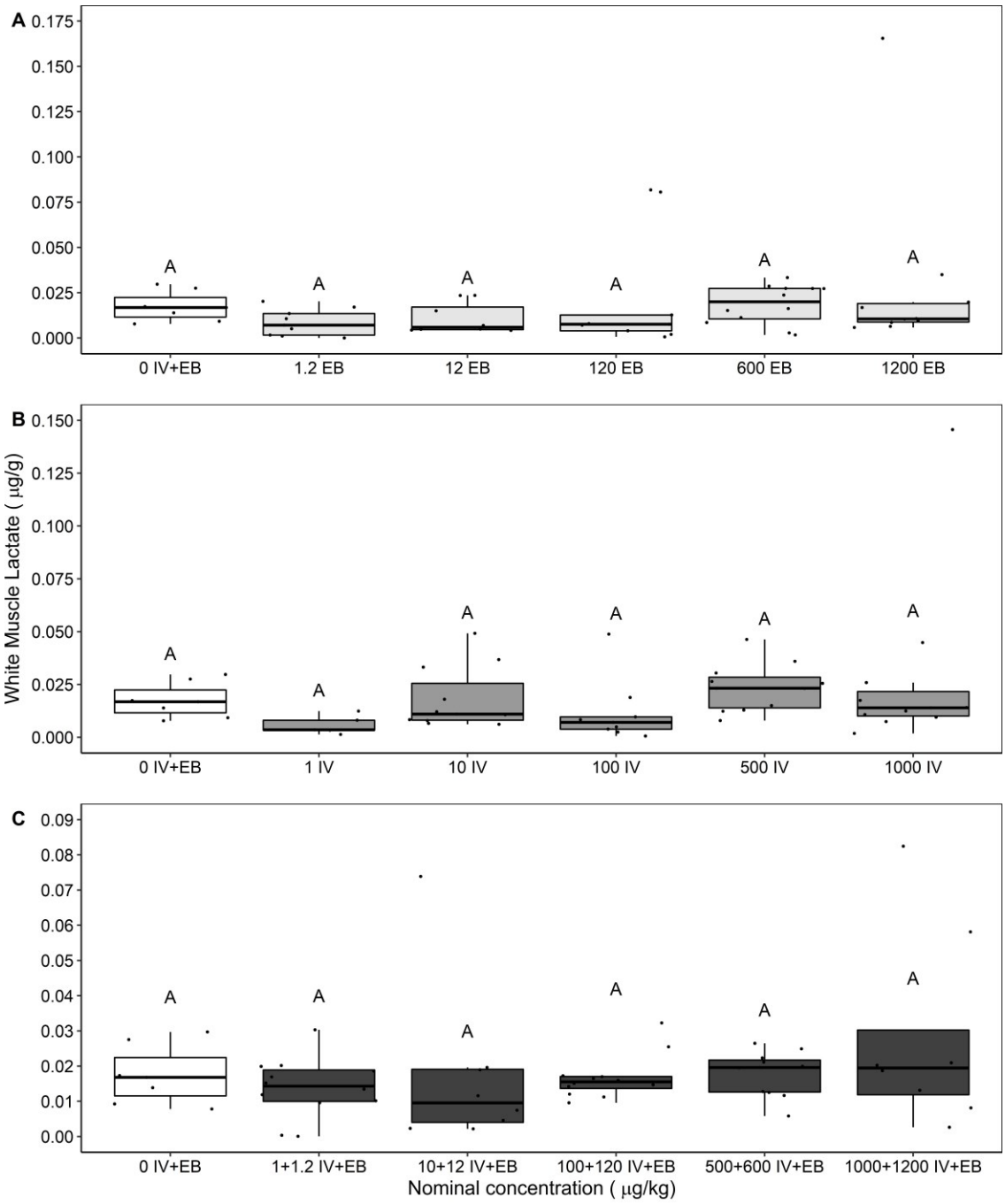

Figure 2-18. White muscle lactate concentration of *P. stellatus* exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (n = 12*, boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish*). Concentrations were 1.2, 12, 120, 600 and 1200 µg EMB/kg sediment (EMB); 1, 10, 100, 500 and 1000 µg IVM/kg sediment (IVM); 1.2+1, 12+10, 120+100, 600+500, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05).

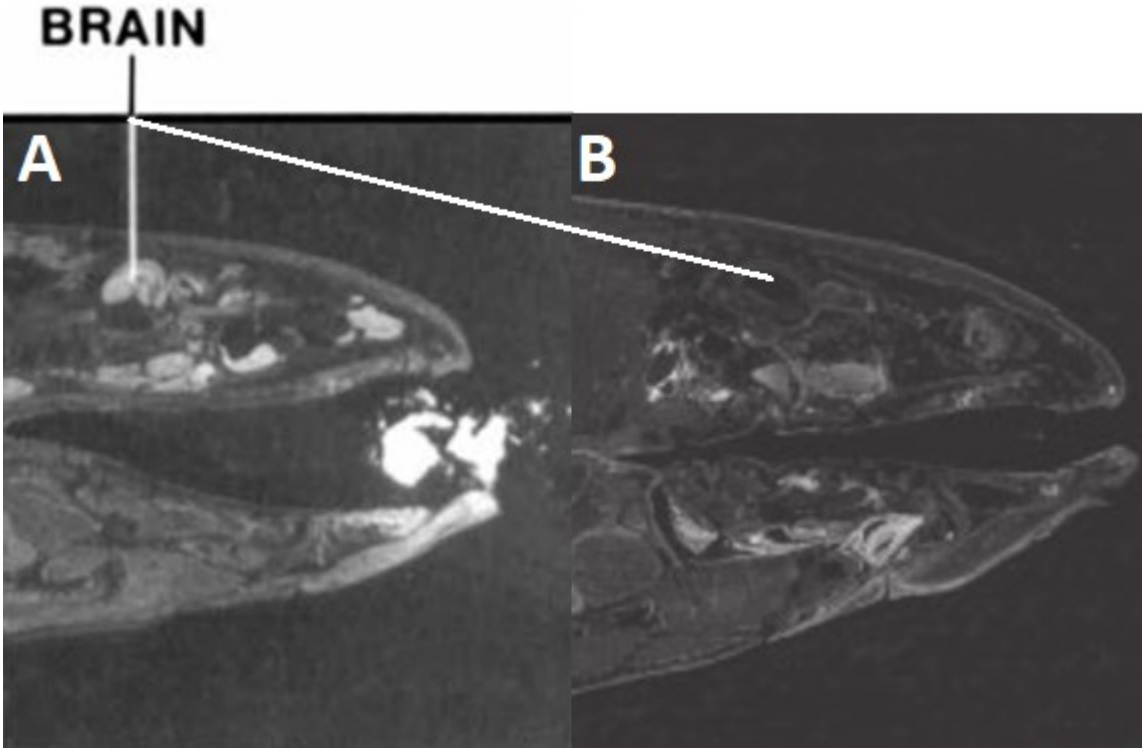

Figure 2-19. Autoradiograph imagery of the distribution of tritium labelled ivermectin (A) and emamectin Benzoate (B) in Atlantic Salmon. Bright areas indicated a high quantity of radioactivity. The first image (A) was taken from Høy et al., (1990) and the second (B) from Sevatdal et al., (2005).

Chapter 3. Avoidance, burying and camouflage behaviours in juvenile starry flounder exposed to SLICE® and ivermectin

3.1. Introduction

Marine aquaculture is an important industry globally, for both economic and food security reasons. In Canada, Atlantic Salmon (*Salmo salar*) is an important species produced in marine aquaculture and is predominantly produced using open-net pen aquaculture. Canada is the fourth largest producer of Atlantic Salmon globally, producing 120,427 t in 2023 (FAO, 2023). This industry is a key sector within Canadian aquaculture, where the value of salmon produced amounted to almost CAD $ 1 B in 2021, with 70% of production coming from British Columbia (BC; Statistics Canada, 2022, 2021a).

By design, Atlantic Salmon open-net rearing pens allow for the exchange of water and nutrients between the pens and the surrounding ocean environment; this has led to concerns associated with the potential environmental impacts of this type of aquaculture. One of the impacts associated with open-net pen systems comes from uneaten feed, feces, pharmaceuticals applied to the feed, and chemicals excreted in the feces, all of which end up in the surrounding environment (Brooks et al., 2002). The density of fish rearing in a relatively small area in these pens can lead to diseases and parasitic infestations and may require pharmaceutical intervention to prevent loss of fish and maintain fish health (Bateman et al., 2022; Mordecai et al., 2021; Wootten et al., 1982). The timely treatment of ectoparasitic infestations such as sea lice is key to maintaining the health of *S. salar* and maintaining the economic viability of the aquaculture operation. Two avermectin-derived compounds have been used to treat sea lice infestations in farmed Atlantic Salmon in Canada; emamectin benzoate (EMB), which is used as the active ingredient (0.2% w/w dry powder) of a formulation in Canada called SLICE®, and ivermectin (IVM), which is used as the active ingredient (0.5% w/v liquid) in a formulation called IVOMEC® (Hamoutene et al., 2022; Haya et al., 2005) . EMB is comprised of a 9:1 ratio of two homologues of avermectin: 4’-epimethyamino-4’deoxyavermectin B1a and 4’epimethyamino-4’-deoxyavermectin B1b benzoate (Schering-Plough, 2000). Ivermectin is a mixture consisting of ≥90% 22,23-dihydroavermectin B1a and ≤10% 22,23-dihydroavermectin B1b (National Center for Biotechnology Information, 2023). SLICE® is the most commonly used chemotherapeutant approved for use to prevent and treat

farmed Atlantic Salmon for sea lice in Canada, and IVM has been used off-label in experimental or emergency situations where resistance to SLICE® was observed (E. Horsberg, 2012; Hamoutene et al., 2022). Both EMB and IVM are administered to Atlantic Salmon in-feed (Davies and Rodger, 2000; Hamoutene et al., 2022); these compounds are taken up by the parasite from fish skin and mucous, and exert their antiparasitic actions through irreversible activation of glutamate-gated chloride channels at invertebrate inhibitory synapses (Cornejo et al., 2014; Grant, 2002; Roy et al., 2000). Activation of chloride channels in inhibitory neurons results in the hyperpolarization of muscle and nervous tissue, and inhibition of neural transmission leading to paralysis and death (Grant, 2002; Roy et al., 2000).

Most of the administered EMB and IVM are released into the surrounding marine environment in uneaten pellets or unmetabolized in Atlantic Salmon feces (Davies and Rodger, 2000; Høy et al., 1990; Olsvik et al., 2008; Shaikh et al., 2007; Wang et al., 2013). Once in the environment, both IVM and EMB partition adsorb to particulates and sediments and settle into the benthic environment due to the low water solubilities and high octanol-water partition coefficients (log K_{ow}) values of 3.2 and 5, respectively) (Bright and Dionne, 2005; Davies and Rodger, 2000). In marine sediments both IVM and EMB can persist for long periods of time with half-lives of 225 d to >404 d observed for EMB and >188 d for IVM (Benskin et al., 2016; Davies et al., 1998; Prasse et al., 2009; SEPA, 2004). Given long sediment half-lives, as well as strategies of multiple applications within a single year, there is the potential for the compounding of environmental concentrations. This can lead to longer-term chronic exposures at relatively high concentrations that may result in toxic effects to non-target benthic organisms (Benskin et al., 2016; Davies et al., 1998; Prasse et al., 2009). Concentrations of IVM and EMB previously found in marine sediments range from non-detectable to as high as 35 to 167 μg kg^{-1} sediment and modelled concentrations range from 366 μg kg^{-1} for EMB to 6.8 - 17.3 μg kg^{-1} for IVM (Cannavan et al., 2000; DFO, 2012, 1996; Ikonomou and Surridge, 2013; SEPA, 1999; Veldhoen et al., 2012). The majority of toxicity studies with EMB and IVM have not used environmentally realistic exposure conditions that mimic the persistence and partitioning of avermectins in the marine benthic environment (Olker et al., 2022). Due to the lack of environmentally realistic exposures of EMB and IVM to benthic marine fish species, substantial gaps in knowledge of the potential effects of these chemotherapeutants on these teleosts exist.

Although concentrations of EMB and IVM administered to fish in previous toxicity studies cannot be directly compared to concentrations observed in marine sediments, the reported effects indicate the potential effects that may be expected from an extended exposure. Toxic effects of avermectins in fish include lethargy, altered swimming behaviour or reduced swimming ability, loss of appetite, loss of equilibrium, darkened skin coloration, paralysis and death (Collymore et al., 2014; Domingues et al., 2016; Johnson et al., 1993; Katharios et al., 2001; Kennedy et al., 2014; Ogueji et al., 2019; Oliveira et al., 2016a; Palmer et al., 1996; Thiripurasundari et al., 2014; Ucán-Marín et al., 2012), effects which could potentially lead to population-level impacts.

There exists concern that open-net pen Atlantic salmon farms act as fish aggregating sites where wild marine teleost species accumulate in the surrounding area to feed on released pellets and on prey species feeding on the detritus and feed (Brooks, 1994; Brooks et al., 2002; Costelloe et al., 1998). Atlantic salmon farms also increase the sedimentation and finer-grained sediments in the benthic areas surrounding the pens, which several species of flatfish have been shown to prefer (Moles and Norcross, 1995). Several features of flatfish life history traits and morphological characteristics suggest the potential of a higher exposure compared to other benthic or benthopelagic fish. Flatfish are negatively buoyant and spend most of their time in contact with the substrate (Gibson et al., 2015). The timing and duration in contact with benthic substrates vary among species but most flatfish species spend a considerable amount of time in direct contact, and often buried within, sediments. The feeding strategies of most flatfish might also lead to the unintended ingestion of contaminated sediments, feces, or uneaten food (Gibson et al., 2015). These life history traits, their abundance and population stability, proven rearing in a lab environment, and a range overlapping with Atlantic Salmon aquaculture make the Starry Flounder (*Platichthys stellatus*) a model organism for the study of the effects of avermectin anti-sea lice chemotherapeutants on non-target benthic teleost fish (Burton, 2010; Gibson et al., 2015; Orcutt, 1950; Ralson, 2005).

It is unknown if flatfish can perceive and avoid avermectin insecticides in marine sediments, by swimming or reducing their burying behaviour. Some flatfish have external olfactory systems on their bodies for non-visual detection of prey, in addition to potential detection and avoidance by ingestion and respiration (Gibson et al., 2015) which could facilitate the perception and avoidance of avermectins. To truly assess the risk of exposure of IVM and EMB to non-target benthic teleosts, their preference to avoid these chemotherapeutants should be considered.

The potential avermectin-induced darkened skin colouration consistently observed in exposed fish is of particular concern for flatfish species, due to the importance of crypsis (Burton, 2010; Gibson et al., 2015). Cryptic colouration is one of the most effective mechanisms to avoid predation and capture prey in flatfishes (Gibson et al., 2015). Flatfish chromatic biology and their ability to match the pattern and coloration of their surrounding substrates is a critical component of the life history of flatfish feeding and predator avoidance (Burton, 2010). The objective of this study was to evaluate potential sublethal effects EMB (using the formulation SLICE® 0.2% premix) and IVM (or a combination) at environmentally relevant concentrations on starry flounder following a subchronic contaminated sediment exposure using morphometrics and growth, avoidance and burying behaviour, and camouflage ability.

3.2. Methods

3.2.1. Fish

Juvenile Starry Flounder were captured in Boundary Bay, BC in a low-gradient intertidal zone with mixed tides of up to a 3.5-m range. Fish were collected during daytime hours at low tide ± 4 h depending on the elevation of the tide using a beach seine (10 m long, 1.2 m high, 3.18 mm mesh size [stretched]). Mean fish weight was 4.00 ± 2.11 g (range 0.76 – 12.35 g) and mean total length of 69.6 ± 12.0 mm (range 45.0 – 104.7 mm). Fish were transported on the same day as collection in 45 L coolers filled with seawater collected at the sampling location, with aeration, and topped with dechlorinated ice to maintain a temperature of approximately 8-10°C. This method of transport was tolerated well by the fish with no transport-related mortalities. Fish were transferred to 180 L (152 cm length x 58 cm width x 20 cm depth) long shallow fiberglass tanks. Seawater was obtained from the Vancouver Aquarium (VA, Vancouver, BC) sourced directly from Burrard Inlet, BC. At the VA, the water was slow sand filtered and disinfected with ultraviolet (UV) radiation. storage tank for use. Holding tank seawater was kept at 29.37 ± 3.19 ‰, 11 ± 1°C under a 12:12-h photoperiod. Water quality was maintained using Hagen® Fluval® FX6 mechanical and biological filters, Coralife® hang-on-back protein skimmers, and Coralife® ultraviolet sterilizers. Water temperature was maintained by a Coralife 1/4 HP flow-through aquarium chiller. Holding density did not exceed 0.025 ± 0.013 g L^{-1} of seawater. Seawater changes (50-75%) were conducted 3-4 times per week.

Fish were fed with Cargill® EWOS 1.2 mm farmed fish salmon pellets 3-4 times weekly *ad libitum* and any excess food was removed during water changes. Fish were acclimated for at least 2 weeks prior to use in an experiment. Individuals were not size selected for experiments. All experiments were approved by the Simon Fraser University Animal Care Committee (protocol # 1245B-17) which abides by regulations set by the Canadian Council for Animal Care.

3.2.2. Contaminated sediments

SLICE® 0.2% Premix (Merck Animal Health, Itervet Canada Corp., Kirkland, QC), which contained 0.2% EMB w/w, was obtained from Fisheries and Oceans Canada (DFO). IVM (22,23-Dihydroavermectin B1, CAS Number 70299-86-7 95% purity) was obtained from Sigma-Aldrich (Oakville, ON).

Sediment was collected from Centennial beach (Tsawwassen, BC) which is considered an acceptable uncontaminated reference site based on results from the Boundary Bay Assessment and Monitoring Program (BBAMP) (2009 – 2015), completed by Hemmera (2017). Sediment from this region has an organic carbon content of 0.02 – 0.2 % (Hemmera, 2014). Sediment was collected from the upper 10 cm, sieved during collection using 1 mm metal sieves to remove debris and dried prior to experimental use. Sediment was air dried and then stored in 19 L food grade plastic buckets with a lid in the dark at 4°C.

Target concentrations of EMB- and IVM-treated sediment were prepared by creating a stock sediment with a nominal concentration of 12,000 µg EMB kg^{-1} dry sediment or 10,000 µg IVM kg^{-1} dry sediment, and serially diluting the stock with clean, dry sediment to achieve target concentrations. The stock and other concentrations were created by hand mixing the SLICE® and IVM with a small amount of clean, dry sediment in a 17 L stainless steel bucket with a stainless-steel garden trowel, and then by adding the remaining sediment and hand mixing. The hand-mixed sediment was then stirred at low speed vigorously for 2 min using a ½" 2-speed hammer drill with a 67 mm Jiffy Mixer HS-1 stainless steel mixing head. Preparations were stored in the stainless-steel bucket at 4°C in the dark for a maximum of 2 weeks before use.

The sediment concentrations used in the current study are nominal, however in a recent study where sediments were treated in a similar manner chemical analyses for EMB and IVM were performed on representative samples (range 1–1000 µg kg^{-1}

[Strachan and Kennedy, 2021; Xie et al., 2011]). Sediments were air-dried and extracted three times with dichloromethane, followed by centrifugation at 1500 *xg*. Supernatants were combined and cleaned by elution through anhydrous sodium sulfate and dried by rotary film evaporation. The evaporated residues were reacted with derivatization reagents according to Xie et al., (2011). The derivatization solutions were dried down under N_2, and the residue was taken up in 1 mL of high-performance liquid chromatography (HPLC) grade acetonitrile (Xie et al., 2011). Samples were analyzed by HPLC on a Hewlett-Packard model 1050 with an HP model 1046A programmable fluorescence detector and HP 3396 Series II Integrator (Hewlett-Packard). The detection limit for EMB and IVM was between 10 and 25 ng kg^{-1}. Measured concentrations were 86%–95% of all target concentrations. Recoveries were determined by comparing five spiked samples in sediment with one another. Recoveries from sediment were 87%–96%, with a between-day variability of 5%.

3.2.3. Exposures

Range-finding trials were performed for EMB and IVM to determine the range of concentrations of IVM and EMB that would result in a mortality of <20%; these mortality data in addition to environmental concentration data were used to select the appropriate concentration ranges for subsequent sublethal exposures.

Spiked sediment (1 kg) was added to 10 L glass aquaria (31 cm length x 16 cm width x 21 cm height) which were filled with aerated seawater (~9 L). The sediment and seawater in the aquaria were then left to settle for 12 to 24 h prior to the addition of *P. stellatus*. Fish (n=6) were randomly selected from their holding tank and lightly anaesthetized using 0.1 g L^{-1} buffered MS222. During anaesthesia fish were measured for total length (mm), weight (g), and then tagged with a Visible Implant Elastomer (VIE) tag according to the manufacturers protocol (Northwest Marine Technology Inc., Anacortes, WA) on the ventral surface immediately under the dorsal fin to allow for individual fish identification. Fish were recovered in aerated seawater and gently added to aquaria and exposed to contaminated sediments for 30 d.

For each experiment there were 3 exposure concentrations and a negative control that contained uncontaminated sediment. For each concentration there were 3 replicate tanks each with 6 fish in each tank. Nominal treatment concentrations in sediments were 12, 120, and 1200 µg kg^{-1} EMB; 10, 100, and 1000 µg kg^{-1} IVM, and 12/10, 120/100,

1200/1000 µg kg^{-1} EMB/IVM for the combination exposures. Animals were fed Cargill® EWOS 1.2 mm farmed fish salmon pellets 3-4 times weekly *ad libitum* and any excess present during the subsequent feeding food was removed by vacuum suction during each water change. The density of the food was lower than the treated sediment which allowed for the removal of excess food while avoiding the removal of any treated sediment. Water quality (salinity, dissolved oxygen, pH, ammonia [Ammonia Alert®, Seachem Laboratories, Madison, GA] and temperature) were measured prior to each water change of 75% of seawater 3-4 times each week. All exposure tanks were kept in temperature-controlled water baths and held at 11 ± 1°C under a 12:12 h photoperiod. Following the exposure, 3 randomly selected fish were used immediately for an avoidance assay, and 3 fish used for a camouflage assay. An overview of the experimental design can be seen in Figure 3-1.

3.2.4. Avoidance Assay

Two groups of *P. stellatus* were used in a 48-h avoidance assay. The first, a "naïve" group were fish that had not been previously exposed to the chemicals. The other, is a "chronic" group which was exposed to one of the treatments of EMB, IVM, the combination of both, or the negative control. Avoidance assays were conducted in 10 L glass aquaria (31 cm length x 16 cm width x 21 cm height) divided in half with a removable plexiglass divider. The assay methodology was partially designed based on the ISO Guideline 17512-1 avoidance test for earthworms (ISO 527-2, 2003) and from Møhlenberg and Kiørboe (1983) and Mole et al (1994) for observing the avoidance behaviour of a flatfish exposed to dosed sediment. One kilogram of sediment was added to each assay chamber, 500 g of clean sediment to one randomly chosen side (unseeded side) and 500 g of sediment on the other side (seeded side) treated with either 12, 120 or 1200 µg kg^{-1} EMB; 10, 100 or 1000 µg kg^{-1} IVM or a combination 12/10, 120/100, or 1200/1000 µg kg^{-1} IVM/EMB for the chronic treatment groups. For the naïve fish, this side was contained either 12 or 1200 µg kg^{-1} EMB; 10 or 1000 µg kg^{-1} IVM or a combination 12/10 or 1200/1000 µg kg^{-1} IVM/EMB. For the negative control, 500 g of clean sediment was added to both sides. Unexposed (naïve) fish were randomly assigned a sediment concentration, including the clean sediment control, for the assay. For the chronically exposed fish, the concentration and chemical (e.g., 100 µg kg^{-1} IVM) they were exposed to for the previous 30-d was used on the seeded side of the assay chamber.

After the sediment was added, clean and filtered seawater was used to fill the aquaria by filling on the clean sediment side of the chamber to minimize disturbance to the treated sediment. The filled aquaria were left for 12-h with aeration to maintain the dissolved oxygen level, to allow for the sediment to settle prior to the addition of fish to the assay chamber. To initiate the assay, the plexiglass divider was removed, and 3 fish were added to the middle of the assay chamber to avoid biasing fish movement to either side. For the duration of the assay the temperature of the seawater in each assay chamber was maintained at maintained at 11 ± 1°C with a 12:12-h photoperiod and aerators were turned on intermittently to maintain adequate dissolved oxygen levels, while still allowing observers to track the location of fish within the tank. Trial assays using this protocol were well tolerated by fish with no mortalities. Fish movement was recorded for 48-h using a Yi Home 2 cloud-based consumer-grade home security camera with infrared recording for the dark photoperiod (Yi Technology Inc., Shanghai, CN). Black plastic was used to allow light into aquaria during the day but block any additional glare or hotspots that might affect the ability to observe fish movement during the recording. Video was manually reviewed for the side of the assay chamber fish were on and whether they were buried at each 2 h period. Burying was recorded as a general behaviour whether or not the fish were on the contaminated side of the assay chamber as other mechanisms of avermectin exposure other than direct avoidance could affect burying behaviour. The metric used for avoidance was the mean proportion of fish on the seeded (contaminated) side over the 48-h assay and for burying the endpoint was the mean proportion of fish buried over 48-h. Animals were euthanized at the end of the assay using buffered MS222 (1 g L^{-1}) in seawater, and then measured and weighed.

3.2.5. Camouflage assay

Camouflage Assay Protocol

The camouflage assay used was a modified protocol based on Iwanicki (2016). The assay was conducted in a dark room in a 62 L plastic storage box (17.78 cm h x 100.33 cm l x 51.435 cm w) containing recirculating, aerated and temperature-controlled sea water maintained with a Hagen® Fluval® FX6 mechanical and biological filter, and a Coralife® ultraviolet sterilizer. The assay chamber was a 30 cm diameter, 50 cm tall plastic tube with four XLamp neutral white 4000K LEDs (CreeLED, Inc., Durham, NC) with 0.6 amp output, mounted on top of the tube in pairs at opposite ends. Neutral white foam core

was used to reflect light into the arena to reduce hotspots and shadows. Images of laminated substrates of three different sizes (cobble, gravel and sand) recorded at Crescent Beach, BC were used to line the inside and bottom of the cylindrical chamber (see Fig. 3-2). For the assay, all pumps and filtration for the assay chamber were turned off to avoid noise, vibration, and disturbance. Three randomly selected fish were added to the chamber (noting the individual VIE identification colour) on a neutral grey background for 30 min and then exposed to the cobble, gravel and sand substrate for 10 min, recording images at 30 sec, 2.5, 5, 7.5 and 10 min for each. Photos of fish were captured using a Sony a6300 Digital SLR camera with a sigma 30 mm f1.4 lens. The camera settings remained constant for the duration of the experiment (aperture = F4.5, shutter speed = 1/5", ISO = 400, exposure compensation = +0.7). Images were captured in RAW file format. The camera was mounted on a tripod approximately 1.5 meters above the assay arena. After the camouflage assay, animals were euthanized using buffered MS222 (1 g L^{-1}) in seawater, and then measured and weighed.

Image Analysis

Multispectral images were generated using the Image Calibration and Analysis Toolbox (Troscianko and Stevens, 2015) plugin for ImageJ (Schneider et al., 2012). All images were calibrated to a standard (PTFE sheet) to control for variation in light reflecting off the different substrates. Fish camouflage response was characterized from a cropped image (for each substrate background), to create a polygon representing the region of interest (ROI) for each fish starting at the base of the anal fin near the caudal peduncle. From the caudal peduncle at the posterior end of the dorsal fin, the polygon extended along followed the outline of the fish body excluding the dorsal, caudal, and pelvic fins (See Fig. 3-3). A second ROI was then created for a 5 cm band for the area surrounding the flounder body, including the fins (See Fig. 3-3). Once the ROIs were selected the multispectral images were filtered at 7 spatial frequency bands, or bandpass filters (i.e., 2, 4, 6, 8, 16, 32, 64, 128 pixels). The mean pattern energy (power) across the spatial frequency bands of the fish body ROI and the background ROI for the surrounding 5 cm of substrate background were calculated using the Image Calibration and Analysis Toolbox. The difference in mean pattern energy ($Pattern_{diff}$) and difference in luminance ($Luminance_{diff}$; perceived brightness) between the fish body and surrounding substrate were calculated and used as metrics to indicate background pattern and brightness matching, respectively, to indicate camouflage. A smaller difference in pattern energy

equated to a closer background match and better camouflage, and a larger pattern energy difference indicated the opposite. The same is indicated by luminance (perceived brightness) difference ($Luminance_{diff}$) between the fish's body and the background substrate.

3.2.6. Calculations and Statistics

For body condition, Le Cren's Relative condition (K_n; Le cren, 1951) was used to determine body condition over Fulton's Condition (K, Fulton, 1904). Relative condition of individuals was calculated using $K_n = W_0\ W^{-1}$ where K_n is relative condition, W_0 is the observed weight and W is the expected weight. Expected weight was calculated using the fish total length in the linear regression equation from the length-weight relationship of initial lengths and weights for the wild-caught population used in these exposures. Expected weight was calculated according to the equation W = log a + b log L where W is the body weight (g), L is the total length of the fish (mm), a is the intercept of the regression and b is the growth coefficient related to body form, determined from the slope off the regression curve. Percent growth was calculated according to the equation $G_{\%} = (W_F - W_0)\ W_0^{-1}\ 100$ where $G_{\%}$ = percent growth, W_F = final weight (g) and W_0 = initial weight (g).

Prior to statistical analysis, a 'tank' effect was tested using a restricted maximum likelihood (REML) estimation treating exposure tank as a random factor. If tank effect was determined to be statistically significant, the statistical analysis was completed using exposure tank as the experimental unit with individual fish values within that exposure tank averaged. If tank effect was not statistically significant between replicate tanks for a given endpoint, then statistical analysis was completed using individual fish within an exposure tank as the experimental unit. For all endpoints, whether at the tank- or fish-level, a quantile outliers test was completed (0.1 and 3 times the interquartile range [IQR]) and if an outlier was detected it was removed. Endpoint values were then tested for a normality using an Anderson-Darling test, and the Levene test was used to determine homogeneity of variance. If the data exhibited a normal distribution and equal variances then a one-way analysis of variance (ANOVA) was completed using exposure concentration as the factor, and Tukey's post hoc multiple comparison test. If the data were not normally distributed and, or, exhibited unequal variances, then the Kruskal-Wallis test was used with the Steel-Dwass test for multiple comparisons. For all statistical tests significance was determined using $p<0.05$. For the analysis of the proportion of *P. stellatus* buried or on the treated side

of the avoidance chamber over time for the 48-h assay, simple linear regression was used for each treatment concentration. Multiple comparison of the slope values of each regression line between controls and treatments for each of EMB, IVM and the combination exposure was completed using an ANCOVA according to McDonald (2014). For the camouflage analysis, initially a restricted maximum likelihood estimation was used to evaluate whether time, substrate background or treatment had an effect on the two camouflage metrics used (pattern difference and luminance difference). If a factor had a significant effect on the camouflage metrics then they were included in subsequent analyses. All data analyses were performed using JMP 15 software (SAS Institute, Cary, NC) and all figures were prepared using R (R Core Team, 2021) and the package ggplot2 (Wickham, 2016).

3.3. Results

3.3.1. Water quality

During the 30 d exposures, camouflage assays and 48-h avoidance assays, the seawater was consistently measured at 11 ± 1°C, pH of 7.6 ± 0.3, dissolved oxygen 8.5 ± 0.9 mg L^{-1}, ammonia <0.02 ppm and salinity 27 ± 3 ‰. These ranges of water quality parameters were well within normal ranges for this species were expected to support the health of *P. stellatus.*

3.3.2. Mortality

The exposure concentration ranges were chosen from range-finding trials previously performed with *P. stellatus* exposed to sediment treated with EMB, IVM and a combination of the two to reduce potential mortality to <20% at the highest concentration used. Mortality was monitored for the duration of the 30 d exposures and did not exceed 20% cumulative mortality for the EMB, IVM and for the EMB/IVM exposures (Table 3-1). One replicate from each of the 100 µg kg^{-1} IVM and 120+100 µg kg^{-1} IVM+EMB treatments were compromised part-way through the exposure due to a temperature spike in the seawater supply identified during a water change which resulted in some mortalities. Fish subjected to the temperature increase were excluded from the subsequent analyses and no other replicates were affected by the temperature spike. At the treatment level, the two highest concentrations of IVM and the combination did exceed 20% mortality, where the

1000 µg kg^{-1} IVM and 1200+1000 µg kg^{-1} IVM+EMB had 42% and 28% mortality, respectively. This exceeded the mortality rates observed at these treatment concentrations for another exposure of *P. stellatus* evaluating the effect on swimming and respirometry (see Ch. 2). These results confirmed that the ranges selected were sublethal to *P. stellatus* with the exception of the highest concentrations of IVM and the combination of IVM and EMB.

3.3.3. Growth and condition

Growth

All treatment groups for EMB, IVM and combination exposures (including negative controls) lost weight, and therefore showed negative growth over the 30-d exposure period. In negative controls, the change in fish mass was -17.5 ± 1.9 %. None of the mean percent change in mass values for any concentrations were significantly different from controls, with the exception of the highest 1000 µg kg^{-1} IVM concentration (-26.7 ± 2.7 % [Fig. 3-4; $p = 0.03$, $\chi^2 = 3.16$]). There was no evidence of a concentration-dependent response of percent change in mass of *P. stellatus* exposed to EMB, IVM or the combination.

Relative condition

Mean relative condition (K_n) values decreased over the 30-d exposure period for all treatment groups including the negative controls, which is consistent with the negative percent change in mass. The mean K_n for the negative control group was 0.80 ± 0.02. Similar to the results for percent change in mass, the mean K_n values for all treatments were not significantly different from the negative control, except for the IVM 1000 µg kg^{-1} treatment (See Fig. 3-5; $p = 0.04$, $F = 4.92$).

3.3.4. Avoidance Assay

Avoidance

For the control assays, both sides of the assay chamber contained uncontaminated sediment, so the proportion of fish on the right side of the chamber were arbitrarily selected as a reference point for which to compare the naïve (unexposed) and chronic (exposed) treatment group fish. The arbitrary selection was used because fish

would be expected to be approximately evenly distributed between sides over time (i.e., between 40-60%). The mean proportion of *P. stellatus* on the right side of the avoidance chamber over 48 h was determined and compared between the naïve and chronic control groups to assess whether, or not, there was an effect of the 30-d exposure alone on avoidance behaviour within the arena. There was no significant difference in the mean proportion of fish on the right side of the assay chamber between chronic (0.46 ± 0.09) and naïve (0.46 ± 0.07) fish, and therefore no effect of the 30-d exposure on avoidance behaviour ($p = 0.96$, $t = 0.05$; see Fig. 3-7). The control treatments were also necessary to demonstrate that under the assay conditions that fish did not demonstrate a preference for one side of the assay chamber.

For naïve fish used in the avoidance assay, no evidence of avoidance for any concentration of EMB was found ($p = 0.13$, $\chi^2 = 4.01$; see Fig. 3-7). For the IVM treatment, avoidance was observed at the lowest concentration but there was no evidence of a concentration-dependent effect on avoidance ($p = 0.0029$, $F = 6.17$; see Fig. 3-7). In the combined treatment assay at the highest concentration (1200+1000 µg kg^{-1}), there was a significant increase in the proportion of fish on the contaminated side, which indicated attraction rather than avoidance ($p = <0.0001$, $F = 12.0$; see Fig. 3-7). The avoidance behaviour over time for the 48-h assay period was also assessed and no clear concentration-dependent trends in avoidance over the assay period were observed for EMB (see Fig. 3-8), IVM (see Fig. 3-9) or the combination treatment (see Fig. 3-10).

For the avoidance assays using previously exposed fish, significant avoidance of contaminated sediment was observed for the 12 (0.23 ± 0.03) and 1200 µg kg^{-1} EMB (0.31 ± 0.03) treatment groups ($p<0.0001$, $\chi^2 = 35.7$; see Fig. 3-7). The avoidance was not concentration-dependent as no significant avoidance was found for the intermediary concentration (120 µg kg^{-1}; see Fig. 3-7). For the IVM treatment group, significant avoidance was observed at the 10 and 100 µg kg^{-1} concentrations, but not the 1000 µg kg^{-1} indicating avoidance at lower doses but not at the highest dose, with no concentration-dependent effect on avoidance ($p<0.0001$, $\chi^2 = 44.7$; see Fig. 3-7). For all combination treatment concentrations significant avoidance was observed, however, there was a similar level of avoidance for each treatment, indicating that avoidance response was not concentration-dependent ($p<0.0001$, $F = 15.5$; see Fig. 3-7). For these exposed fish, the avoidance over the 48-h assay period for EMB was variable with no concentration-dependent trend or trend in opposition of the average avoidance over the 48-h period (see Fig. 3-8). The IVM (see Fig. 3-9) and combination (see Fig. 3-10) treatments also showed

a variable avoidance response over time, however, both of the highest concentrations showed an increasing trend in fish on the contaminated side, which would indicate potential attraction. This was not consistent with the findings of the mean avoidance values with the highest IVM concentration showing no significant avoidance and the highest combination showing significant avoidance, not attraction (see Fig. 3-7).

Burying

The mean proportion of *P. stellatus* buried over the 48-h test period was compared between naïve and previously exposed fish from the control treatments to assess whether there was an effect of a 30-d exposure on burying behaviour. There was a significant difference between the mean proportion of fish buried for the naïve (0.66 ± 0.03) and chronic (0.31 ± 0.03) control groups, where burying decreased for the previously exposed fish (See Fig. 3-6; $p = 0.03$; $t = 2.19$). A similar effect of the naïve fish having a higher mean number of fish buried compared to the equivalent concentration of previously exposed fish was observed for all treatment concentrations, matching the effect observed in the control groups (see Fig. 3-11).

For the EMB exposures there was no change in burying behaviour for the naïve ($p = 0.29$, $\chi^2 = 2.50$) and exposed groups treatment ($p = 0.09$, $\chi^2 = 6.47$; see Fig 3-11). For the change in burying over time over the 48-h assay period, each treatment concentration showed an increase in burying over time for the naïve and chronic treatments, with no concentration-dependent difference in the rate of increase (see Fig. 3-8).

For the IVM and combination exposures the highest concentrations of 1000 µg kg^{-1} IVM ($p = 0.003$, $F = 6.17$) and 1200+1000 µg kg^{-1} EMB+IVM ($p<0.0001$, $F = 12.0$) (in naïve and exposed fish) significantly lower burying behaviour compared to the negative controls was observed (see Fig. 3-11). The decrease in *P.* stellatus burying appeared to be concentration-dependent for all of the aforementioned exposure groups except for the exposed IVM fish (see Fig. 3-11). For the exposed fish in the IVM (see Fig. 3-9) and combination (see Fig. 3-10) exposure groups, burying increased over time over the 48-h assay period for all treatments with no concentration-dependent difference observed. For the naïve fish, a similar trend was observed over the 48-h assay period for the IVM and combination exposure. The changes in burying over time increased in the control group, were neutral or slightly decreased for the lowest concentrations (10 µg kg^{-1}IVM or 12+10 µg kg^{-1}EMB+IVM), and burying significantly decreased compared to the control for the highest 1000 µg kg^{-1} IVM ($p = 0.0002$, $F = 8.81$; see Fig. 3-9) and 1200+1000 µg kg^{-1} EMB+IVM ($p = 0.002$, $F = 6.25$; see Fig. 3-10) treatments.

3.3.5. Camouflage Assay

Camouflage assays were completed following exposure using images recorded over 3 substrate backgrounds. For mean pattern difference, time showed no significant effect ($p = 0.42$, $F = 0.64$), whereas treatment ($p = 0.0007$, $F = 5.01$) and substrate background ($p<0.0001$, $F = 2177.29$, see Fig. 3-12) showed a significant effect. Similarly for mean luminance difference (perceived brightness), time showed no significant effect ($p = 0.99$, $F = 0.08$), while treatment ($p = 0.001$, $F = 4.52$) and substrate background ($p<0.0001$, $F = 44.84$, see Fig. 3-12) did have significant effects. For all subsequent analyses time was excluded as a factor and values over time were pooled, and the treatment effect was analyzed separately for each substrate background.

The mean pattern difference ($Pattern_{diff}$; difference between the flounder body pattern and the substrate background pattern) was assessed for each treatment concentration and substrate background. Flounder $Pattern_{diff}$ was significantly different between substrate backgrounds ($p<0.0001$, $F = 283.02$, see Fig. 3-12) where fish could match their body pattern best to the sand background, followed by cobble and then gravel. The substrate background also had a significant effect on the luminance difference ($Luminance_{diff}$) between the flounder body and background for all treatments ($p<0.0016$, $F =6.56$, see Fig. 3-12). Fish were able to best match the luminance of the sand substrate, and less for the cobble and gravel backgrounds (see Fig. 3-12). Due to the inherent differences in pattern and luminance for each substrate background, the camouflage metrics were assessed separately for each.

The camouflage ability of *P. stellatus* was reduced compared to the control group over the sand substrate background for the 120 µg kg^{-1} EMB treatment group with significant increases in $Pattern_{diff}$ ($p = 0.0008$, $F = 6.69$; see Fig. 3-13) and luminance difference ($p = 0.04$, $\chi^2 = 8.06$; see Fig 3-13). Over the gravel background there was a significant reduction in pattern matching ($p = 0.01$, $F = 4.15$; see Fig. 3-13) but not luminance, and over cobble there was no evidence of an effect on camouflage (see Fig. 3-13). Overall, there was not a concentration-dependent effect on camouflage for the EMB exposure.

Camouflage ability was significantly reduced for the 100 µg kg^{-1} IVM treatment with significantly higher $Pattern_{diff}$ and $Luminance_{diff}$ than the control over all substrate backgrounds (see Table 3-1 and Fig. 3-13). $Luminance_{diff}$ was also significantly higher for the 1000 µg kg^{-1} IVM over sand and gravel, indicating a reduced ability to match the

brightness of those substrate backgrounds (see Table 3-1 and Fig. 3-13). There was no clear concentration-dependent effect of IVM on *P. stellatus* camouflage ability and the responses were similar across substrate backgrounds.

There was reduced camouflage ability indicated by significantly higher $Pattern_{diff}$ and $Luminance_{diff}$ compared to the control over all substrate backgrounds for the highest combination treatment concentration (1200+1000 µg kg^{-1} EMB+IVM; see Table 3-1 and Fig. 3-13). The trends in response varied based on substrate background, with a concentration-dependent reduction in both $Luminance_{diff}$ and $Pattern_{diff}$ over the cobble background (see Fig. 3-13).

3.4. Discussion

The aim of the present study was to determine if sublethal effects on the growth, condition, avoidance, burying and camouflage behaviour of *P. stellatus* occurred with exposures to the anti-sea lice chemotherapeutants SLICE® (AI: EMB), IVM or a combination of both. The assays provided the first evidence of sublethal effects of avermectins on the cryptic colouration and avoidance behaviours of a flatfish exposed to contaminated sediment at environmentally relevant concentrations of these chemicals used at Atlantic Salmon farms.

3.4.1. Mortality

Cumulative mortality did not exceed 20% for any exposure, indicating that the sediment concentrations used sublethal. to *P. stellatus*. A recent study by Strachan and Kennedy (2021) exposed adult Tidepool Sculpin (*Oligocottus maculosus*) to sediment treated with EMB for 10 d, with a reported LC_{50} value (95% CI) of 1980 (1249-3750) µg EMB kg^{-1} sediment. Sahota et al. (2022) exposed Pink Salmon fry, a pelagic fish species, to EMB in sediment for 10 d and reported an LC_{50} value (95% CI) of 2065 (1384-3720) µg EMB kg^{-1} sediment. These results are consistent with the findings of this study with the maximum treated used (1200 µg kg^{-1}) being below the LC_{50} found by Strachan and Kennedy (2021). Other studies from the literature used other exposure methods, so direct comparison to the current study cannot be made. Based on the findings of the current study, the previously observed environmental concentrations of EMB (167 µg kg^{-1}) and IVM (17.3 µg kg^{-1}) observed in marine sediments below open-net pen Atlantic Salmon

farms (Cannavan et al., 2000; DFO, 2012, 1996; Ikonomou and Surridge, 2013; SEPA, 1999; Veldhoen et al., 2012) would not be directly lethal to *P. stellatus*.

3.4.2. Growth and morphometrics

The relative change in mass over the 30-d exposure period in all groups was negative with the highest mass loss occurring at the highest IVM concentration. A range-finding exposure was completed prior to this experiment using the same experimental conditions and feed, where fish exhibited positive growth and feeding were observed, though the scope was substantially less than the current study. With previously observed feeding and growth, and the suitable water quality parameters recorded throughout the 30-d exposure, it is unclear why negative growth was observed in control fish. It was clear that fish were not eating enough to gain mass, which precluded the ability to elucidate any effects on growth related to the avermectin exposure.

In fish exposed to IVM (Domingues et al., 2016; Ucán-Marín et al., 2012), EMB (Skilbrei et al., 2015) and avermectin (Du et al., 2023) negative growth in a concentration-dependent manner has been previously observed. Matching what was observed for all other treatment concentrations, no significant changes in growth for fish exposed to EMB and IVM have also been shown previously(Ananda Raja et al., 2020; Bowker et al., 2013; Katharios et al., 2001; Kent et al., 2019). In several fish species, IVM and EMB caused significant reductions in appetite and feeding response, which may explain the general trend of reduced growth observed (Azevedo and Kennedy, 2022; Bowker et al., 2013; Domingues et al., 2016; Johnson et al., 1993; Roy et al., 2000; Ucán-Marín et al., 2012). The few effects on change in mass observed in this study are consistent with previous findings showing no growth-related effects following abamectin-type chemotherapeutant exposure (Bowker et al., 2013; Katharios et al., 2001; Kent et al., 2019; Kilercioglu et al., 2020). Given the novelty of sediment-based exposures to EMB and IVM in fish, the uptake and tissue concentrations accrued over the 30-d exposure period are unknown. Perhaps at the sublethal concentrations used, or the duration of the exposure (30-d) effects on growth in a concentration-dependent manner could not be observed, where growth was not a sensitive enough endpoint to demonstrate a sublethal effect in *P. stellatus*. In future sediment-based exposures of avermectin to a flatfish species, the scope of the range-finding trial or growth trial should be increased with additional fish and replicates to confidently determine conditions and feed at which growth will be observed. Additionally,

a control group with fish that are not anaesthetized and tagged should be included to assess if this process at the beginning of the exposure had an effect on appetite.

Starry Flounder are a commercially important species with aquaculture production in China and Korea, and studies assessing optimum dietary composition, intake and rearing conditions to maximize *P. stellatus* health and growth exist (Bögner et al., 2018; Ding et al., 2010; Kim, 2012; Ko et al., 2019; Lim et al., 2013). The rearing conditions of several *P. stellatus* growth studies were compared to those used in the current study; in previous studies, biomass density ranged from 5.4-15.7 g L^{-1}, a range higher than that used in this study (2.4 g L^{-1}), indicating that density was not the cause of the lack of growth. The feed type varied but most were with an extrusion feed, which would float, compared to the pelleted feed used in this study which would sink. However, Bögner et al (2018) used a commercial pellet feed that was a similar size and nutritional composition to the feed used in the current study, and reported postive growth. Given the benthic life history of *P. stellatus* and known prey species being benthic invertebrates that bury in sediments, it is unlikely that the cause of the lack of feeding is sinking feed pellets, rather than floating extrude (Orcutt, 1950). The key differences identified between the current study and other *P. stellatus* growth studies was the feeding frequency, which was either daily or twice daily, compared to every 2 d in the current study. The anaesthesia and tagging used at the start of the exposures in the current study may also be a factor (Bögner et al., 2018; Ding et al., 2010; Kim, 2012; Ko et al., 2019; Lim et al., 2013). Fish in this study were fed to satiety every 2 d and excess feed was always found and removed, so it appeared that the feeding rate was enough, however a higher feeding frequency was perhaps needed. It is possible that the use of hatchery-reared (as in Chinese and Korean flatfish aquaculture) rather than wild fish would standardize life-stage and size and habituation to laboratory conditions, would be more suitable for toxicological studies. Bögner et al (2018) used this approach, acquiring Starry Flounder from a Korean hatchery for use and found success in survival, growth and fish health.

Avermectin exposure did not affect relative condition values which were reduced from initial values, consistent with the observed mass loss. Similar to what was suggested for mass loss, future studies assessing the effect of avermectin or other chemotherapeutants on *P. stellatus* should first optimize exposure conditions and feeding regiment in order to confidently use growth and condition as metrics of sublethal toxicity. In a study assessing avermectin effects on fish condition, Lozano et al (2021) found sub-optimal condition values for *Prochilodus lineatus* exposed to IVM. Ucán-Marín et al (2012)

exposed *S. salar* to IVM applied to feed and found that weight and condition factor were significantly reduced in exposed fish (Ucán-Marín et al., 2012). There is the potential that the exposure concentration was not sufficiently high, or the duration was not long enough to elucidate an effect on change in mass and condition. To account for the different exposure route and simulate the long half life (>100 d) in the benthic marine environment for EMB and IVM, a longer exposure duration might be necessary. However, with the loss in mass and reduced condition over the 30-d exposure period for the control group it is difficult to determine an effect of avermectin exposure on growth and condition.

3.4.3. Avoidance

The ability of flatfish to perceive and avoid these chemotherapeutants in sediments would limit exposures and subsequent toxicity. Naïve *P. stellatus* showed no tendency to avoid EMB, avoided IVM, and showed a trend of attraction at the highest combination treatment concentration. Previously exposed fish, however, avoided EMB, IVM and the combination treatments at lower concentrations than naïve fish. Opposing results were found for the highest IVM concentration where the naïve fish avoided the contaminated sediment, but exposed fish did not. For the highest combination treatment, the opposite effect was observed where significant attraction was found in the naïve group, and significant avoidance observed for the exposed fish. The significant avoidance observed at lower concentrations in previously exposed fish compared to unexposed fish, indicates that previous exposure to avermectins potentially increases the sensitivity to detect and avoid subsequent exposures. Significant avoidance by both groups when both EMB and IVM are combined was seen for previously exposed groups, which means that there is a potential chemosensitization on *P. stellatus* when exposed to both chemotherapeutants that increases their ability to perceive and avoid avermectins.

The distribution of the fish from the naïve and exposed control groups within the assay chamber was found to be the same, thus confirming that the 30-d exposure itself did not influence fish movement within the assay chamber (see Fig. 3-6). Thus, any avoidance effects observed for the exposed fish, above what was observed for the naïve fish, could be attributed to exposure to avermectins.

Previous assessments teleost avoidance of avermectins are limited, with only one study showing that Pink Salmon (*Oncorhynchus gorbuscha*) that had not been previously exposed to EMB avoided the chemical at concentrations in seawater above 300 µg L^{-1}

EMB (Sahota et al., 2022). These results are not directly comparable to the current study, however the confirmation that significant avoidance of EMB was observed in a teleost species is valuable and corroborates the findings of the exposed fish avoidance assay.

Fish chemosensory inputs are a critically important means by which they interact with their aquatic environment. Responses to external chemical stimuli can be mediated by olfaction (smell), solitary chemosensory cells, or gustation (taste) on epithelial cells on the head, gills, mouth or elsewhere on the epithelium, but for the most part, it is thought that avoidance or attraction responses are mediated by olfaction (Hara, 1994; Tierney, 2016). Behavioral responses are intended to improve an organism's position with respect to survival. Unpleasant or painful stimuli will typically evoke avoidance behaviour. Organisms rely on chemical defense mechanisms to avoid the potential toxic effects of foreign compound exposure (Tierney, 2016). There is the potential that the avoidance response was mediated by detection and response by the *P. stellatus* chemosensory organs.

Both EMB and IVM are thought to cause neurotoxicity in teleosts, in part, by agonistically bind to γ-Amino butyric acid A receptors ($GABA_AR$), causing hyperpolarization of GABAergic neurons and inhibitory neurotransmission (Estrada-Mondragon and Lynch, 2015; Stevens and Charles, 2001; Xu et al., 2016). GABAergic neurons have been shown in the olfactory bulb of several teleost species, and that GABAergic neurotransmission acts as the "gatekeeper" by strongly modulating the neuronal signalling and behavioural responses associated with olfaction (Costa et al., 2022; Daghfous et al., 2018; Song et al., 2017). The reception of chemical signals in the aquatic environment by olfaction, the subsequent processing and integration of this information in the fish central nervous system (CNS), and the physiological and behavioural responses are all part of a complex system that is vulnerable to the disruptive effects of toxicants (Tierney et al., 2010). Given that GABA is typically associated with inhibitory neurotransmission, it would be expected that binding and activation of $GABA_A$ receptors shown to be present in the teleost olfactory epithelium might inhibit an aversive response to these chemotherapeutants (Costa et al., 2022). However, Costa et al (2022) demonstrated that exposure of the olfactory epithelium of Gilthead Seabream (*Sparus aurata*) to a $GABA_A$ receptor agonist, muscimol, increased and decreased the apparent olfactory sensitivity to some odorants. Thus, there is the potential that the agonistic action of avermectins in sediment on GABAergic neurotransmission in the olfactory bulb is what mediated an aversive response to avermectins and thereby led to avoidance.

Signalling associated with direct $GABA_A$ receptor binding and activation by avermectins could lead to the development of an aversive response to exposure to these chemotherapeutants and therefore avoidance. In Gilthead Seabream (*Sparus aurata*) exposure of the olfactory epithelium to a known $GABA_AR$ agonist, muscimol, led to an increased olfactory sensitivity to some odorants (Costa et al., 2022). It has been shown in mice that activation of GABAergic neurotransmission by the agonistic binding of extra synaptic $GABA_ARs$ is associated with learning behaviours related to avoidance and aversion (Creed et al., 2014). Repeated intraperitoneal administration of the $GABA_A$ receptor agonist gaboxadol has been shown to reward aversive behavioural responses, such as avoidance (Vashchinkina et al., 2014b, 2014a, 2012). Thus, there is the potential that olfactory or CNS-based activation of GABAergic neurons associated with the ventral tegmental area (VTA) reward centre of the animal brain by avermectins could promote aversive behaviours such as avoidance of the original chemical stimulus. In another study by Tews et al (1984), assessing direct avoidance of GABA added to feed, rats showed direct avoidance of GABA in food, likely through both olfaction and gustation. It was suggested that activation of olfactory neurons leading to an avoidance response could be mediated by GABA agonists. Although the avoidance and aversive behaviour associated with $GABA_A$ receptor binding and the subsequent GABAergic neurotransmission in the VTA was assessed in mice and rats, and not teleosts, it has been shown that GABAergic neurotransmission and neurons are highly conserved across taxa (Bouché et al., 2003; Grone and Maruska, 2016).

The presence of $GABA_A$ receptors in the olfactory bulb of zebrafish has been well documented, and has been suggested that the over activation of $GABA_A$ receptors by IVM has led to the inhibition of signalling in the olfactory bulb (Azevedo and Kennedy, 2022; McLean and Fetcho, 2004; Monesson-Olson et al., 2018). Azevedo and Kennedy (2022) showed a concentration-dependent reduction in olfactory response to food stimulus in IVM exposed zebrafish. In Pink Salmon exposed to EMB in sediment for 10-d, as well as unexposed fish, there was no effect of EMB exposure on olfactory response, which is somewhat contradictory to the effect of IVM on olfaction observed previously (Sahota et al., 2022). This differing response to IVM and EMB might explain the difference in avoidance response observed in the current study between the two chemotherapeutants for previously exposed *P. stellatus*. Both EMB and the combination exposures showed significant avoidance at lower and the highest concentrations, whereas IVM did not show avoidance at the highest concentration. There is the potential that above a certain IVM

concentration, olfactory inhibition occurred to a level that precluded the ability of *P. stellatus* to detect and avoid IVM, as was suggested and observed by (Azevedo and Kennedy, 2022) hence the lack of avoidance at the highest concentration. The avoidance of EMB with no effect on olfaction that was observed by Sahota et al (2022) would be consistent with the findings of the current study with avoidance observed at the highest EMB and combination exposures.

There is also the potential that the difference in avoidance response between IVM and EMB is due to GABAergic effects within the CNS, not just in the olfactory bulb. IVM has generally been observed as more toxic than EMB, and this is potentially due to the fact that IVM accumulates within the teleost brain substantially more than EMB (Høy et al., 1990; Sevatdal et al., 2005). The additional accumulation of IVM in *P. stellatus* CNS compared to EMB, could be responsible for the lack of avoidance, as agonistic action on $GABA_AR$ in other brain regions, or on other binding sites (e.g., benzodiazepines), can inhibit the neuronal signalling that leads to the aforementioned aversive conditioning and avoidance (Creed et al., 2014; Høy et al., 1990; Vashchinkina et al., 2014b, 2014a, 2012). If this was a mechanism behind the lack of avoidance, it would explain the continued avoidance at high concentrations of EMB due to the lack of accumulation of EMB in the CNS of exposed fish (Sevatdal et al., 2005). The lack of avoidance observed at the highest IVM concentration could also be due to neurotoxicity associated with excessive GABAergic inhibitory neurotransmission that leads to sedative-like effects including reduced movement, but if that were the case, it would be expected in the combination exposure as well.

The difference in response between naïve and exposed fish at the same avermectin concentrations could also be explained by the prior exposure to avermectins leading to a conditioned aversion to these chemotherapeutants that would lead to avoidance. This would explain the significant avoidance observed in the EMB and combination treatments for exposed fish, but no avoidance for the naïve fish. Similarly, for IVM, no significant avoidance was observed for naïve fish but was found in for lower concentrations of IVM for exposed fish.

Overall, the results of the effect of IVM and EMB on *P. stellatus* avoidance behaviour from the 48-h avoidance assay are mixed and somewhat difficult to interpret, however, several general trends were observed. First, unexposed (naïve) fish generally did not avoid the avermectin-treated sediment over the 48-h assay period. Second, chronically exposed fish avoided treated sediments in each of the IVM, EMB and

combination exposures at environmentally relevant sediment concentrations; but not necessarily in the expected concentration-dependent manner. Lastly, the results potentially suggest that *P. stellatus* are more sensitive to the detection and avoidance response to EMB than to IVM as evidenced by the avoidance observed at the highest EMB and combination exposure concentration, but not the highest IVM concentration. It is encouraging that previously exposed fish demonstrated an avoidance of the chemotherapeutants, however it is unknown in the wild whether the exposure concentration and duration required for chemosensitization that might lead to avoidance, would cause other sublethal effects such as reduced swimming ability in *P. stellatus*.

In summary, it appears that *P. stellatus* do exhibit an avoidance response to avermectins in marine sediment. Results associated with teleost toxicant avoidance can be variable and substantial knowledge gaps exist on teleost avoidance of contaminants in sediment, particularly for flatfish (Tierney, 2016). Additional studies should be completed on other benthic teleost taxa to assess if this response is consistently observed and, generally, avoidance response should always be considered when assessing exposure, toxicity and risk to aquatic organisms. Also, studies should seek to determine the mechanism behind the avoidance behaviour (i.e., olfactory, neurological, or both) and the threshold at which avoidance will occur to help inform targeted environmental concentrations of avermectins in marine sediments. Often avoidance assays are completed using organic contaminants dissolved in water, which is again, an unrealistic simulation of what would occur in the aquatic environment. A standardized assay using teleost fish and treated sediment should be developed for use to assess avoidance for toxicants that partition into the sediment compartment within an aquatic ecosystem to provide a true estimate of avoidance response.

3.4.4. Burying

The flatfish order of teleost fish (Pleuronectiformes) encompasses a diverse group of taxa including flounder, sole, turbot, plaice and halibut. Several morphological and life history characteristics common across flatfish taxa make this order of fish particularly susceptible to organic contaminants in marine sediments. One of these behaviours is the ability and preference to bury themselves within sediments (Gibson et al., 2015). The frequency and duration spent buried within sediments varies, but can make up a substantial portion of their post-metamorphosis life stage and is a key behaviour for

predator avoidance, ambush prey capture, avoiding shear currents, as well as recovery and digestion (Gibson et al., 2015). The second endpoint assessed from the 48-h avoidance assay was the effect of chronic and naïve exposures to IVM and EMB on *P. stellatus* burying behaviour, as this could also be a behavioural change indicating avoidance and a reduction in burying could have a negative effect on flatfish survival, were it to occur in the wild.

Similar to avoidance, a comparison between the control groups of the naïve and chronic exposures was completed for the proportion of fish buried to determine if there was a prior exposure effect on burying, independent of any effects caused by avermectin exposure. There was a significant difference in fish burying between the previously exposed and naïve fish; with a significantly lower proportion of exposed fish buried (see Fig. 3-6). There is not a study of flatfish burying behaviour directly comparable to the current study conditions, however flatfish burying behaviour in a laboratory setting has been studied. Laan (2015) used burying behaviour and camouflage as indicators of fish health compared to more conventional metrics. Burying behaviour was measured at the beginning and end of a 10-d survival period for three species of flatfish and there was no difference in burying over the 10-d period. Other studies using hatchery-reared turbot and flounder species assessed the effect of conditioning to sediment, on burying efficiency or frequency compared to naïve fish and found no significant differences between groups (Ellis et al., 1997; Iglesias-Estévez and Rodríguez-Ojea, 1994; Kristensen et al., 2014). The findings of these studies assessing flatfish burying behaviour following conditions or acclimation to study conditions did not match the findings of the current study, where previous exposure conditions reducing burying behaviour. The significant difference between the exposed and naïve control group burying was likely lack of energy and muscle mass needed to actively bury themselves due to the loss in mass and reduction in body condition (see Figs 3-4 and 3-5), as burying in sediments is an active process requiring multiple fin and body movements (Gibson et al., 2015). Another potential cause is acclimation to the exposure tank conditions and to human movement leading to a reduced startle response and burying.

Without prior exposure to avermectins, the naïve *P. stellatus* showed no change in burying behaviour when exposed to EMB-treated sediment but did show a significant reduction in burying for IVM and the combination of both (see Fig. 3-11). The extent to which burying was reduced for IVM and the combination exposures was similar. This result indicates that any changes in burying behaviour for a naïve exposure to IVM and EMB

likely caused by IVM and not EMB. Similar to the naïve fish, *P. stellatus* previously exposed to EMB showed no significant change in burying behaviour at any concentration during the avoidance assay. For fish exposed to IVM and the combination, significant reductions in burying behaviour were observed, matching what was observed for the naïve fish. Although the burying results compared to their respective control groups were similar between naïve and exposed fish, the exposed group generally buried less than the naïve group. The difference in response for burying and avoidance, suggest that any effect on burying was by a different mechanism than what caused avoidance of avermectins and that decreased burying behaviour wasn't necessarily used as a means of avoidance.

Burying mostly increased over the 48-h assay period, meaning that regardless of exposed or naïve fish, and treatment concentration, *P. stellatus* would increase their burying over the 48-h assay period. This was likely a result of fish acclimating to the assay chamber conditions. However, the highest IVM and IVM/EMB treatment concentrations for naïve fish differed and reduced burying over the 48-h assay period (see Fig. 3-9 and 3-10). This difference in short term response compared to the other treatment concentrations could be due to detection and avoidance, though it would be expected for the naïve EMB treatment as well if that were the case, and similar trends in avoidance were not observed over time for naïve fish (see Figs. 3-8 to 3-10). The immediate decline in burying behaviour over time for naïve fish in the highest IVM and IVM/EMB concentrations, as well as the significant reduction in the mean proportion of fish buried for the exposed and naïve IVM and combination exposures could be due to reduced movement or swimming ability from inhibitory GABAergic neurotransmission caused by IVM, and not EMB accumulation in the CNS (Høy et al., 1990; Sevatdal et al., 2005).

IVM accumulation in the CNS can lead to decreased swimming and movement ability, which would impact burying (Azevedo and Kennedy, 2022; Domingues et al., 2016; Ezenwaji et al., 2017; Johnson et al., 1993; Katharios et al., 2001; Kennedy et al., 2014; Ogueji et al., 2019; Oliveira et al., 2016b; Palmer et al., 1996; Thiripurasundari et al., 2014; Ucán-Marín et al., 2012). Burying engages both fin and muscular movement along the entire flatfish body. The process consists principally of vigorous beats of the head against the sediment accompanied by a wave of muscular contraction that travels with decreasing amplitude down the body (Gibson et al., 2015). The process of burying requires active muscle engagement, similar to swimming, and swimming performance has been significantly reduced or swimming behaviour altered following exposure to IVM and the combination of IVM and EMB (see Ch. 2; Collymore et al., 2014; Domingues et al., 2016;

Johnson et al., 1993; Katharios et al., 2001; Kennedy et al., 2014; Ogueji et al., 2019; Oliveira et al., 2016; Palmer et al., 1996; Thiripurasundari et al., 2014; Ucán-Marín et al., 2012). For exposed fish, it is likely that the same sedative-like effects and reduced swimming performance, thought to be caused by ivermectin accumulation in fish CNS and agonistic binding to $GABA_A$Rs, had a similar effect on burying activity in *P. stellatus* (Estrada-Mondragon and Lynch, 2015; Høy et al., 1990). This mechanism of toxicity for reduced burying would also explain the lack of a significant change in burying observed for the EMB chronic exposure, as EMB does not cross the teleost BBB as readily as IVM and therefore does not accumulate in the CNS (Sevatdal et al., 2005).This result is consistent with Sahota et al (2022) and *P. stellatus* (see Ch.2) finding no effect of EMB on swimming, and significant reductions in Starry Flounder burst swimming performance observed for the IVM and combination exposures (see Ch. 2).

Overall there was a clear effect of IVM and the combination of IVM/EMB on burying behaviour, but not EMB alone. The lack of response of EMB and the fact that IVM more readily accumlates in teleost brains indicate a neurotoxic effect reducing burying behaviour in *P. stellatus*. There appeared to be no increase in effect on burying from the combination exposure compared to the IVM exposure which indicates that the effect on burying behaviour is likely due to IVM. Significant reductions in *P. stellatus* burying behaviour at one enviornmentally relevant concentration of IVM (10 μg kg^{-1}) was observed, and other significant reductions in burying were observed above sediment concentrations that have been previously observed around open-net pens.

3.4.5. Camouflage

Flatfish camouflage, through the use of cryptic skin coloration and patterns, is a key behaviour for survival (Burton, 2010; Gibson et al., 2015). Cryptic colouration in flatfish has been studied for over a century, and recent technological advances in multi- and hyper-spectral image analysis have allowed the detailed analysis of flatfish body pattern, brightness, colouration and perception using predator visual models (Akkaynak et al., 2017; Price et al., 2019; Sumner, 1911; Troscianko et al., 2016; Troscianko and Stevens, 2015), but this methodology has not been used to assess the effects of a toxic chemicals (Akkaynak et al., 2017; Iwanicki, 2016).

Prior to assessing the concentration-response effect of avermectins, the substrate, avermectins in general, and time were assessed for whether, or not, they had a significant

effect on *P. stellatus* body pattern and luminance. Time had no significant effect on those metrics of camouflage. Flatfish chromatophore responses to background patterns may be a “physiological”, rapid or short-term responses (sec to h) or longer term (d to weeks) “morphological” increases or decreases in pigments and chromatophores (Burton, 2010). Since cryptic body pattern and brightness occurred rapidly and was independent of time over the assay period, *P. stellatus* cryptic body pattern for camouflage is likely a physiological, fast-acting cryptic response over a hormone-mediated morphological one. This pathway of camouflage for *P. stellatus* was also suggested by Iwanicki (2016). It is important to identify the mechanism by which crypsis takes place in the organism to understand mechanisms by which the process might be impacted from toxicant exposure.

In general, *P. stellatus* showed the best camouflage ability, indicated by both pattern and luminance, over the sand background. This is unsurprising given that fine-grained substrates are their preferred habitat (Orcutt, 1950). The poorest ability to camouflage was for the gravel substrate background is likely due to the wide range of substrate pattern, sizes and colouration, making pattern matching more complex and less likely. In a study evaluating the camouflage of Summer Flounder (*Paralichthys dentatus*) and Windowpane Flounder (*Scophthalmus aquosus*) over a similar range and size of substrate backgrounds found that the pattern was best matched by uniform substrates and was reduced with increasing substrate size and complexity (Akkaynak et al., 2017).

Avermectin exposure significantly reduced *P. stellatus* camouflage ability, with similar reductions in both pattern and luminance, though the response was not always concentration-dependent. Both IVM and EMB showed a non-monotonic response with the highest reduction in camouflage ability observed at the intermediary concentrations of IVM (100 μg kg^{-1}) and EMB (120 μg kg^{-1}). The reduction in camouflage ability for EMB and IVM were similar, but IVM showed a slightly more pronounced reduction in ability over sand and cobble. Broadly, the concentration-dependent trends in camouflage responses were similar across substrates for the IVM and EMB treatments, with minor differences in significance observed. Curiously, the combination exposure did not present with the same non-monotonic concentration-response. The highest combination concentration (1200+1000 μg kg^{-1}EMB+IVM) showed the largest effect on *P. stellatus* camouflage with significant reductions in both $Pattern_{diff}$ and $Luminance_{diff}$ across all substrates. Unlike the EMB and IVM exposures, the second highest concentration of the combination had no significant effect on camouflage metrics.

There are several potential explanations for the effect of reduced camouflage ability for EMB, IVM and the combination of both based on avermectins known agonistic action on $GABA_A$Rs. The inhibition or alteration of neuroendocrine processes that regulate the release of α-melanophore stimulating hormone (α-MSH) and melanin concentrating hormone (MCH) through inhibitory GABAergic neurotransmission caused by the action of IVM and EMB as $GABA_A$ agonists, is one potential mechanism. $GABA_A$ receptors and neurons have been found in the brain regions where MCH is released and have been shown to inhibit MCH release (Eggermann et al., 2003; Toossi et al., 2016; van den Pol et al., 2004; Xie et al., 2006). MCH is responsible for reducing dark melanophore pigments and modulates and inhibits the melanophore dispersal effect of α-MSH, thereby lightening skin tone, and inhibition of MCH release and signalling could lead to unbalanced release and binding of α-MSH which could potentially cause skin darkening. However, $GABA_A$-mediated neurotransmission has also been shown to inhibit α-MSH release (Molagoda et al., 2021). Given that MCH and α-MSH is a process influenced and regulated by several factors, perhaps the GABAergic neurotransmission caused by IVM and EMB exposure potentially inhibiting the release of both neuropeptides alters the balanced release and modulation of both allowing the signal transduction of α-MSH to dominate over the lightening effect of MCH, thereby explaining the skin darkening observed in *P. stellatus* (Burton, 2010; Burton and Vokey, 2000).

The non-monotonic concentration-response observed for IVM and EMB was unexpected and could also be related to the balance of the MCH-α-MSH signalling to control chromatophore movement. With the MCH-α-MSH signalling being a tightly regulated system, controlled by several neuroendocrine processes, and inhibitory GABAergic neurotransmission can affect the release of both, there is the potential that different levels of $GABA_A$ receptor activation caused by changes in avermectin concentration could lead to a non-monotonic concentration-response effect rather than a classical concentration-dependent response. MCH inhibition might occur until a certain level of GABAergic neurotransmission caused by a threshold dose of avermectins, above which α-MSH inhibition might also occur leading to a balance between the two hormones and less of an effect on camouflage at higher concentrations. This could also potentially explain the different effect observed in the combination exposure, where the intermediary combination concentration is above that threshold, thereby lessening the effect on camouflage and then the highest combination concentration again leads to an unbalanced signalling response control camouflage leading to the highest level of skin darkening and

most affected camouflage ability. Overall, it is difficult to elucidate the mechanism behind the range in effects of avermectin on camouflage ability within the scope of the current study, however it is clear there was some effect. Future work should endeavor to increase the range and number of concentrations tested to gain a complete understanding of the effect of avermectin concentration on *P. stellatus* camouflage ability.

Reduced camouflage ability could also be explained by direct action on the visual response to background visual stimuli in *P. stellatus*. Flatfish camouflage is directly influence by visual ques from their surrounding environment, and the action of IVM and EMB on GABAergic neurotransmission could have an effect on teleost visual stimuli and the subsequent response on camouflage and skin pigment. It has been shown that GABA-mediated feedback in the retina has an important role in converting trichromatic cone response into colour opponency in horizontal cells (Marc et al., 1978). Exposure of fish to darkness induced depolarization of cone horizontal cells and increased release of GABA (Marc et al., 1978). If GABA is the neurotransmitter associated with the response to dark visual stimuli in fish, then there is potential that uncontrolled or excessive GABA activation and release by IVM and EMB binding to $GABA_A$Rs could lead to continuous “dark” visual stimulus response and therefore darkened body pigment and pattern. By this mechanism, it would be expected that IVM would have a more pronounced effect on camouflage ability than EMB, given the increased tendency of IVM to accumulate in the CNS, but this was not the observed effect (Høy et al., 1990; Sevatdal et al., 2005). It would also be expected with the indirect mechanism of reduced camouflage ability due to visual inhibition, that the highest combination concentration would have the most pronounced effect due to IVM inhibition of Pgp leading to increased CNS concentrations of both avermectins, which was observed (Griffin et al., 2005; Kennedy et al., 2014; Stevens and Charles, 2001; Xu et al., 2016). Given the increased effect of the combined treatment on *P. stellatus* camouflage and the physicochemical properties of avermectins leading to persistence in marine sediments, the administration of both IVM and EMB at an Atlantic Salmon aquaculture site should be approached with caution.

One common effect observed across studies and taxa exposing teleosts to IVM and EMB at high concentrations was fish presented with darkened skin pigmentation, which was also observed in the current study (Domingues et al., 2016; Johnson et al., 1993; Katharios et al., 2001; Ogueji et al., 2019; Palmer et al., 1996; Roy et al., 2000; Ucán-Marín et al., 2012). In another study exposing *P. stellatus* to similar avermectin concentrations for 30-d (see Ch. 2) a binomial endpoint of whether darkened skin

colouration occurred, or not, was assessed. Though the results of this are not directly comparable, as specific metrics of camouflage such as pattern and luminance were not assessed, it can give some indication of which dose camouflage might be affected from avermectin exposure. In the previous study, no significant increase in darkened skin colouration was observed for any EMB treatments. A significant increase in darkened skin pigmentation was observed at the two highest concentrations of IVM and the highest concentrations of the combination (see Ch.2). The EC_{50} for individuals with darkened skin colouration was 267 ± 1068 µg kg^{-1} for IVM and 255 ± 151 µg kg^{-1} for the combined EMB and IVM concentration. The observational results of no darkened skin pigmentation following EMB exposure do not align with the effects of EMB on pattern and luminance in the current study. This discrepancy is potentially due to multispectral image analysis of pattern and luminance are more sensitive endpoints to detect camouflage effects than the visual, qualitative observation of darkened skin pigmentation. The significant effects of IVM and the combination and camouflage do align with the results of the other study (see Ch. 2) and the concentration at which IVM effects were observed is within the EC_{50} standard error values from the previous study.

The current study assessed metrics of camouflage within the human visual spectrum. Although spatial visual processing is thought to be similar between vertebrate taxa and studies comparing human and non-human visual spectra have been shown to be similar, assessing Starry Flounder camouflage using visual spectrum models for mono-, di- and trichromatic predators of *P. stellatus* to assess camouflage ability following avermectin exposure would help to inform the effect on camouflage in the wild as it relates to predation (Caves and Johnsen, 2018; Godfrey et al., 1987; Stevens et al., 2013; Troscianko and Stevens, 2015). In addition to quantifying camouflage using predator visual spectra, additional metrics of camouflage that have been shown to be important such as colour matching and edge disruption would provide a more holistic estimation of *P. stellatus* camouflage and changes to camouflage performance following exposure to a toxicant such as avermectins in sediment (Akkaynak et al., 2017; Troscianko et al., 2017). Future experiements assessing toxicant exposure on flatfish, or other teleost, camouflage should include these additional metrics and use a camera capable of capturing wavelengths of light outside of the human visual spectrum (e.g., UV light).

Flatfish are exceptional among animals in their responses to differences in background patterns, an important adaptation for flat-bodied fish with demersal life histories (Parker and Brown Jr., 1948). There is a myriad of stimuli and neuroendocrine

process involved in regulating crypsis in flatfish, presumably the mechanism of avermectins causing darkened colouration in teleosts could be multifaceted. Overall, it is clear that a 30-d exposure to avermectin in sediments reduced camouflage ability at environmentally relevant concentrations, which is cause for concern (Cannavan et al., 2000; DFO, 2012, 1996; Ikonomou and Surridge, 2013; SEPA, 1999; Veldhoen et al., 2012). Reduction in the flatfish ability to camouflage can lead to an increase in conspicuousness. This increase in conspicuousness can then affect feeding, growth, predator avoidance and survival (Burton, 2010; Sumner, 1935, 1934). Reduction in individual survival and fitness by avermectin exposure could in-turn lead to local population- and ecosystem-level impacts (Burton, 2010; Sumner, 1935, 1934).

3.5. Conclusions

The avermectin-type insecticides EMB (in the SLICE® formulation) and IVM are currently in use in Canada to treat sea lice infestations in open-net pen Atlantic Salmon aquaculture operations in both the Pacific and Atlantic regions, with sequential applications of SLICE® and IVM sometimes (Hamoutene et al., 2022). The tendency of EMB and IVM to persist in the benthic marine sediments for long periods of time (half-life >188 d) and the potential combined or subsequent applications of EMB and IVM is concerning due to the potential sublethal toxic effects to non-target benthic teleosts from both EMB and IVM individually and the combination of both. In the current study, significant increases in avoidance of contaminated sediment and reductions in *P. stellatus* burying were observed using a 48-h avoidance assay. Substantially more avoidance and reduced burying was observed in fish previously exposed to avermectins for 30-d, than in naïve fish not previously exposed. However, significant reductions in burying and some avoidance were still observed for the naïve group of fish. For avoidance, significant effects were observed for all avermectin exposures (i.e., EMB, IVM and EMB+IVM), whereas for burying significant reductions were only observed for the IVM and combination treatments, which might mean that only IVM caused a reduction in burying. The difference between avoidance and burying response to EMB potentially indicates different mechanisms behind avermectin-induced avoidance and reduced burying. The 30-d exposure to avermectins also had a significant effect on *P. stellatus* camouflage ability, indicated by the difference in flounder body pattern and luminance (perceived lightness) from three substrate backgrounds. The effects on camouflage for EMB and IVM were similar with

what appeared to be a non-monotonic concentration-response of camouflage metrics to both. The same concentration-response was not observed for the combination exposure and the largest reduction in camouflage ability was found for the highest combination concentration group, which suggests that high doses of IVM and EMB combined would have the most significant effect on flatfish camouflage. The behavioural effects on avermectin exposure observed in the current study at previously reported concentrations found in marine sediments have concerning implications for sublethal effects on flatfish that could affect survival and fitness in the wild. Future research should seek to expand the scope of the assays used in the current study, in both the number and range of concentrations and the duration of the exposures and assays to completely capture the range of sublethal effects of avermectins to these flatfish behaviours. Additionally, other metrics of camouflage such as colour matching, edge disruption and camouflage ability should be considered in the context of the visual spectra of *P. stellatus* predators.

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3.7. Figures and Tables

Table 3-1. Endpoints of Platichthys stellatus measured following a 30-d exposure to sediment treated with emamectin benzoate (EMB) prepared from SLICE® 0.2% premix, ivermectin (IVM) or a combination of both (EMB+IVM), and naïve fish not previously exposed to the chemotherapeutants for the 48-h avoidance assay. Endpoints measured were mortality, growth (% change in mass), relative condition (Kn), mean proportion of fish on the seeded (treated) side (naïve and chronic), proportion of fish buried (naïve and chronic), mean pattern difference (power) between flounder body and substrate background for cobble, gravel and sand; and mean luminance difference between flounder body and substrate background for cobble, gravel and sand. Statistical tests selected based on satisfying required assumptions (e.g., normality for ANOVA) and statistical significance from the negative control was denoted by p<0.05. SE=standard error; µg kg-1=µg chemotherapeutant for each kg of sediment; NOEC=no observed effect concentration; LOEC=lowest observed effect concentration; ANV=ANOVA, KW=Kruskal-Wallis, P. = proportion, *=value of difference in slope vs control ±SE of the diference. NOEC and LOEC are based on no effects or observed effects significantly different from the negative control.

Chemical	Endpoint	Significant Endpoint Value ± SEM (Control value)	Test	Statistic (p-value)	NOEC/LOEC (µg kg^{-1})
EMB	Mortality	<20%	-	-	-

IVM		<20%	-	-	-
EMB+IVM		<20%	-	-	-
EMB	Growth (% Δmass)	-	KW	χ^2=3.93 (p=0.27)	1200/-
IVM		-26±2.7 (-17±2.0)	ANV	F=3.16 (p=0.03)	100/1000
EMB+IVM		-	ANV	F=0.46 (p=0.70)	1200+1000/-
EMB	K_n	-	ANV	F=1.61 (p=0.19)	1200/-
IVM		0.71±0.03 (0.81±0.02)	ANV	F=4.92 (p=0.0036)	100/1000
EMB+IVM		-	ANV	F=2.04 (p=0.12)	1200+1000/-
Avoidance					
EMB	P. Seeded Side (Naïve)	-	KW	χ^2 =4.01 (p=0.13)	1200/-
IVM		0.58±0.03 (0.46±0.02)	ANV	F=6.17 (p=0.0026)	1000/10
EMB+IVM		0.68±0.03 (0.46±0.02)	ANV	F=12.0 (p<0.0001)	12+10/ 1200+1000
EMB	P. Seeded Side (Chronic)	0.31±0.03 (0.46±0.02)	KW	χ^2 =35.7 (p<0.0001)	120/1200
IVM		0.25±0.03 (0.46±0.02)	KW	χ^2 =44.7 (p<0.0001)	1000/10
EMB+IVM		0.27±0.03 (0.46±0.02)	KW	F=15.5 (p<0.0001)	-/10+12
CTRL	P.Seeded Side Chronic vs Naive	-	t-test	t=0.05 (p=0.96)	-
EMB	Slope P.Seeded vs Time (Naïve)	-0.08±0.04*	t-test	t=-2.04 (p=0.04)	12/1200
IVM		0.12±0.03*	t-test	t=3.52 (p=0.0005)	1000/10
EMB+IVM		0.22±0.04*	t-test	t=5.78 (p<0.0001)	12+10/ 1200+1000
EMB	Slope P.Seeded vs Time (Chronic)	-0.23±0.04*	t-test	t=-5.74 (p<0.0001)	-/12
IVM		-0.21±0.04*	t-test	t=-5.18 (p<0.0001)	1000/10
EMB+IVM		-0.21±0.04*	t-test	t=-5.84 (p<0.0001)	-/ 12+10
Burying					
EMB	P. Buried (Naïve)	-	KW	χ^2 =2.5 (p=0.29)	-/1200

IVM		0.47±0.04 (0.66±0.05)	ANV	F=9.8 (p<0.0001)	10/1000
EMB+IVM		0.48±0.06 (0.66±0.05)	KW	χ^2 =15.6 (p=0.0004)	-/ 1200+1000
EMB	P. Buried (Chronic)	-	KW	χ^2 =6.5 (p=0.09)	-/1200
IVM		0.18±0.04 (0.31±0.03)	ANV	F=8.14 (p<0.0001)	100/1000
EMB+IVM		0.17±0.04 (0.31±0.03)	ANOVA	F=5.83 (p0.0008)	-/12+10
CTRL	P. Buried Chronic vs Naïve	0.31±0.13 (naïve) 0.66±0.10 (chronic	t-test	t=2.19 (p=0.03)	-
EMB	Slope P.Buried vs Time (Naïve)	-	-	-	1200/-
IVM		-0.20±0.05*	t-test	t=-4.20 (p<0.0001)	10/ 1000
EMB+IVM		-0.19±0.05*	t-test	t=-3.47 (p=0.0006)	12+10/ 1200+1000
EMB	Slope P.Buried vs Time (Chronic)	-	-	-	1200/-
IVM		0.11±0.04*	t-test	t=2.60 (p=0.0096)	10/100
EMB+IVM		-0.14±0.04*	t-test	t=-3.15 (p=0.0018)	120+100/ 1200+1000
Camouflage					
EMB	Pattern Difference (Power)	Cobble	KW	χ^2 =5.2 (p=0.15)	1200/-
		Gravel	ANV	F=4.2 (p=0.01)	12/120
		Sand	ANV	F=6.7 (p=0.0008)	1200/120
IVM		Cobble	ANV	F=3.6 (p=0.02)	1000/-
		Gravel	ANV	F=6.4 (p=0.0015)	1000/100
		Sand	ANV	F=8.4 (p=0.0003)	1000/100
EMB+IVM		Cobble	ANV	F=3.6 (p=0.03)	1200+1000/ 120+100
		Gravel	ANV	F=5.3 (p=0.005)	1200+1000/ 120+100
		Sand	ANV	F=3.5 (p=0.03)	120+100/ 1200+1000
EMB	Luminance Difference	Cobble	KW	χ^2 =2.0 (p=0.57)	1200/-

		Gravel	ANV	F=1.3 (p=0.27)	1200/-
		Sand	KW	χ^2 =8.1 (p=0.04)	1200/-
IVM		Cobble	ANV	F=3.1 (p=0.04)	1000/100
		Gravel	ANV	F=3.6 (p=0.02)	1000/-
		Sand	ANV	F=4.7 (p=0.008)	1000/-
EMB+IVM		Cobble	KW	χ^2 =8.6 (p=0.04)	120+100/ 1200+1000
		Gravel	KW	χ^2 =13.9 (p=0.003	120+100/ 1200+1000
		Sand	ANV	F=4.9 (p=0.007)	1200+1000/ 120+100

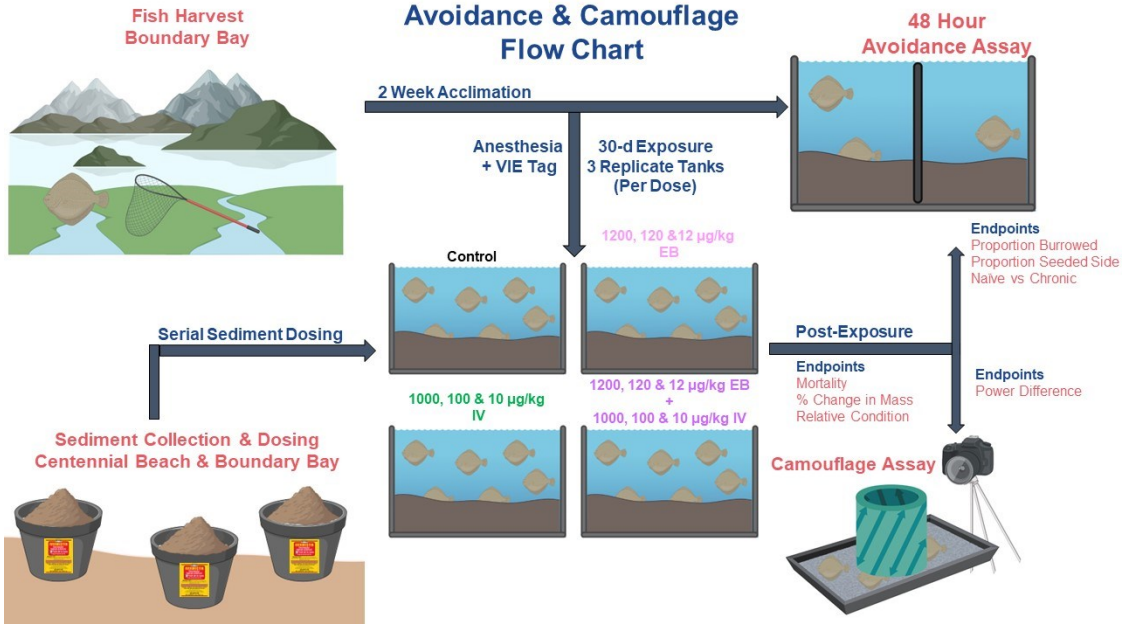

Figure 3-1. Exposure design and results summary for avoidance, burying and camouflage effects in Platichthys stellatus exposed to IVM, EMB and the combination of both in marine sediments.

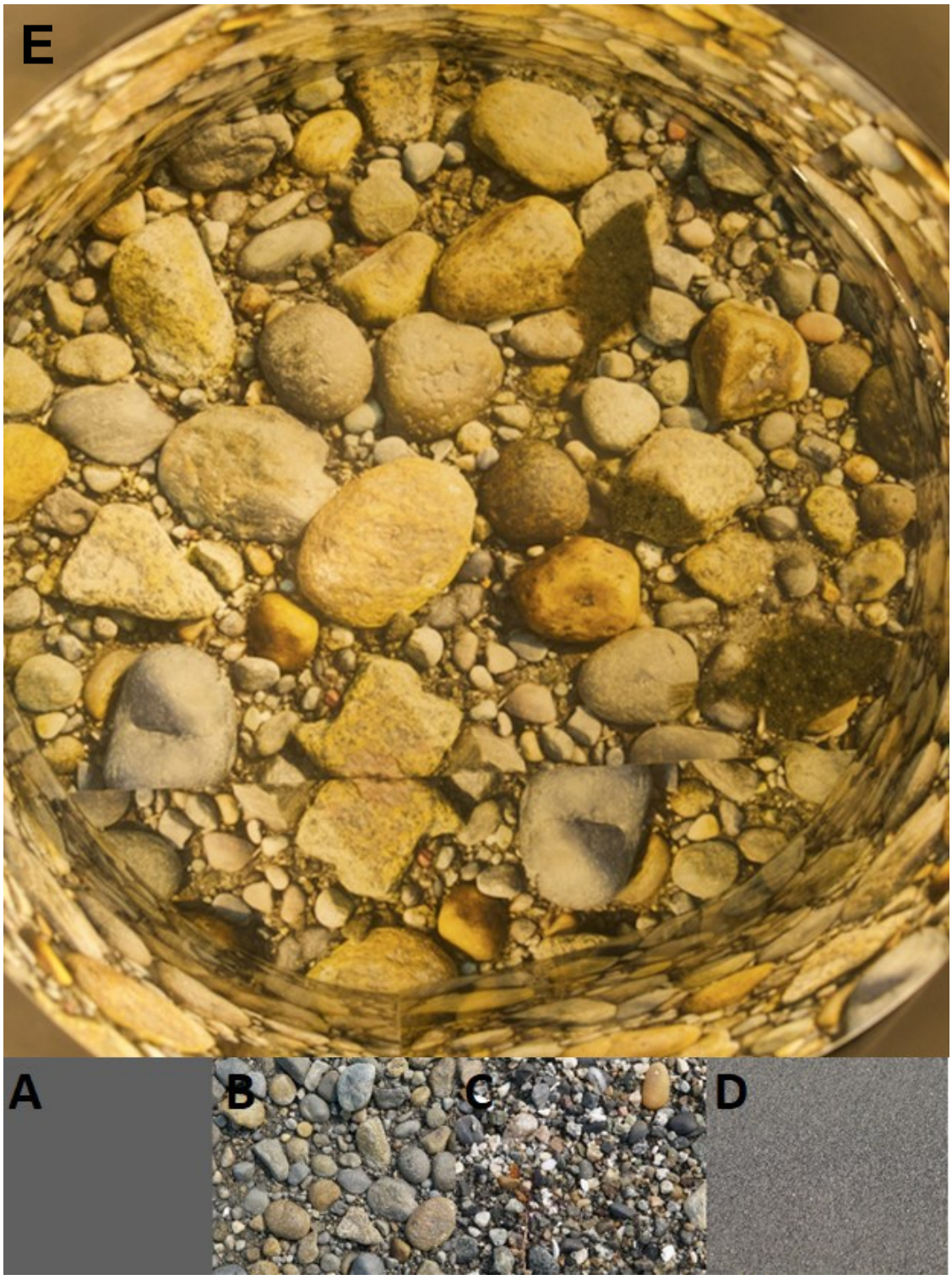

Figure 3-2. **Images of laminated backgrounds used to compare Platichthys stellatus body pattern and perceived brightness. Images include the grey acclimation background (A), a cobble background (B), gravel background (C) and sand background (D); as well as an image of three P. stellatus in the camouflage assay chamber (E).**

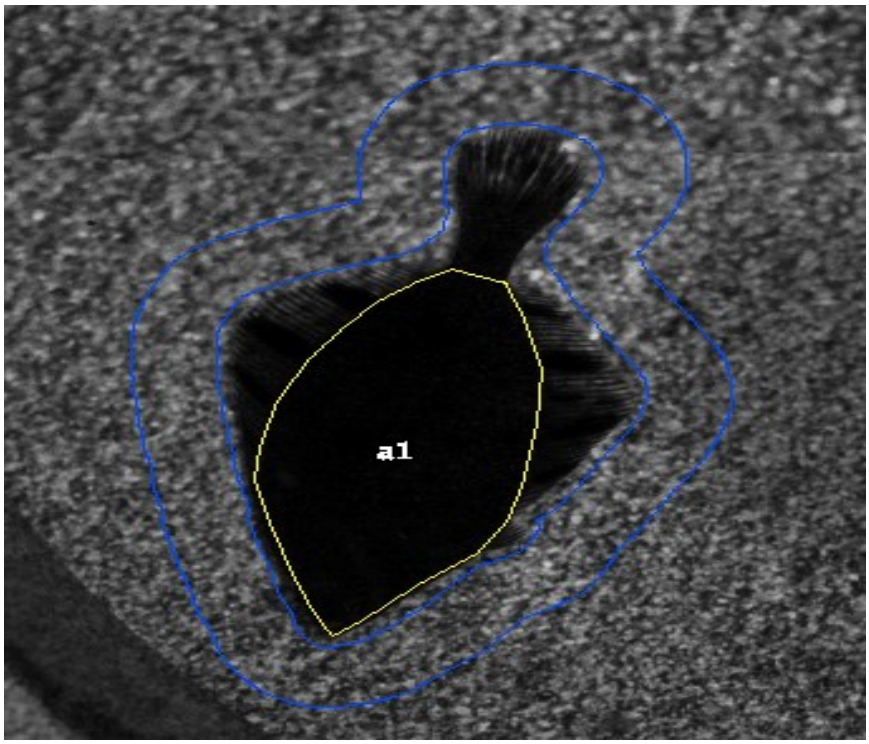

Figure 3-3. **Image of the selection of two regions of interest (ROIs) of the flounder body area excluding the dorsal and anal fins and a 5 cm wide band of substrate background surrounding the flounder body. The ROIs were selected in the multispectral image analysis software in imageJ which were compared to quantify the difference between Platichthys stellatus body pattern (power) and perceived brightness (luminance) and the surrounding substrate background.**

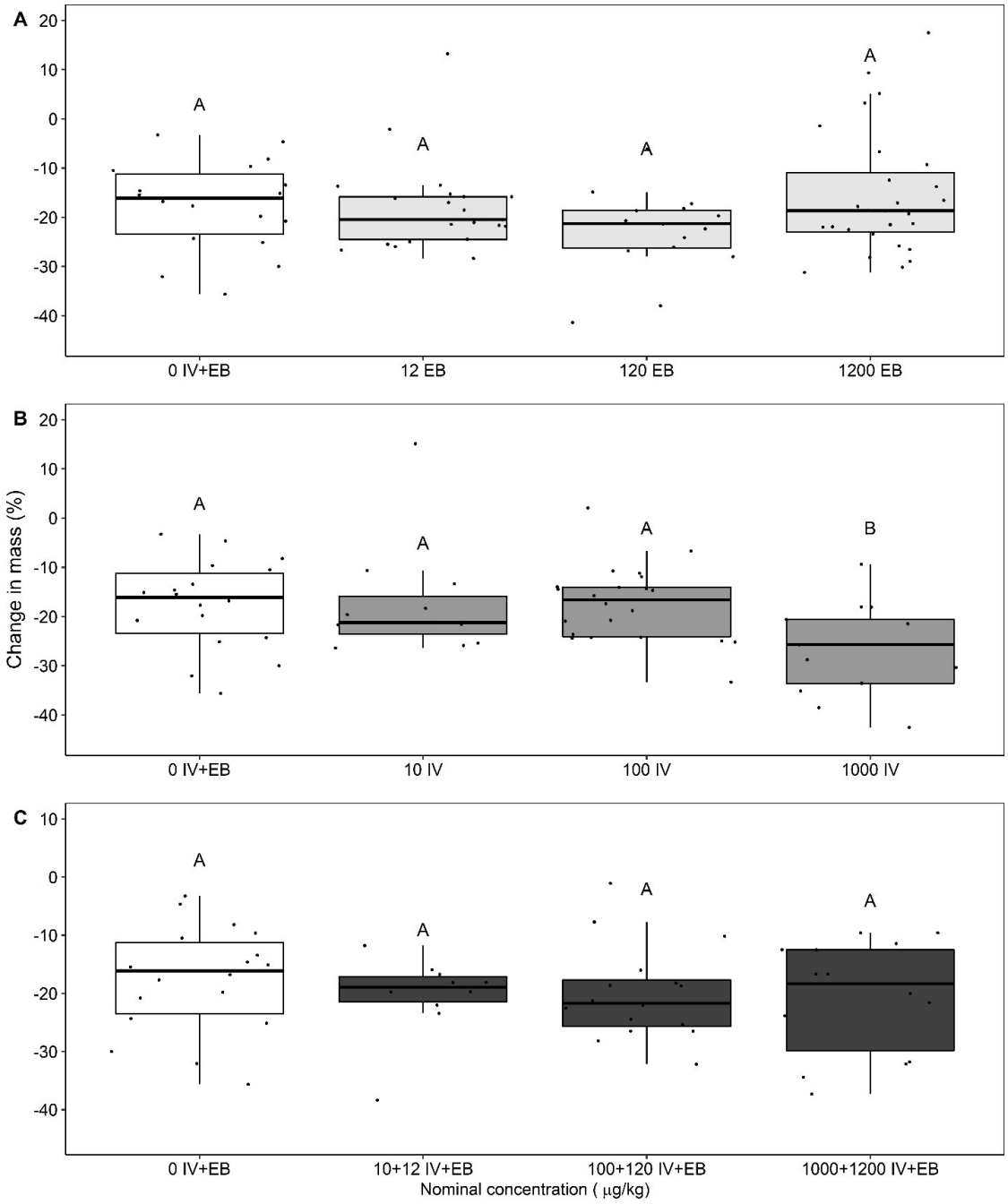

Figure 3-4. Percent change in mass of P. stellatus exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (boxes = interquartile range (IQR), bolded line = median, whiskers = 1.5*IQR, dots = individual fish). Concentrations were 12, 120, and 1200 µg EMB/kg sediment (EMB); 10, 100 and 1000 µg IVM/kg sediment (IVM); 12+10, 120+100 and 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups ($p < 0.05$).

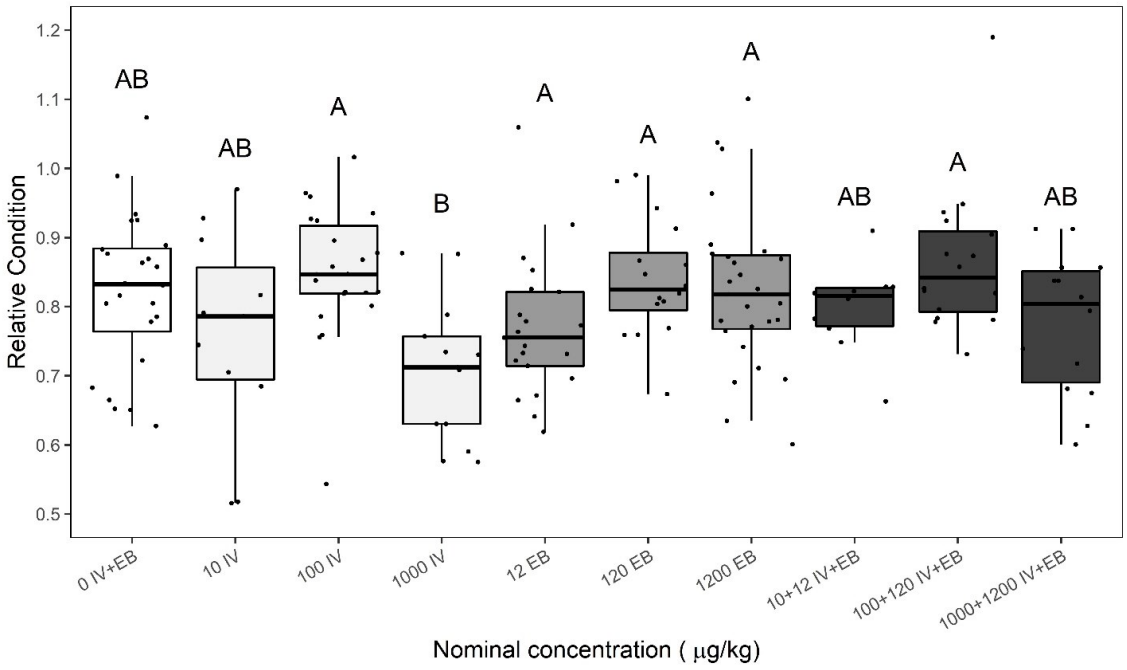

Figure 3-5. **Relative condition (K_n) of P. stellatus exposed for 30-d to sediment spiked with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) Ivermectin (IVM) or (C) a combination of both EMB+IVM (boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish). Concentrations were 12, 120and 1200 µg EMB/kg sediment (EMB); 10, 100and 1000 µg IVM/kg sediment (IVM); 12+10, 120+100, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Upper case letters represent statistically different groups (p < 0.05).**

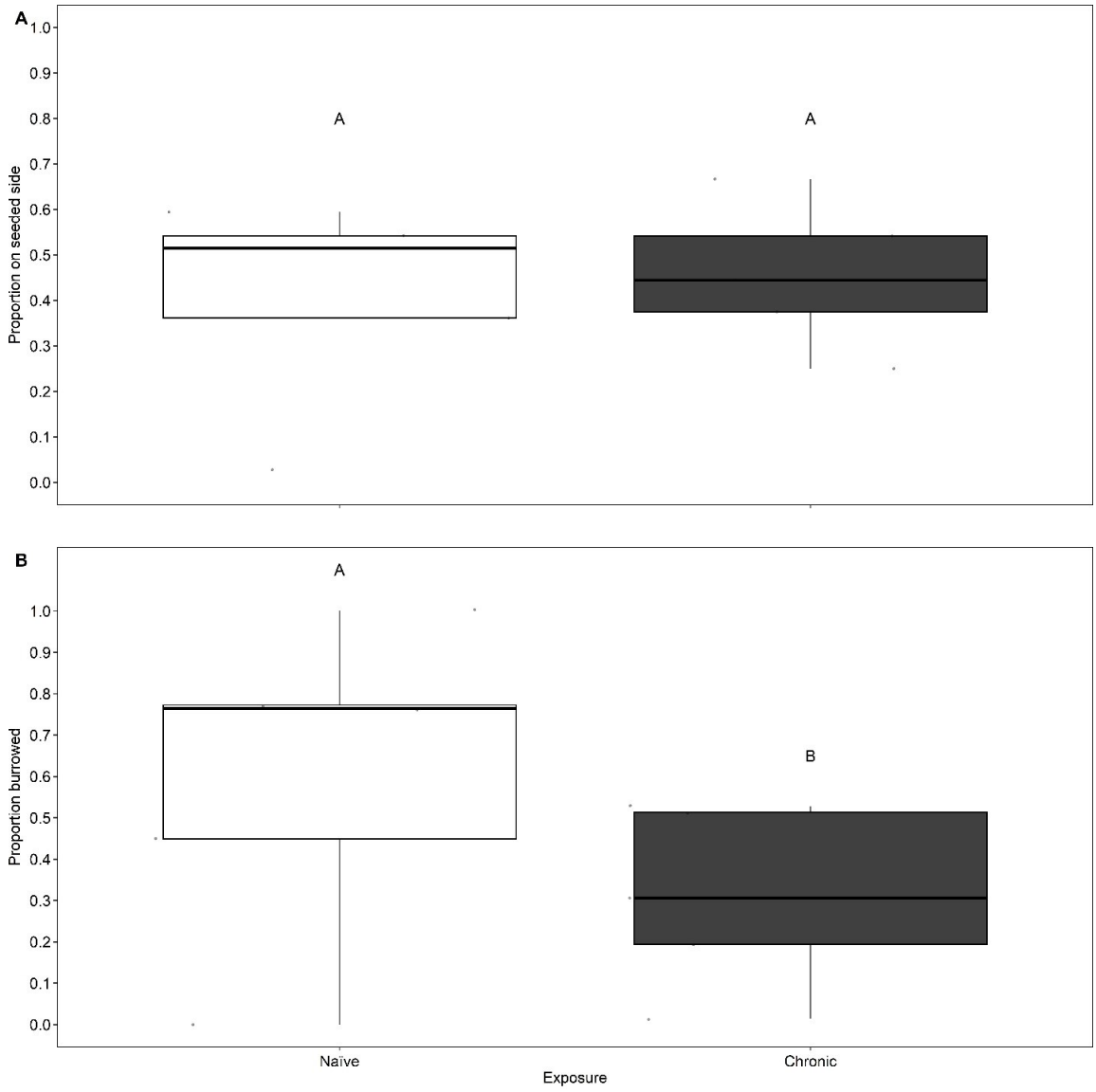

Figure 3-6. **Mean proportion of P. stellatus on the seeded (treated) side of the assay chamber (A) and mean proportion of P. stellatus buried over 48-h avoidance assay. Average values were from the negative control groups of a naïve (□) group not previously exposed to the sea lice chemotherapeutants and a chronic group (■) exposed for 30-d prior to the 48-h avoidance assay to control (clean) sediment. Boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR. Upper case letters represent statistically different groups ($p < 0.05$).**

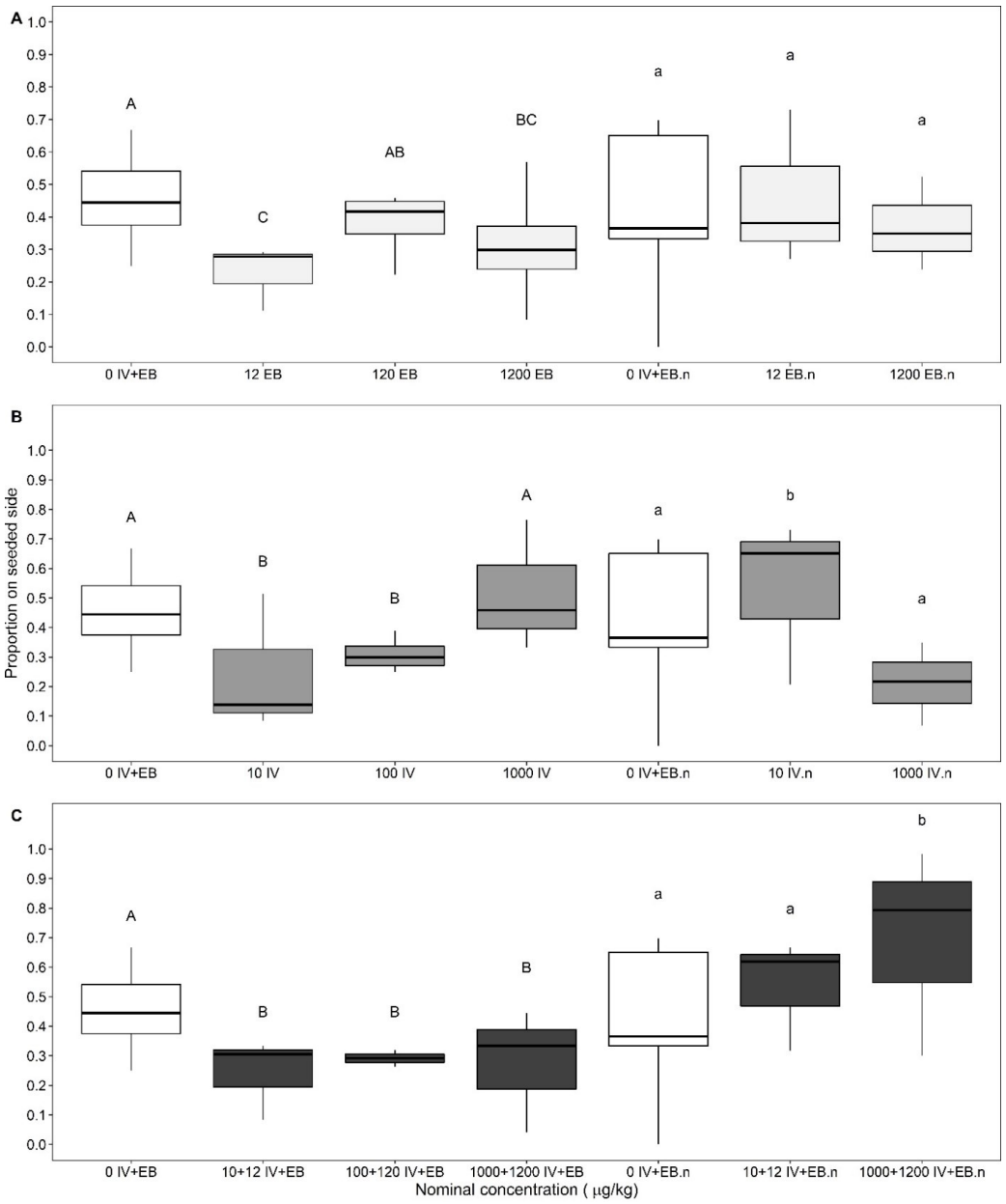

Figure 3-7. Mean proportion of P. stellatus on the seeded (treated) side of the 48-h avoidance assay chamber. Concentrations were 12, 120 and 1200 µg EMB/kg sediment for the chronic assay and 12 and 1200 µg EMB/kg sediment for the naïve exposure (EMB); 10, 100 and 1000 µg IVM/kg sediment for the chronic exposure and 10 and 1000 µg IVM/kg sediment for the naïve exposure (IVM); and 12+10, 120+100, 1200+1000 µg EMB+IVM/kg sediment for the chronic exposure 12+10, 1200+1000 µg EMB+IVM/kg sediment for the naïve exposure (EMB+IVM); or to a clean sediment negative control □. Boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR. The '.n' next to the treatment concentrations indicates a naïve exposure. Letter differences indicated statistically significant groups with the naïve group using lowercase letters and the chronic exposures upper case letters (p<0.05).

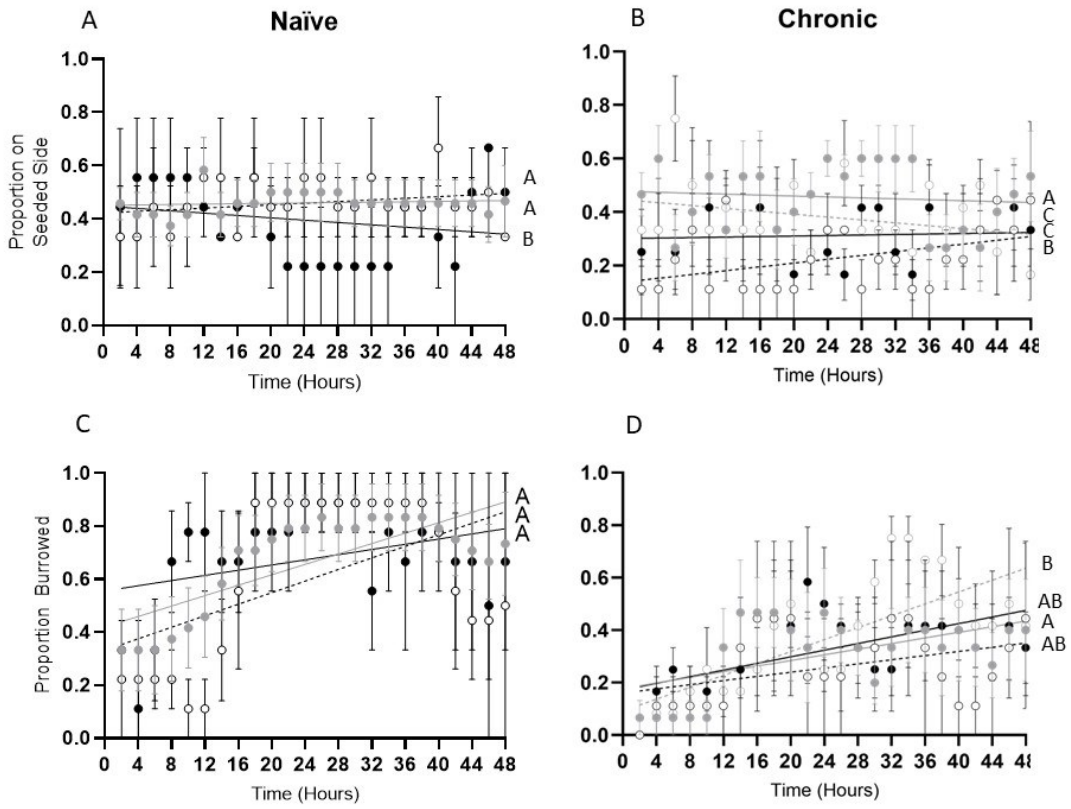

Figure 3-8. **Linear regressions of the proportion of P. stellatus on the seeded (treated) side of the 48-h avoidance assay chamber over time for the Naïve (A) and Chronic (B) exposure groups; and of the proportion of fish buried over time for the same assay period for the Naïve (C) and Chronic (D) exposure groups. Naïve fish were not previously exposed to EMB and the Chronic fish were exposed to the same concentrations of EMB used in the 48-h avoidance assay for 30-d. Concentrations of EMB were 0 (●), 12(○), 120(○), and 1200(●) µg EMB/kg sediment. Error bars represent the standard error of the mean and each point represents the mean proportion of fish at a given time point. Statistically significant differences between treatment concentrations for each exposure and endpoint were indicated by upper case letters on the right of each plot ($p<0.05$).**

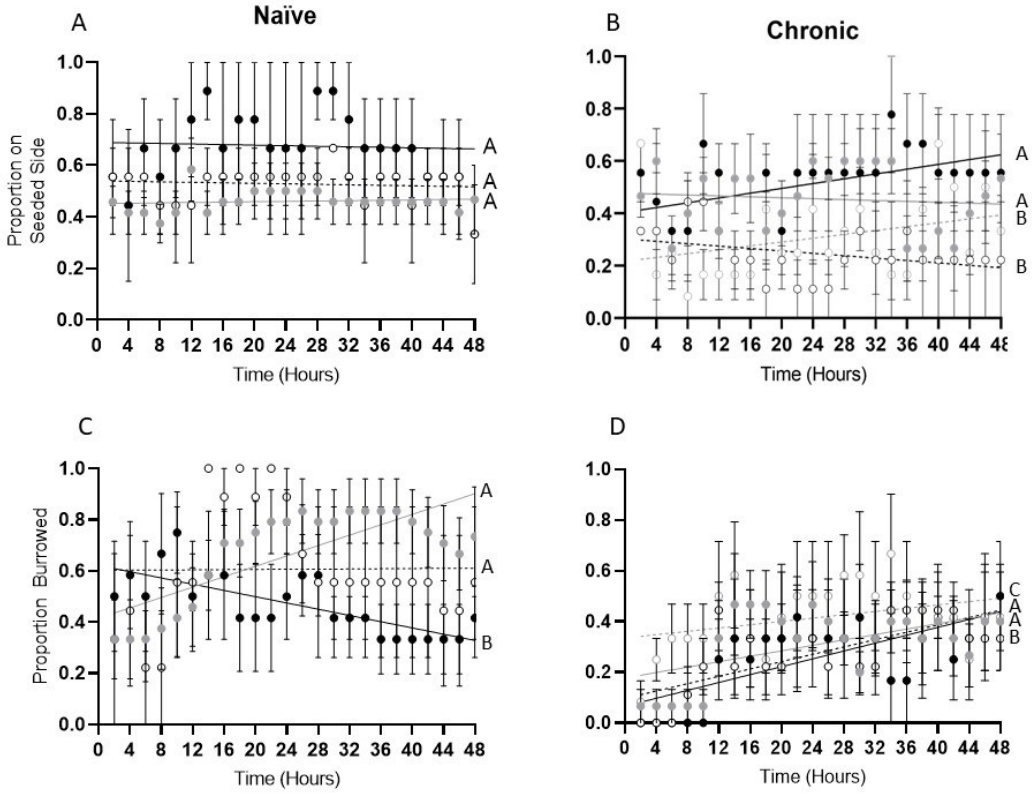

Figure 3-9. **Linear regressions of the proportion of P. stellatus on the seeded (treated) side of the 48-h avoidance assay chamber over time for the Naïve (A) and Chronic (B) exposure groups; and of the proportion of fish buried over time for the same assay period for the Naïve (C) and Chronic (D) exposure groups. Naïve fish were not previously exposed to IVM and the Chronic fish were exposed to the same concentrations of IVM used in the 48-h avoidance assay for 30-d. Concentrations of IVM were 0 (●), 10(○), 100(○), and 1000(●) µg EMB/kg sediment. Error bars represent the standard error of the mean and each point represents the mean proportion of fish at a given time point. Statistically significant differences between treatment concentrations for each exposure and endpoint were indicated by upper case letters on the right of each plot ($p<0.05$).**

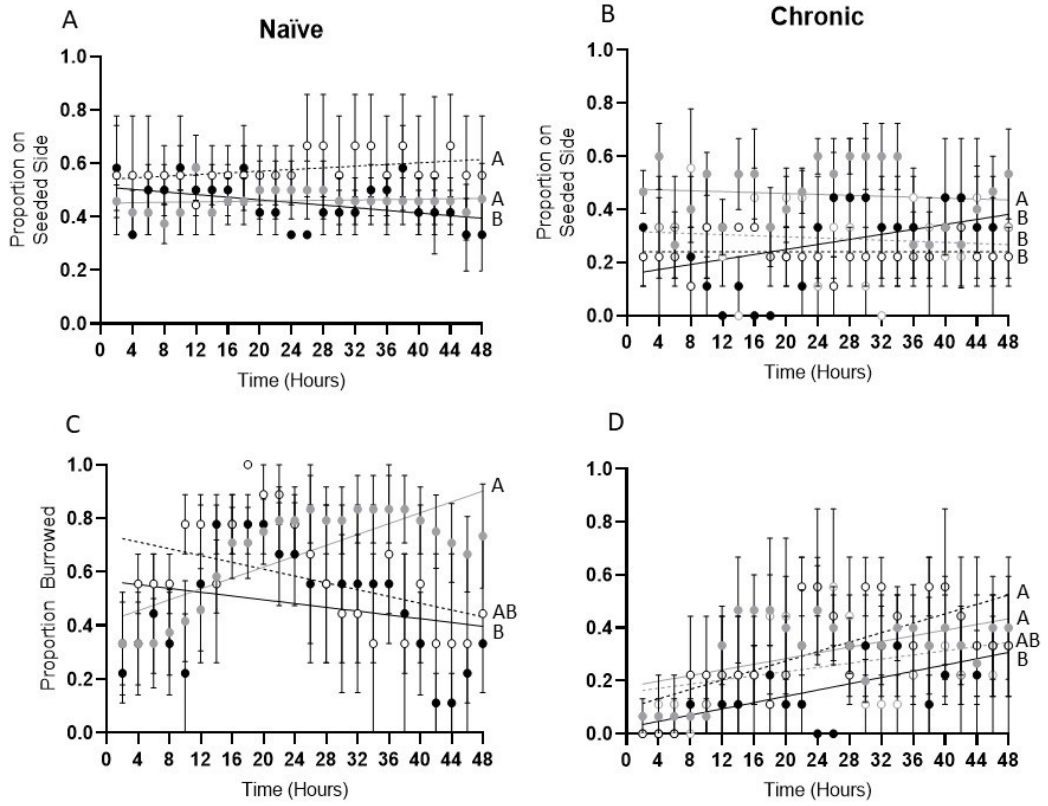

Figure 3-10. Linear regressions of the proportion of P. stellatus on the seeded (treated) side of the 48-h avoidance assay chamber over time for the Naïve (A) and Chronic (B) exposure groups; and of the proportion of fish buried over time for the same assay period for the Naïve (C) and Chronic (D) exposure groups. Naïve fish were not previously exposed to the combination concentrations of EMB and IVM and the Chronic fish were exposed to the same concentrations of EMB and IVM used in the 48-h avoidance assay for 30-d. Concentrations of EMB+IVM were 0 (●), 12+10(○), 120+100(○), and 1200+1000(●) µg EMB+IVM/kg sediment. Error bars represent the standard error of the mean and each point represents the mean proportion of fish at a given time point. Statistically significant differences between treatment concentrations for each exposure and endpoint were indicated by upper case letters on the right of each plot (p<0.05).

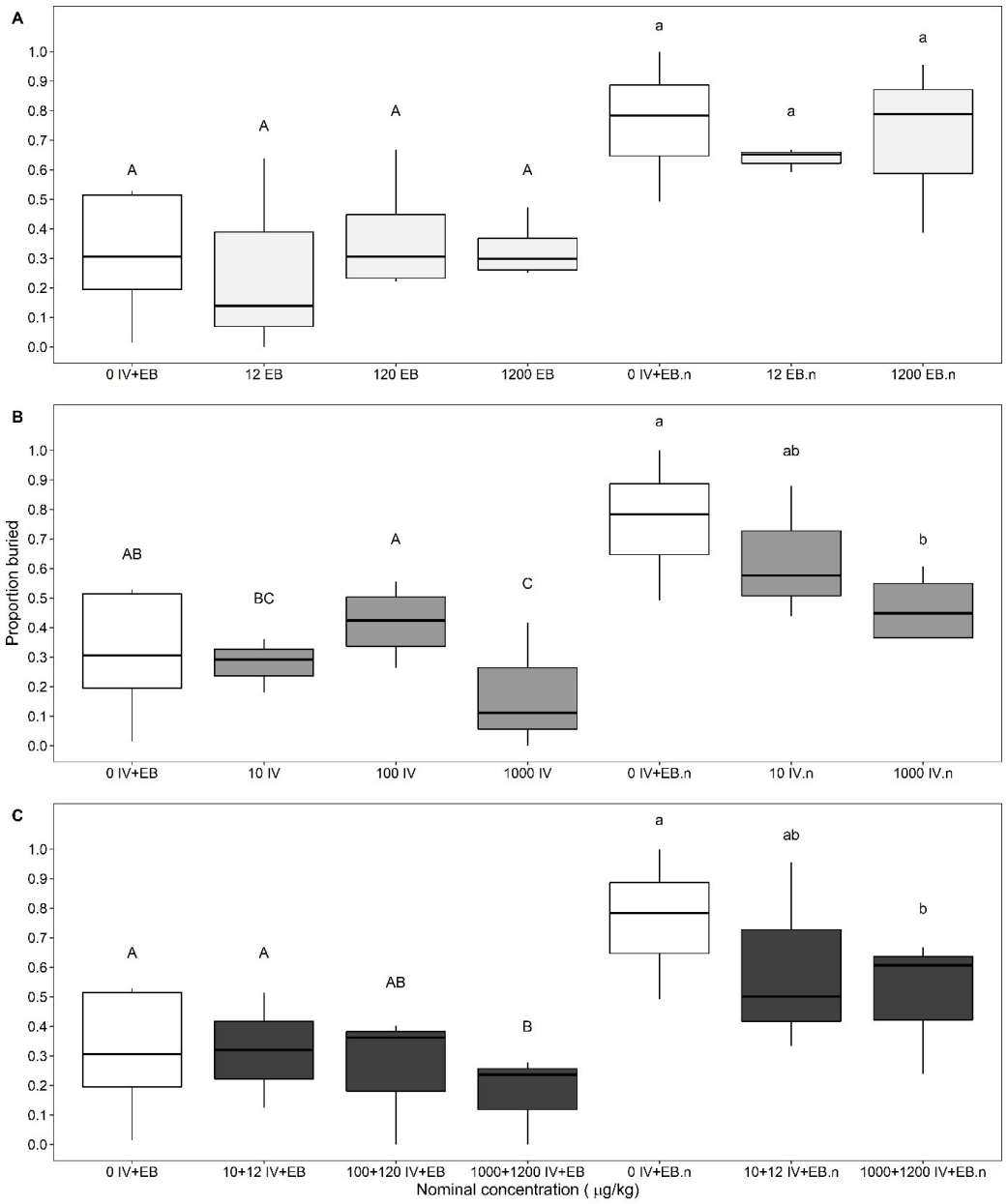

Figure 3-11. Mean proportion of P. stellatus buried averaged over the 48-h avoidance assay period. Concentrations were 12, 120 and 1200 µg EMB/kg sediment for the chronic assay and 12 and 1200 µg EMB/kg sediment for the naïve exposure (EMB); 10, 100 and 1000 µg IVM/kg sediment for the chronic exposure and 10 and 1000 µg IVM/kg sediment for the naïve exposure (IVM); and 12+10, 120+100, 1200+1000 µg EMB+IVM/kg sediment for the chronic exposure 12+10, 1200+1000 µg EMB+IVM/kg sediment for the naïve exposure (EMB+IVM); or to a clean sediment negative control □. Boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR. The '.n' next to the treatment concentrations indicates a naïve exposure. Letter differences indicated statistically significant groups with the naïve group using lowercase letters and the chronic exposures upper case letters (p<0.05).

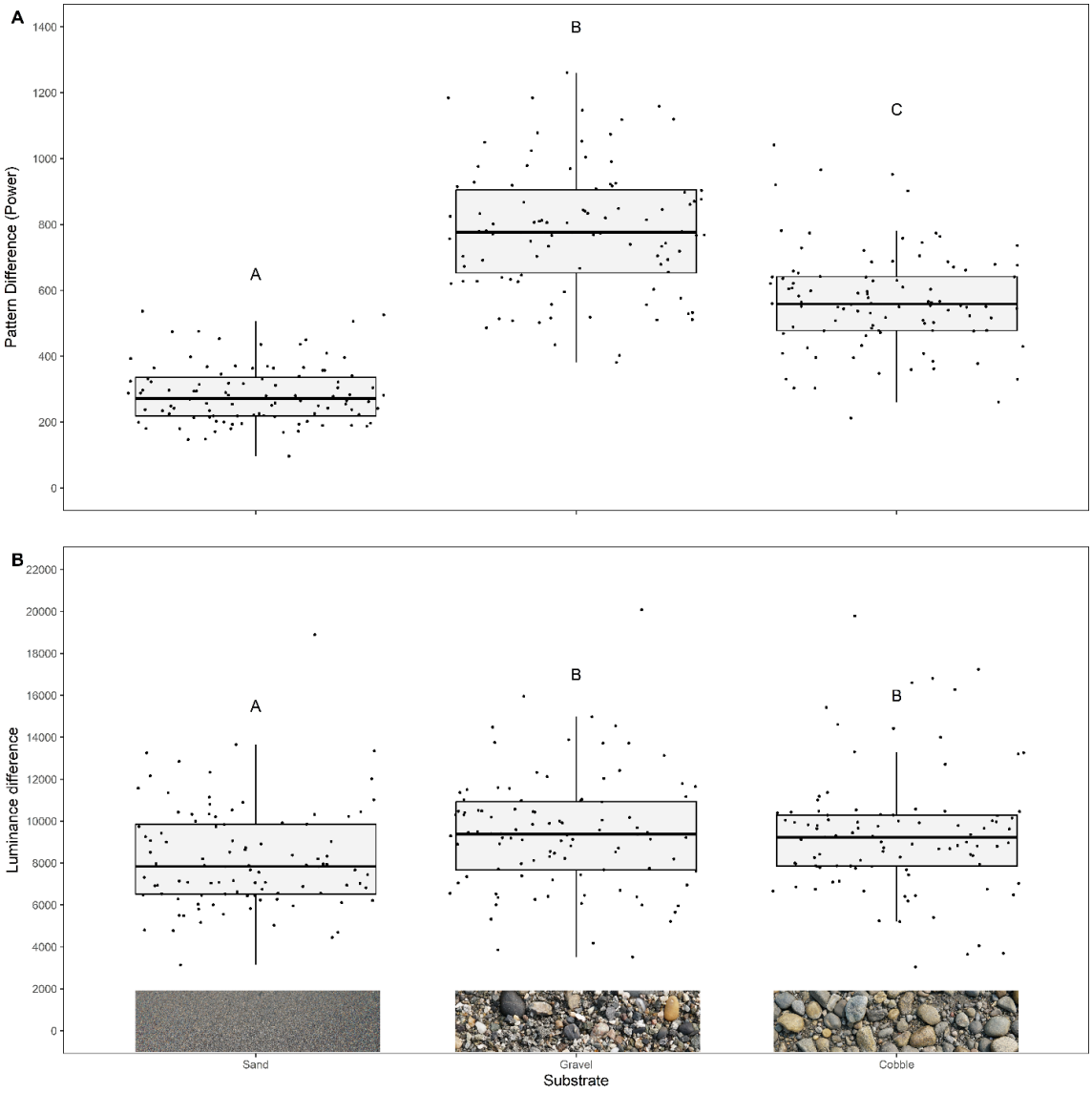

Figure 3-12. Pattern difference (A; power) and Luminance difference (B;perceived brightness) between P. stellatus body and each substrate type (i.e., sand, gravel and cobble) background used in a camouflage assay. Values were pooled for fish across all treatment concentrations including the control to observe a substrate-level effect. Boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots=individual fish. Upper case letters indicate statistically significant differences ($p<0.05$).

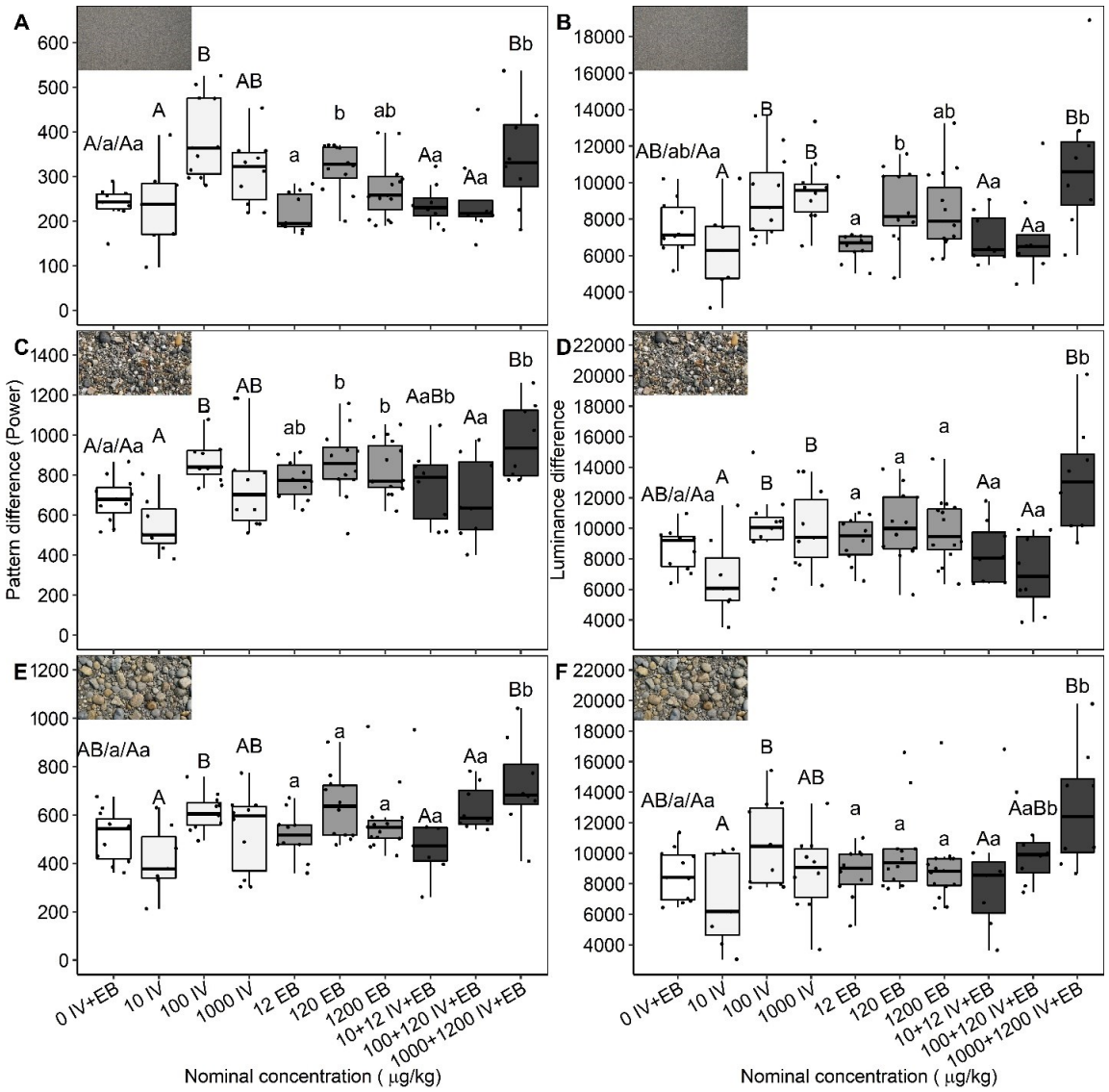

Figure 3-13. Mean pattern difference (power) of P. stellatus body pattern compared to a sand (A), gravel (C) and cobble (E) substrate background and mean luminance (perceived brightness) difference for flounder body compared to sand (B), gravel (D), and cobble (E) substrate background. Fish were exposed for 30-d to sediment treated with 12, 120 and 1200 µg EMB/kg sediment (IVM); 10, 100 and 1000 µg IVM/kg sediment (EMB); and 12+10, 120+100, 1200+1000 µg EMB+IVM/kg sediment (EMB+IVM); or to a clean sediment negative control □. Boxes = interquartile range [IQR], bolded line = median, whiskers = 1.5 x IQR, dots = individual fish. Letter differences indicated statistically significant groups with upper case letters used for the IVM exposure group, lower case letters for the EMB exposure and a combination of uppercase and lower case for the combination EMB+IVM exposure (p<0.05).

Chapter 4. Conclusions and Future Research

This thesis assessed the sublethal effects of two anti-sea lice chemotherapeutants used in open-net pen Atlantic Salmon aquaculture on juvenile Starry Flounder (*Platichthys stellatus*). *P. stellatus* were exposed to sublethal concentrations of emamectin benzoate (EMB) from SLICE ® (0.2% Premix), ivermectin (IVM), and a combination of both in marine sediments at, or near, concentrations observed in the marine environment. Two experiments were conducted to assess the effects of avermectins over several levels of biological organization including biochemical, physiological, and behavioural. First, an evaluation of the effects of IVM, EMB and a combination of both on growth and morphometrics, physiological and behavioural endpoints including aerobic scope (AS), burst swimming performance (U_{burst}), burying and skin pigmentation; as well as biochemical endpoints including tissue glucose, lactate and cortisol. Second, an assessment focused on the sublethal effects of avermectin chemotherapeutants on behavioural endpoints with important implications for contaminant exposure and survival in the wild. These endpoints included contaminant avoidance, burying behaviour and camouflage using the metrics of the difference between *P. stellatus* body pattern ($Pattern_{diff}$) and luminance (perceived lightness, $Luminance_{diff}$) and three substrate backgrounds.

Significant concentration-dependent reductions in aerobic scope and U_{burst} were found for *P. stellatus* exposed to IVM and a combination of both EMB and IVM, but not those exposed to only EMB. Following a 30-d exposure a significant reduction in burying and increase in individuals with darkened skin were observed for the IVM and combination exposures. The behavioural and physiological endpoints were shown to be more sensitive sublethal endpoints than growth, morphometrics or biochemical endpoints. Prior exposure increased the avoidance behaviours to these chemicals as well but did not affect burying behaviour which was reduced even without past exposure. Significant reductions in *P. stellatus* camouflage ability, indicated by both reductions in pattern and light matching were seen, which was different depending on the substrate fish were matching. Across experiments, some of most impacted endpoints (camouflage, U_{burst} and aerobic scope) occurred with the combination exposure, suggesting a potential additive effect of EMB and IVM. Overall, these experiments demonstrated adverse sublethal effects to a marine

flatfish species at environmentally relevant concentrations of current-use anti-sea lice chemotherapeutants.

4.1. Future Research

Although the findings of the current thesis provided novel information of the effects of avermectins on a benthic teleost species using an environmentally realistic exposure scenario, information gaps remain on effects of these chemotherapeutants to non-target marine teleosts. The effects of avermectins on similar endpoints used in the current study should be assessed for other benthic or semi-benthic teleost species occupying different trophic levels such as Pacific Sand Lance (*Ammodytes hexapterus*) as a lower trophic organism and cods (order Gadiformes) as a higher trophic organism. The effects should be assessed for a range of life stages as well, including reproductive and developmental toxicity. *P. stellatus* could be wild caught (e.g., angling, seining or trawling) or sourced from a hatchery when gravid and fertilization could be completed in a laboratory setting. The larval and pelagic life stage of *P. stellatus* could also be assessed as they may prove more sensitive. The assessments of multiple taxa and life stages within those taxa will allow a more accurate evaluation of population- and even ecosystem-level impacts of avermectin exposure to non-target wild teleosts. A combination of *in-situ* exposures below net pen operations and laboratory exposures for early-life stages of *P. stellatus* with measured sediment concentrations for each would better inform what the effects might be in a wild setting. Future studies should seek to optimize culture conditions to ensure and facilitate *P. stellatus* growth and health. Although the exposure conditions were more environmentally realistic than previous studies with avermectins, additional changes could be made that make the exposure and effects even more relevant. The duration of exposures could be expanded to match the half-lives of IVM and EMB in marine sediments (>188 d) and fish could be exposed to treated feed as well as sediment. Future studies could include effects assessments at additional levels of biological organization such as the use of *omics* (e.g., genomics, proteomics and metabolomics), expanded conventional biochemical assays including blood and tissues, and the effects on olfaction and the heart using an echo-olfactogram and echocardiogram, respectively, to provide a better understanding of the underlying mechanisms behind the observed effects on behaviour and physiology. If swim performance and aerobic scope assays were repeated, pre-and post- exercise biochemical parameters would help inform any effects on energy

transduction. Repeated swimming and aerobic assays, allowing for recovery to evaluate the effects on fish recovery should be included as well. For the avoidance assay, a larger assay chamber and extended assay duration would be valuable in confirming whether or not avoidance occurs. For assessing effects on camouflage, other parameters such as edge disruption and colour matching would prove useful. As well, using hyperspectral imagery, an evaluation of camouflage in the context of the visual spectra of *P. stellatus* predators and prey to better assess potential effects on survival would be novel.

The body of evidence on the effects of avermectin chemotherapeutants used in open-net pen Atlantic Salmon aquaculture, on non-target wild marine fauna is increasing. With additional information of the effects of these chemotherapeutants, regulatory bodies can be better informed to provide acceptable environmental concentrations that are both protective of non-target fauna and allow for therapeutic administration to protect the health and economic viability of *S. salar* in net pen aquaculture. Given the persistence of EMB and IVM and potential additive effects of the two chemotherapeutants together found in the current study, the use of both should be avoided if possible. EMB has been shown to be less toxic than IVM in most taxa tested, including *P. stellatus* in the current study, so preference is for the use of SLICE® over IVM, if one is used. Alternatives to the use of chemotherapeutants for sea lice control has been successfully tested and implemented and should be considered for first-line treatments for sea lice infestations before the use of chemotherapeutants to minimize the effects to non-target organisms. The strategic placement of a culture of filter feeders within a net pen to filter the pelagic microscopic early-life stage of sea lice (Trigo and Mondéjar, 2020), as well as lumpfish used as a cleaner fish for mechanical removal of the sea lice (Imsland et al., 2018; McEwan et al., 2018) were both shown to be effective. Mechanical and thermal delousing techniques have also been used (Burridge et al., 2010).

4.2. References

Burridge, L., Weis, J.S., Cabello, F., Pizarro, J., Bostick, K., 2010. Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. Aquaculture 306, 7–23. https://doi.org/10.1016/j.aquaculture.2010.05.020

Imsland, A.K.D., Hanssen, A., Nytrø, A.V., Reynolds, P., Jonassen, T.M., Hangstad, T.A., Elvegård, T.A., Urskog, T.C., Mikalsen, B., 2018. It works! Lumpfish can significantly lower sea lice infestation in large-scale salmon farming. Biol. Open 7. https://doi.org/10.1242/bio.036301

McEwan, G.F., Groner, M., Cohen, A.A.B., Imsland, A.K.D., Revie, C., 2018. Modelling sea lice control by lumpfish on Atlantic salmon farms: Interactions with mate limitation, temperature and treatment rules. Dis. Aquat. Organ. 133. https://doi.org/10.3354/dao03329

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