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Bioaccumulation of selected veterinary medicinal products (VMPs) in the blue mussel (*Mytilus edulis*).

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Abstract

Veterinary medicinal products (VMPs) are widely used within the fish farming industry to control sea lice infestations. There is concern that wild and farmed mussels in the vicinity to these fish farms may be exposed and subsequently bioaccumulate these chemicals, which could pose a threat to human health. To understand the fate of these chemicals in the environment, controlled laboratory exposures were performed to establish the uptake and depuration of selected VMPs in the blue mussel (*Mytilus edulis*). The VMPs included teflubenzuron, emamectin benzoate and deltamethrin. The effects of salinity on the bioaccumulation of teflubenzuron were also investigated to see whether mussels in brackish waters exhibit different bioaccumulation dynamics. Salinity had no significant effect on the uptake or depuration curves for teflubenzuron down to 15‰. The uptake rate constants (k_1) for teflubenzuron, emamectin benzoate and deltamethrin in mussels were 192, 4.82 and 2003, with kinetic bioconcentration factors (BCFs) of 1304, 49 and 2516. Depuration rate constants (k_2) were also found to differ between the three VMPs at 0.147, 0.048 and 0.796 for teflubenzuron, emamectin benzoate and deltamethrin, with calculated elimination half-lives ($t_{1/2}$)of 4.7, 14 and 0.87 days. The longer elimination half-lives for teflubenzuron and emamectin benzoate, suggest that these chemicals accumulate in blue mussels and therefore have the potential to bioaccumulate in wild and farmed mussel populations in the environment.

Keywords:

Teflubenzuron, deltamethrin, emamectin benzoate, bioconcentration factor, elimination rates

1. Introduction

The farming of Atlantic salmon (*Salmo salar*) has over recent years expanded to fulfil the demand for fish consumption, and in Norway alone over one million tonnes of fish are produced annually from over 500 active farms (Fiskeridirektoratet). One of the main challenges to the salmon farming industry is the control of ectoparasites such as *Lepeophtheirus salmonis*. These parasites attach to the external surface of fish and feed off the mucus, skin and blood resulting in potentially severe fish health effects. Historically, the use of veterinary medicinal products (VMPs) have been by far the most common method for the control of salmon lice in salmonids. Data published from the Norwegian Institute for Public Health shows an increase in VMPs usage since 2008, with more than 10 tonnes of active ingredient (A.I.) employed in 2016 (Norwegian Institute for Public Health, 2016). In 2017, due to stricter regulations on the use of VMPs in Norwegian fish farms, and the development of non-chemical methods for salmon lice removal, there was a significant reduction in the quantities of VMPs used. However, despite this reduction, the sum of VMPs (as A.I.) used still amounted to approximately two tonnes annually. VMPs are a major environmental concern, particularly since some VMPs, such as chitin synthesis inhibitors, have a very high acute to chronic toxicity ratio in aquatic crustaceans, indicating that they are highly biologically active molecules that can have adverse effects on non-target organisms at very low concentrations (Macken et al., 2015; Lillicrap et al., 2015; Samuelsen et al., 2014).

The VMPs are administered to the fish either via the food, or in bath treatments. The chitin inhibitors diflubenzuron and teflubenzuron, and the chlorine channel GABA gate receptor antagonist emamectin benzoate are all dosed via the feed. Whereas other VMPs, such as hydrogen peroxide, the acetylcholine esterase inhibitor azamethiphos, and the synthetic pyrethroid insecticides cypermethrin and deltamethrin, are applied via bath treatments.

Concerns over the reduced effectiveness of VMPs, has led to a gradual increase in the use patterns of VMPs to enable the same therapeutic response. This has led researchers to implicate chemical resistance of the parasite to hydrogen peroxide, azamethiphos, emamectin benzoate, deltamethrin and cypermethrin (Treasurer, 2000; Lees et al., 2008; Kaur et al., 2015; Carmona-Antoñanzas et al., 2017; Helgesen, 2014; Sevatdal, 2005). Chemical resistance has forced the pharmaceutical industry to develop alternative chemical products and combinations of products. Mechanical (external scrubbing of the fish) and biological (cleaner fish) treatments have also started to be increasingly used to reduce the need for chemical treatment.

There is concern that VMPs may pose a threat to the health of non-target species (Burridge et al., 2010; Haya et al., 2005; Macken et al., 2015; Lillicrap et al., 2015; Lillicrap, 2018). For example, chitin inhibitors such as teflubenzuron and diflubenzuron are expected to be particularly toxic to crustaceans that undergo moulting. This includes commercially important crustaceans such as lobster, crab, and shrimp (Samuelsen et al., 2014), although the full extent of the impact on these commercial species in the environment is not known. Exposure of copepod eggs (*Acartia tonsa*) to 1 µg/L diflubenzuron reduced hatching success, caused structural abnormalities in those that did hatch, and resulted in moulting failure at the next stage of development (Tester and Costlow, 1981). However, the relative toxicity of VMPs to other marine taxa such as molluscs, fish, echinoderms compared to crustaceans is low. Conversely, low toxicity of VMPs to mussels may lead to high chemical body burden concentrations and increased biomagnification in vertebrates including humans.

Maximum Residue Levels (MRLs) have been established for the different VMPs in fish tissue to limit human exposure. However, the density of the Norwegian aquaculture farms, and the proximity of fish farms to mussel farms, has raised the question whether the VMPs used could bioaccumulate in neighbouring mussel populations. Particularly since mussels are well-known biomonitoring species that filter large volumes of seawater and accumulate a wide range of chemicals within their tissues (Beyer et

al., 2017). Farmed mussels that are grown for human consumption and have been exposed to anthropogenic substances may pose a concern for public health.

The need to establish the uptake and depuration of these VMPs in mussels is therefore necessary to understand the bioaccumulative potential and the threat imposed to human health or marine organisms that consume mussels. The aim of the present study was to determine the uptake and depuration of three selected VMPs used in the salmonid fish farming industry. These VMPs include: teflubenzuron, emamectin benzoate and deltamethrin, with log K_{ow} partition coefficients of 5.4, 5.0 and 6.2 (Marsella et al., 2000; MacBean, 2010; Hansch et al., 1995,). Based on these partition coefficients, all three chemicals would be expected to bioaccumulate in aquatic organisms. However, the uptake and depuration rates of these three chemicals in blue mussels were not available from the scientific literature.

An additional aspect of the study was to determine if different salinities affect the uptake and depuration of VMPs in blue mussels. Blue mussels inhabit both coastal regions and estuaries with freshwater inputs that reduce the salinity. The higher biological energy demands through active regulation of the ionic blood composition of mussels may influence uptake and depuration and was considered an important aspect of the study.

Finally, measured concentrations of VMPs in farmed mussels from known locations, in the vicinity to coastal fish farming industries, were also investigated to determine if measurable concentrations of selected VMPs can be detected in farmed mussels which may pass on through the food chain.

2. Materials and methods

The blue mussels used in the experiment were obtained from the lower inter-tidal region of the outer Oslo fjord near the NIVA marine research station in Solbergstrand, Drøbak, Norway. This region of coastline has no fish farming activities and therefore were considered suitable for the experiment. The species of *Mytilus* was not determined in this study, although previous studies have identified this population to consist entirely of *M. edulis* (Brooks and Farmen, 2013). All mussels were salinity acclimated for at least two weeks prior to starting the bioaccumulation experiments.

2.1. Laboratory exposure

A flow-through seawater exposure system was used to deliver stable concentrations of the test compounds over a 14-day uptake phase. Due to the low solubility of teflubenzuron and deltamethrin a saturation column was used (see Figure 1), whilst emamectin benzoate was dosed directly into a mixing vessel using a concentrated stock solution.

2.1.1. Preparation of the saturation column

Saturation columns were prepared for teflubenzuron and deltamethrin only. The amount of test chemical required to provide stable concentrations during a 14-day uptake phase was calculated using the following equation (EQ. 1).

$$Amount\ of\ test\ chemical\ (mg) = Solubility\ (mg/L) * flow\ rate\ (L/h) * dosing\ duration\ (h)$$ – EQ. 1.

The solubility of teflubenzuron and deltamethrin were taken from the literature to be 9.4 µg/L (0.0094 mg/L, Marsella et al., 2000) and 20 ng/L (0.00002 mg/L, Fairchild et al., 2010) respectively. The flow rate through the saturation column was established at 1.2 L/ h and the dosing duration, including a 2-day stabilisation phase, was set at 16 days (384 h). The amount of test chemical calculated was multiplied by a factor of 25, and the total amount of test chemical dissolved in 130 mL of acetone. The

chemically spiked acetone was poured over 600 mL of washed pumice and stirred thoroughly for five minutes until it had fully absorbed. The treated pumice was spread out on a tray in a fume cupboard for a minimum of 48 h to allow the acetone to evaporate.

A 2 L glass saturation column was packed firstly with 4 mm diameter glass beads to a height of approximately 4 cm (Figure 1). Approximately 500 mL of tap water was added to prevent the formation of air pockets, before 600 mL of the treated pumice was added. Clean pumice of approximately 4 cm thick was added above the treated pumice and finally 4 cm of glass beads (4 mm) were added on top to keep the pumice in place. The central glass tube was inserted carefully into the bottle so that the end of the tube was below the line of the treated pumice. Four saturation columns were constructed in total, three for teflubenzuron for the different salinities (35, 25, 15‰), and one for deltamethrin.

2.1.2. Dosing system

The saturation column was used within the dosing system as shown in figure 1. Temperature (8 ± 1°C) and salinity adjusted (35, 25 and 15 ‰) seawater was forced through the saturation column using a piston pump at a flow rate of 1.2 mL/ min. The design of the saturation column ensured that the seawater flowed through the entire volume of the treated pumice. The dissolved concentration of the selected VMP in the seawater, as it left the saturation column, was equal to the solubility of the selected VMP in seawater. This was diluted with clean seawater within a mixing chamber before flowing equally into three separate 10 L tanks. Only glass was used for the tubing and tanks that came into direct contact with the VMPs. The dosing system was run for a period of 48 h before the addition of the mussels. A total of 30 mussels were placed in each treatment tank, which initiated the start of the uptake phase of the experiment. Mussels were fed every other day with Shellfish diet® (Reed Mariculture Inc.), a concentrated liquid feed containing a mixture of 6 marine algae. The exposure

146 treatments included teflubenzuron dosed at a nominal concentration of 1 µg/L at three different
147 salinities of 15, 25 and 35‰. Deltamethrin uptake and depuration was performed in full seawater (34 ±
148 1 ‰) using the saturation column to obtain nominal concentration of 20 ng/L. Due to the higher
149 solubility of emamectin benzoate a stock concentration of 1 mg/L was used for dosing to achieve a
150 nominal exposure concentration of 1 µg/L.

151

152 2.1.3. Water and biota sampling

153 Water samples were collected in 7 mL glass containers at specific time points during the 14-day uptake
154 phase on days 0, 1, 3, 7, 10 and 14. The glass containers were sealed and stored at 4°C in the dark until
155 analysed for concentrations of the VMP. One water sample was taken from each of the three mussel
156 exposure tanks so that three water samples were measured at each time point.

157

158 Mussel samples (three pooled whole mussel homogenates) were collected on the same days as the
159 water samples during the uptake phase. Mussels were sampled by removing three random individuals
160 from each of the three exposure tanks (9 mussels sampled per time point). The external surface of the
161 mussels were rinsed in clean seawater and opened by cutting the posterior adductor muscle with a
162 sterile scalpel. The mussels were left to drain the excess internal fluid for a few minutes before the
163 whole soft tissue was removed and placed in separate heat-treated glass jars. The mussel samples were
164 frozen at -20°C before analysed for the selected VMP.

165 After the final water and mussel samples were taken at the end of the uptake phase (day 14), the
166 remaining mussels were removed from the exposure tank, rinsed well in separate acclimation seawater
167 (i.e. 35, 25 or 15‰) and placed into new clean 10 L tanks of flowing (~2 L/ min) acclimation seawater for

the start of the depuration phase. During the depuration phase, mussels were sampled at specific time points up to a maximum of 21 days to establish chemical depuration curves. Samples for the depuration phase were taken on days 15, 16 and 21 for emamectin benzoate and the salinity effects on teflubenzuron experiments; and on days 15, 18, 21, 28 and 35 for deltamethrin and the extended teflubenzuron experiments.

2.2. Collection of farmed mussels for presence of VMPs.

Commercial farmed mussels were purchased from outlets in Oslo for the chemical assessment of VMPs. Information regarding location and date of harvesting, was provided by the mussel processing plant in Rissa, Norway and is shown in figure 2. In all cases, mussels were dissected within 5 days of harvesting (removed from the water). Whole mussel homogenates from five pooled samples were analysed for the following VMPs, teflubenzuron, diflubenzuron and emamectin benzoate.

2.3. Chemical analysis in water and mussel samples

2.3.1. Reagents and chemicals

Standards of teflubenzuron (CasNo: 83121-18-0), diflubenzuron (CasNo: 35367-38-5), emamectin benzoate (CasNo: 155569-91-8), deltamethrin (CasNo: 52918-63-5) as well as HPLC grade, acetonitrile, formic acid, ammonium acetate, sodium sulphate, sodium acetate, Supelclean PSA sorbent and florisil (SPE-FL) column were purchased from Sigma-Aldrich (Steinheim Germany).

HPLC grade diethylether, cyclohexane, dichlomethane and acetone were obtained from Rathburn Chemicals (Walkerburn Scotland). The d6-cyfluthrin was obtained from LGC Standards (Wesel, Germany) and Costar nylon Spin-X filters from Corning (Salt Lake City USA).

Standard stock solutions were prepared in acetone and diluted further to appropriate concentrations with acetonitrile or cyclohexane. All standard solutions were kept in the dark at -20°C.

2.3.2. Teflubenzuron, Emamectin benzoate

A 1 g sample of pooled homogenised mussel tissue was extracted twice with 5 mL acetonitrile (ACN). After centrifugation the extracts were combined. The water was salted out by adding 1 g of NaCl and the final ACN extract was diluted to 10.0 mL of ACN prior to analysis.

For the seawater samples, a 2 mL sample in a 7 mL glass vial was shaken with 3 mL ACN and 1 g NaCl added to salt out the water. The ACN extract was injected into the LC-MS as described.

Teflubenzuron, diflubenzuron and emamectin benzoate were analysed on a Waters Acquity UPLC system connected to a Quattro Ultima triple quadrupole mass spectrometer. Separation was achieved with a Waters BEH C8 column (2.1 x 100 mm) using a gradient elution with ACN and water (with 5.2 mM ammonium acetate). Teflubenzuron and diflubenzuron were detected in negative ESI mode with mass transitions of 379-339 and 379-359 for teflubenzuron and 309-156 and 309-289 for diflubenzuron. Emamectin benzoate was detected in positive ESI mode with mass transitions 886.5-158 and 886.5-302. The identification and quantification were performed using external standards. The average recovery of the three spiked seawater samples was 87%, 83%, and 95% with RSD of 3.5%, 3.2%, and 2.7% for teflubenzuron, diflubenzuron, and emamectin benzoate, while the average recovery of the three spiked mussel samples was 92%, 95%, and 98% with RSD of 2.1%, 1.8% and 3.1%. Limit of detection was 1.0 ng/g (w.w.) for teflubenzuron, 3.0 ng/g (w.w.) for diflubenzuron, and 0.05 ng/g (w.w.) for emamectin benzoate.

2.3.2. Deltamethrin

212 Internal standard, d6-cyfluthrin, was added to 150 to 200 mL seawater samples and extracted with 30
213 mL of dichloromethane, for one hour under magnetic stirring. Sodium sulphate was added to the
214 extracts to remove water and then concentrated using nitrogen flush and transferred to 0.5 mL
215 cyclohexane prior to the gas chromatography – electron capture detector (GC-ECD). Three blank
216 samples and two spiked samples were analysed alongside the seawater samples as part of the quality
217 assurance.

218 Internal standard, d6-cyfluthrin, was added to 2.5 g of pooled homogenised mussel tissue and extracted
219 with 3 mL acetonitrile acidified with 1% acetic acid for one hour in an ultrasonic bath. Sodium sulphate
220 (1.5 g) and sodium acetate (0.25 g) were added to the extracts and shaken vigorously before
221 centrifugation. A 2 mL volume of the extract was evaporated to near dryness and resolved in 0.5 mL of
222 cyclohexane. To remove interferences, the extracts were cleaned using solid phase extraction – florisil
223 (SPE-FL) column eluted with 20% diethylether in cyclohexane followed by PSA. The extracts were then
224 filtered through 0.2 µm nylon filters prior to the analysis. Three blank samples and three spiked samples
225 were analysed alongside the mussel samples as a part of the quality assurance

226 The analysis was performed using an Agilent 6890N GC-ECD and equipped with 30 m DB-5 column, i.d.
227 0.25 mm and 0.25 µm film thickness. The identification and quantification was performed using external
228 and internal standards. The recovery of the two spiked seawater samples was 110% and 112% The
229 average recovery of the three spiked mussel samples was 109%, Rel.stdev.=2%. The limit of detection
230 was 0.5 ng/g (w.w) for the mussel samples and 4 ng/l for the water samples.

231

232 2.4. Statistical analysis

233 Statistical differences between the groups of data were assessed using analysis of variance (ANOVA). A
234 Levene's test was used to check homogeneity of variance and a one -way ANOVA with Tukey post-hoc

test applied. If homogeneity was not achieved a Kruskal-Wallis test was applied. The level of significance was set at $p<0.05$.

3. Results

3.1. Bioaccumulation and depuration of teflubenzuron, and the effects of different salinities

The uptake and depuration curves for teflubenzuron in salinity acclimated mussels are shown in figure 3. The uptake curves for teflubenzuron for the different salinity acclimated mussels were very similar to each other with an apparent steady state occurring after 10 days. Statistical comparison between the teflubenzuron concentrations measured in mussels for the different salinities at the time points 3, 7 and 14 days revealed no significant difference. A significant difference was found on day 10 between mussels acclimated to 15‰ and mussels acclimated to 25‰ (ANOVA, Tukey $p<0.05$). Furthermore, on day 1, mussels acclimated to 35‰ had undetected concentrations of teflubenzuron and were therefore significantly different from 15‰ and 25‰ acclimated mussels. However, overall there was no noticeable difference between the uptake curves with respect to salinity acclimation.

The depuration curves of teflubenzuron for the different salinity acclimated mussels were almost identical. Statistical comparisons of the teflubenzuron concentrations between the salinity acclimated mussels at the different time points (day 15, 16 and 21) showed no significant difference ($p<0.05$). After 7 days of depuration, the teflubenzuron concentrations were between 265-and 376 ng/g (w.w.).

Teflubenzuron bioaccumulation in seawater acclimated mussels was repeated to include an extended depuration period (Figure 4). The water concentrations of teflubenzuron were measured and a time weighted average of 1.35 ± 0.34 µg/L was calculated based on triplicate water samples measured on day 0, 1, 4, 7, 11 and 14 of the uptake phase. This value of 1.35 ± 0.34 µg/L, compares well with an expected

nominal concentration of 1 µg/L and indicates that the saturation column was successful in delivering a stable concentration of teflubenzuron for the 14-day exposure period.

The depuration rate constant (k_2) for teflubenzuron was calculated by plotting the natural log of the chemical concentration over time (days) (OECD 2012, Figure 4). A depuration rate constant of 0.147 was calculated from the decrease in teflubenzuron in mussels after 1, 4, 7, 14 and 21 days of depuration. The time required to reduce the tissue concentration by half ($t_{1/2}$) was 4.7 days calculated using the equation $t_{1/2} = 0.693/k_2$ (OECD 305). The k_2 value could also be calculated for the salinity acclimated mussels of 15, 25 and 35‰ and were 0.160, 0.138 and 0.198 with $t_{1/2}$ durations of 3.5, 5.0 and 4.3 days respectively (Table 1). This shows good agreement between the teflubenzuron depuration data for the different salinity exposure experiments.

The uptake rate constant (k_1) was calculated as described in the OECD 305 test guideline (OECD, 2012) using the following equation (EQ 2.)

$$k_1 = \frac{C_m.k_2}{C_w\left(1-e^{-k_2 t}\right)} \qquad \text{EQ.2}$$

Where C_m and C_w are the chemical concentrations in the mussels and water at a given time (t), and k_2 is the depuration rate constant (OECD, 2012). Since the water concentration of the salinity acclimated mussels was not measured, the k_1 was calculated with the nominal concentration of the exposure water (1 µg/L teflubenzuron). Based on the nominal water concentrations and measured concentrations in the mussels acclimated to 15, 25 and 35‰, the k_1 values were calculated to be 258, 181 and 312, respectively (Table 1). The steady state and kinetic BCFs calculated by C_m/C_w and k_1/k_2 were comparable for the different salinity acclimated mussels ranging between 1121 and 1610 (Table 1).

For the teflubenzuron exposure with the 21-day (extended) depuration phase, the uptake rate constant (k_1) was calculated based on a measured time weighted average water concentration of 1.35 ± 0.34

279 µg/L. The calculated k_1 for teflubenzuron was 192 (Table 1). The steady state and kinetic BCFs for
280 teflubenzuron calculated by C_m/C_w and k_1/k_2 were 1137 and 1304 respectively.

281

282 3.2. Bioaccumulation and depuration of emamectin benzoate

283 The uptake and depuration curves for emamectin benzoate, in full seawater acclimated mussels, are
284 shown in figure 5. An apparent steady state was achieved after 7 days with mean emamectin benzoate
285 concentrations at days 7, 10 and 14 around 45 ng/g (w.w.). A 7-day depuration phase did not result in a
286 significant reduction of emamectin benzoate concentration below the steady state value.

287 The depuration rate constant (k_2) for emamectin benzoate was calculated by plotting the natural log of
288 the chemical concentration over time (days) (OECD, 2012). A k_2 value of 0.048 was calculated from the
289 decrease in emamectin benzoate in mussels after 1, 2, and 7 days of depuration (Table 1). The time
290 required to reduce the tissue concentration by half ($t_{1/2}$) was estimated as 14 days calculated using the
291 equation $t_{1/2} = 0.693/k_2$ (OECD, 2012).

292 The uptake rate constant (k_1) for emamectin benzoate in mussels was calculated as 4.82 based on the
293 measured concentrations in mussel tissue and a nominal water concentration of 1 µg/L (Table 1). The
294 steady state and kinetic BCFs calculated by C_m/C_w and k_1/k_2 were 49 and 100, respectively (Table 1).

295

296 3.3. Bioaccumulation and depuration of deltamethrin

297 The uptake and depuration curves for deltamethrin, in full seawater acclimated mussels, are shown in
298 figure 6. An increase in deltamethrin concentration in mussel tissue was measured after 1-day exposure,
299 which remained relatively constant after 4, 7 and 11 days exposure until a further increase on day 14.
300 However, there were no significant differences between the deltamethrin concentration measured after

1 day with that measured on day 14. A rapid depletion of deltamethrin was observed after only 1 day of depuration, and was below the limit of detection after 7 days.

The depuration rate constant (k_2) for deltamethrin was calculated by plotting the natural log of the chemical concentration over time (days) (OECD, 2012, Figure 6). A k_2 value of 0.796 was calculated from the decrease in deltamethrin in mussel tissue after 1, 2, and 7 days of depuration (Table 1). The time required to reduce the tissue concentration by half ($t_{1/2}$) was estimated as 0.87 days calculated using the equation $t_{1/2} = 0.693/k_2$ (OECD, 2012).

The water concentrations of deltamethrin were measured and a time weighted average of 47.1 ± 4.4 ng/L (± SD) was calculated based on triplicate water samples measured on day 0, 1, 4, 7, 11 and 14 of the uptake phase. The saturation column was shown to produce a stable concentration of deltamethrin for the 14-day exposure period.

Based on the measured concentrations in mussel tissue and time weighted average water concentrations of 47.1 ng/L, the k_1 value for deltamethrin in mussels was calculated as 2003 (Table 1). The steady state and kinetic BCFs calculated by C_m/C_w and k_1/k_2 for deltamethrin were 2523 and 2516 respectively (Table 1).

3.4. Concentrations of VMPs in commercial mussels

Homogenated samples of pooled mussels were analysed for teflubenzuron, diflubenzuron and emamectin benzoate from five mussel farms located along the Norwegian coast, north of Trondheim (Figure 2). These VMPs were not detected above the limit of detection of 1 ng/g (w.w.) teflubenzuron and 0.05 ng/g (w.w.) emamectin benzoate.

4. Discussion

The dosing of the hydrophobic substances teflubenzuron and deltamethrin using the saturation column proved successful, with stable concentrations of these VMPs achieved over the duration of the 14-day uptake phase. Time weighted mean concentrations of 1.35 ± 0.34 µg/L and 42.6 ± 4.44 ng/L for teflubenzuron and deltamethrin in the test solutions were calculated. These values were close to the nominal concentrations despite the nominal concentration being based on solubility limits in freshwater taken from the scientific literature (Fairchild et al., 2010; Marsella et al., 2000; EPA, 1999). The saturation column is recommended as a stable delivery system for poorly water soluble compounds in ecotoxicity testing.

4.1. Salinity effects on teflubenzuron uptake and depuration.

Based on the uptake and depuration curves for teflubenzuron with respect to salinity acclimated mussels, it appears that salinity had no significant impact on bioaccumulation. It should be noted however, that the solubility and stability of teflubenzuron in the aqueous phase was assumed to be equal between salinities of 15 and 35 ‰. The finding suggests that mussels occupying low salinity waters such as estuaries or fjords with freshwater inputs will bioaccumulate teflubenzuron at the same rate as those in full strength seawater. Mussels are frequently used in national monitoring programmes to infer the environmental status of a waterbody or habitat based on the chemical concentrations in their tissues (Davies and Vethaak, 2012). Therefore, confounding factors that influence chemical bioaccumulation may impact the environmental assessment. It is reassuring therefore to know that salinity acclimation does not affect rates of teflubenzuron bioaccumulation down to a salinity of 15‰. To the authors knowledge this is the first study that has investigated the effects of salinity acclimation on the uptake and depuration of teflubenzuron, or other VMPs, in mussels. It may be reasonable to

assume that other benzoyl urea compounds such as diflubenzuron etc., which are also used as VMPs, would act in a similar way to teflubenzuron and be unaffected by reduced salinity.

4.2. Uptake and depuration curves for teflubenzuron, emamectin benzoate and deltamethrin

The similarity between the steady state BCF, calculated as the chemical concentration in the mussel (C_m) divided by the concentration in the water (C_w), and the kinetic BCF, calculated as the uptake rate constant (k_1) over the depuration rate constant (k_2), suggests that a steady state was achieved for all three chemicals within the 14-day exposure.

The uptake and depuration curves for the three VMPs were found to be very different from each other. Deltamethrin was found to be the most bioaccumulative of the three VMPs with a calculated steady state and kinetic BCF of 2516 and 2523, respectively. Deltamethrin has an octanol-water partition coefficient (Log K_{ow}) of 6.2 and was thus expected to bioaccumulate in mussels.

Prediction of BCFs for deltamethrin based on Log K_{ow} partition coefficients have been found to overestimate the value obtained in fish bioaccumulation studies. This is thought to be due to both metabolism of deltamethrin by the fish, their low water solubility, and the reduced bioavailability of deltamethrin bound to dissolved organic carbon (DOC) and suspended colloids (Arnot et al., 2009, Arnot and Gobas, 2006; Day, 1991). DOC concentrations as low as 2.6 mg/L were found to significantly reduce the bioavailability of deltamethrin to *Daphnia magna* (Day, 1991). The DOC concentration of the test water in the current study was less than 1 mg/L and the effects of DOC on reducing the bioavailability of deltamethrin were considered to be negligible.

Measured fish BCFs for deltamethrin have been reported as 144 in the whole body of the channel catfish (*Ictalurus punctatus*) (Cary, 1978). Based on total ^{14}C-labeled residues, the BCF for deltamethrin in whole fish was found to be between 1400 and 698 (Dietz et al., 2009), whereas other fish BCFs for deltamethrin range from 360 to 6000 (Laskowski, 2002). The large range in fish BCF values for deltamethrin highlight the often large uncertainties in BCF studies and the need for a quality tiered assessment strategy (Lillicrap et al., 2016). Incidentally, the BCF values calculated for the three VMPs in this study were the first to be reported in mussels, and caution should therefore be taken until further validation of these values can be obtained in future studies.

The actual concentration of deltamethrin in the mussel tissue was approximately 10 fold lower than that measured for teflubenzuron, with maximum tissue concentrations of 119 µg/kg w.w. after 14 days. The low solubility of deltamethrin resulted in mussels exposed to a time weighted mean concentration 42.6 ± 4.44 ng/L, which was approximately 200 fold lower than teflubenzuron and emamectin benzoate exposure conditions. This resulted in calculated steady state BCF of 2523, the highest of the three compounds.

The rapid metabolism and elimination of pyrethroids, such as deltamethrin, by the fish have been indicated as a reason why pyrethroid concentrations in fish tissues are at low levels (Dietz et al., 2009). The rapid elimination of deltamethrin in whole soft tissue of mussels in the present study could also be attributed to the rapid metabolism of this VMP. Fish are thought to be deficient in the enzyme system that hydrolyses pyrethroids and the metabolism of deltamethrin is mostly oxidative (Demoute, 1989). This is also thought to be the main route of metabolism of deltamethrin in mussels (Katagi, 2011). Whatever the mechanism in mussels, deltamethrin is rapidly eliminated from their tissues and would be unlikely to be found in wild and/or farmed mussels that may have been exposed to a similar treatment regime in the environment.

The steady state and kinetic BCFs for teflubenzuron in mussels were 1137 and 1304, respectively. Maximum tissue concentrations of teflubenzuron were 1535 ng/g (w.w.) after 14 days exposure to a time weighted mean concentration of 1.35 ± 0.34 µg/L. To the authors knowledge, this is the first published data on the uptake and depuration curves for teflubenzuron in mussels. Data are available on the uptake and depuration curves for diflubenzuron in mussels, where a maximum concentration of approximately 1000 ng/g (w.w.) was measured after 14-day exposure, although the exposure was via the feed (0.5 g/kg w.w.at 1.2% body weight) rather than through the water (Norambuena et al., 2016).

Uptake and elimination rates of teflubenzuron in other marine species are seldom reported in exposure studies. Many studies have focussed on teflubenzuron toxicity to marine species and in particular crustaceans that are extremely sensitive to the chitin inhibiting compound. In the rockpool shrimp (*Palaemon elegans*), teflubenzuron was found to bioaccumulate, where concentrations up to 33 ng/g (w.w.) were detected when exposed to environmentally relevant concentrations over 98 days (Olsvik et al., 2017). The detection of teflubenzuron in the tissues of many other marine species have been reported, including intertidal species such as the amphipod *Gammarus locusta* and the blue mussel (*Mytilus edulis*) (Langford et al., 2014). However, controlled laboratory exposures to determine the uptake and elimination dynamics of teflubenzuron in marine species are limited in the scientific literature.

The elimination rate of teflubenzuron in mussels from our study revealed a half-life of 1.2 days, which indicates a relatively rapid depletion from the mussel. A 67% elimination rate of 12 hours was reported for teflubenzuron in the insect *Spodoptera exigua* (Van Laecke and Degheele, 1991). It appears that despite the high Log K_{ow}, teflubenzuron does not bioaccumulate in insects due to the rapid elimination rates (Coppen and Jepson, 1996a, 1996b), a similar situation seems to occur in the mussel. The fate of teflubenzuron in the marine environment is largely dependent on the organic carbon and particulate load of the water, which it remains bound to (Langford et al., 2014). High organic carbon and

414 particulates in the water column during treatment, such as during an algal bloom, would result in
415 teflubenzuron attaching to these particulates. Under this scenario, mussels in close proximity to fish
416 farms may become exposed to elevated concentrations of teflubenzuron during feeding, potentially
417 increasing bioaccumulation rates in mussel tissues. However, the fast elimination rates of teflubenzuron
418 in mussels would suggest concentrations would reduce rapidly within a few days.

419 The steady state and kinetic BCFs for emamectin benzoate in mussels were 49 and 100. Maximum tissue
420 concentrations of emamectin benzoate were only 49 ng/g (w.w.), after 14 d exposure to nominal
421 concentration of 1 µg/L. To the authors knowledge, this is the first published data on the uptake and
422 depuration curves for emamectin benzoate in mussels. Despite the relatively low bioaccumulation of
423 emamectin benzoate the fact that the depletion rate is slow, with an estimated half-life of 14 days,
424 means that what is bioaccumulated in the mussel will remain there for some time. The 14-day half-life
425 was only based on a depuration phase of 7 days with no significant reduction in concentration over this
426 time. An extended depuration phase would be recommended in future studies to improve the
427 depuration constant and the half-life calculation of emamectin benzoate in mussel tissue.

428 In fish, a rapid uptake of emamectin benzoate was reported in bluegill sunfish (Chukwudebe et al. 1996).
429 Residue levels after 28-days exposure to approximately 1 µg/L emamectin benzoate were 128, 90 and
430 40 ng/g (w.w.) for viscera, whole fish, and fillet, respectively. Depuration rates were rapid with residue
431 levels reduced by 90% after 14-days depuration. Depuration half-lives were 3.9, 3.8 and 4.0 days for
432 whole body, fillet, and viscera, respectively. The BCF for whole fish, fillet, and viscera were 80, 30 and
433 116 respectively, suggesting that emamectin benzoate does not bioaccumulate significantly. Although
434 the emamectin benzoate BCF values were similar to those obtained for the mussel, the elimination
435 during the depuration phase was very different in the bluegill sunfish.

436 The uptake and depuration of ivermectin, which is an avermectin substance like emamectin benzoate,
437 was performed with the mussel (*Mytilus edulis*) (Davies et al., 1997). Mussels were exposed to 6.9 µg/L
438 ivermectin over 6-days and a calculated BCF of 752 was reported, with maximum tissue concentrations
439 of 5.2 µg/g (w.w.) ivermectin and an elimination half-life of 22-days. The BCF for ivermectin was
440 approximately 10 fold higher than the BCF calculated for emamectin benzoate in our study. The large
441 size of the emamectin benzoate molecule, which potentially limits its uptake into animal tissues, is
442 considered as a possible explanation for why it has a lower BCF than other avermectins (SEPA, 1999).
443 The depletion half-lives are relatively similar between the two related compounds and supports the
444 view that emamectin benzoate will be relatively persistent in non-target organisms.

445 Active metabolism of the three VMPs by mussels may be expected to have contributed towards the
446 uptake and depuration rates calculated. Deltamethrin is known to be readily metabolised in mammals,
447 with excretion and elimination within 2 to 4 days (Anand et al., 2006). Fish have a much lower capacity
448 (Glickman and Lech, 1982), whereas the freshwater mussel *Unio elongatulus eucirrus* was also indicated
449 to have a reduced capacity to metabolise deltamethrin (Şimşek Köprücü, 2008). However, some
450 metabolism would have been expected to have contributed to the rapid depuration rates observed in
451 the mussels in the present study.

452 The metabolism of teflubenzuron in the European lobster (*Hommarus gammarus*) revealed that
453 sulfonate conjugation was an important reaction in the metabolism of teflubenzuron (Olsvik et al.,
454 2015). The metabolism of teflubenzuron was not available for mussels, but metabolism was thought to
455 have contributed to the reduction in the tissue residue concentrations over the 21-day depuration
456 phase in the present study.

457 The metabolism of emamectin benzoate has been previous found to be slow in the Atlantic salmon
458 (*Salmo salar*), with the metabolite desmethylemamectin B_{1a} accounting for less than 20% of residue

tissue concentration after 90 days (Kim-Kang et al., 2004). The metabolism of emamectin benzoate in mussels was not available from the scientific literature. However, the stable concentration of emamectin benzoate in mussel tissue over 7 days shown in this study, would suggest that metabolism was equally limited.

4.3. Potential for bioaccumulation in mussel populations wild and farmed

The results from this investigation indicate that the extended half-lives of emamectin benzoate and to a lesser degree teflubenzuron are the mostly likely VMPs of the three tested to be present in neighbouring mussel populations. Measurements of five mussel farms along the Norwegian coast did not find detectable concentrations of these VMPs in their tissues at a detection limit of 1 ng/g (w.w.).

A screening study of diflubenzuron and teflubenzuron in the vicinity to fish aquaculture facilities in Norway, where these products were used, measured concentrations in several different non-target species including crabs, shrimp, and mussels (Langford et al., 2014). The filter feeding mussel is most likely to bioaccumulate these compounds attached to the particulate matter that are filtered and taken in as food. However, the elimination rates of teflubenzuron, and also deltamethrin, would suggest that these chemicals would not remain in the mussel for long and thus unlikely to pose a problem with regard to human consumption assuming sufficient time after exposure and before harvesting occurs.

A monitoring survey that looked at the concentrations of emamectin benzoate in water, sediment and biota around a treated fish farm, found quantifiable concentrations of emamectin benzoate in blue mussels positioned up to 100 m from the treatment cages after 1 week (Telfer et al., 2006). However, after 1-month post-treatment, emamectin benzoate was only found in mussels 10 m from the fish cages, which led the authors to conclude that the emamectin benzoate was mostly depurated. Field monitoring studies to determine the chemical residue concentrations at a fish farm site within a Scottish

Loch revealed emamectin benzoate concentrations in hermit crabs (5 µg/kg), dogfish (1.23 µg/kg) and the crab *Munida rugosa* (1.99 µg/kg) one week after treatment (SEPA, 1999). This would suggest that emamectin benzoate is somewhat persistent in the environment and supports the findings of the slow depuration phase in this study.

The fact that teflubenzuron and emamectin benzoate were not detected above the limits of quantification (1 and 0.5 ng/g w.w.) in the sub samples from five mussel farms in our study, may indicate that the risk to humans through ingestion of contaminated mussels is limited. However, the laboratory studies clearly show that emamectin benzoate is particularly persistent in mussel tissue and is likely to remain for some time after exposure. Therefore, biomonitoring of mussels in areas where VMPs are used is recommended.

5. Conclusions

Salinity had no significant effect on the uptake or depuration curves for teflubenzuron down to 15‰. The uptake rate constants (k_1) for teflubenzuron, emamectin benzoate and deltamethrin in mussels were 192, 4.82 and 2003, respectively, with kinetic bioconcentration factors (BCFs) of 1304, 49 and 2516, respectively. Depuration rate constants (k_2) were also found to differ between the three VMPs at 0.147, 0.048 and 0.796 for teflubenzuron, emamectin benzoate and deltamethrin, with calculated elimination half-lives ($t_{1/2}$) of 4.7, 14 and 0.87 days, respectively. Based on the depuration rates, emamectin benzoate, and to a lesser extent teflubenzuron, were most likely to be present for longer in exposed mussels. However, both VMPs were below LOD levels (1 and 0.5 ng/g w.w.) in the commercially farmed mussels measured in this study.

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507

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630

631 Tables and Figures

632

633 Table 1. Calculated uptake and depuration rate constants for the three veterinary medicines in mussels.
634 Concentration in the water (C_w), concentration in the mussel (C_m), Time weighted mean (TWM),
635 Bioconcentration Factor (BCF), uptake rate constant (K_1), depletion rate constant (K_2), elimination
636 half-life ($t_{1/2}$), day 14 (d14).

	C_w (TWM)	C_m (d14)	Uptake rate constant	Depletion rate constant	Steady state BCF	Kinetic BCF	elimination half-life ($t_{1/2}$)
Teflubenzuron	µg/L	ng/g	(K_1)	(K_2)	= C_m/C_w	= K_1/K_2	days
15‰	*1.0	1439	258	0.160	1439	1610	3.5
25‰	*1.0	1121	181	0.138	1121	1312	5.0
35‰	*1.0	1480	312	0.198	1480	1579	4.3
35% extended	**1.35**	**1535**	**192**	**0.147**	**1137**	**1304**	**4.7**
Emamectin benzoate	*1.0	49	4.82	0.048	49	100	14
Deltamethrin	0.047	118.6	2003	0.796	2523	2516	0.87

637 * nominal concentrations.

638

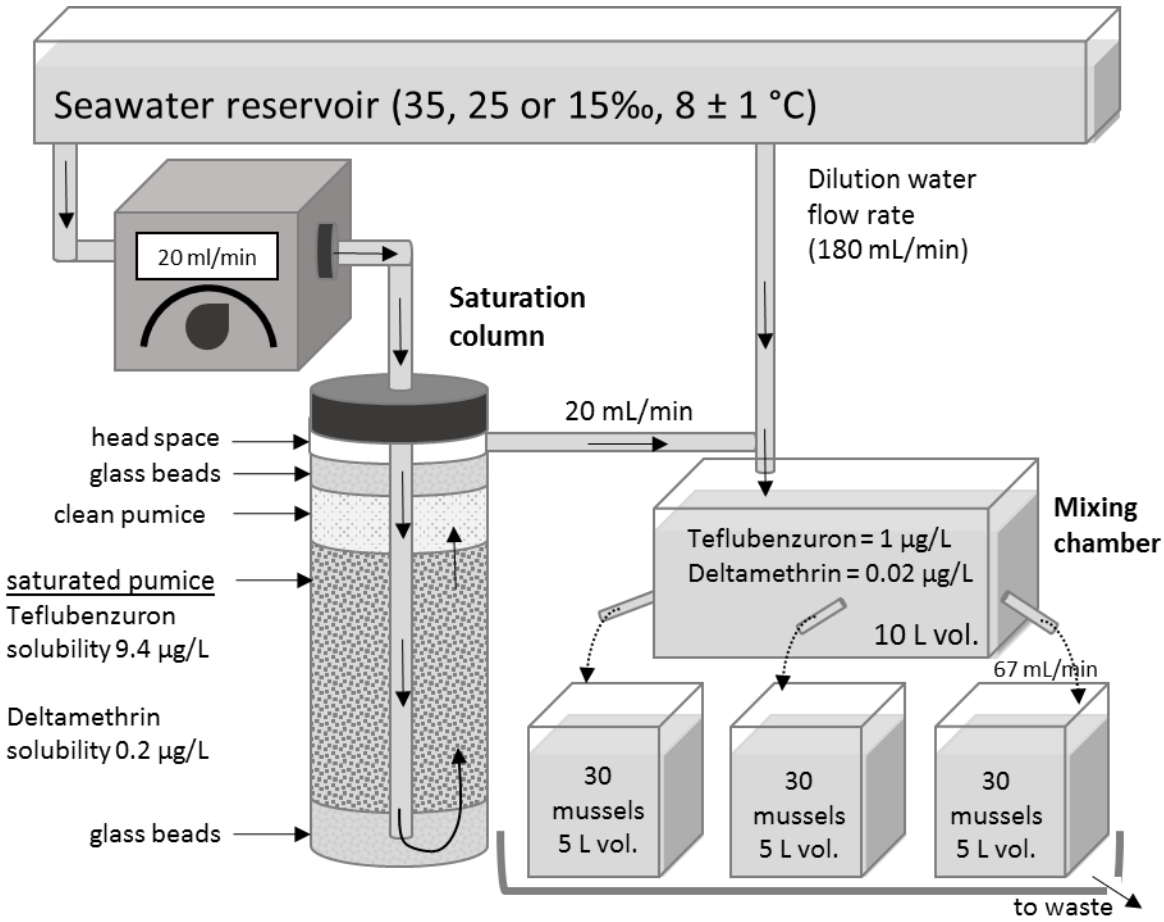


Figure 1. The flow-through dosing system including the saturation column for the individual dosing of teflubenzeron and deltamethrin. The chemical concentration of the water leaving the saturation column is equivalent to the solubility limit, this is then diluted 10 fold with dilution seawater into the mixing chamber before exposed to the mussels. Emamectin benzoate was dosed into the mixing chamber from a concentrated stock solution diluted 1000 fold to achieve a final exposure concentration of 1 µg/L.

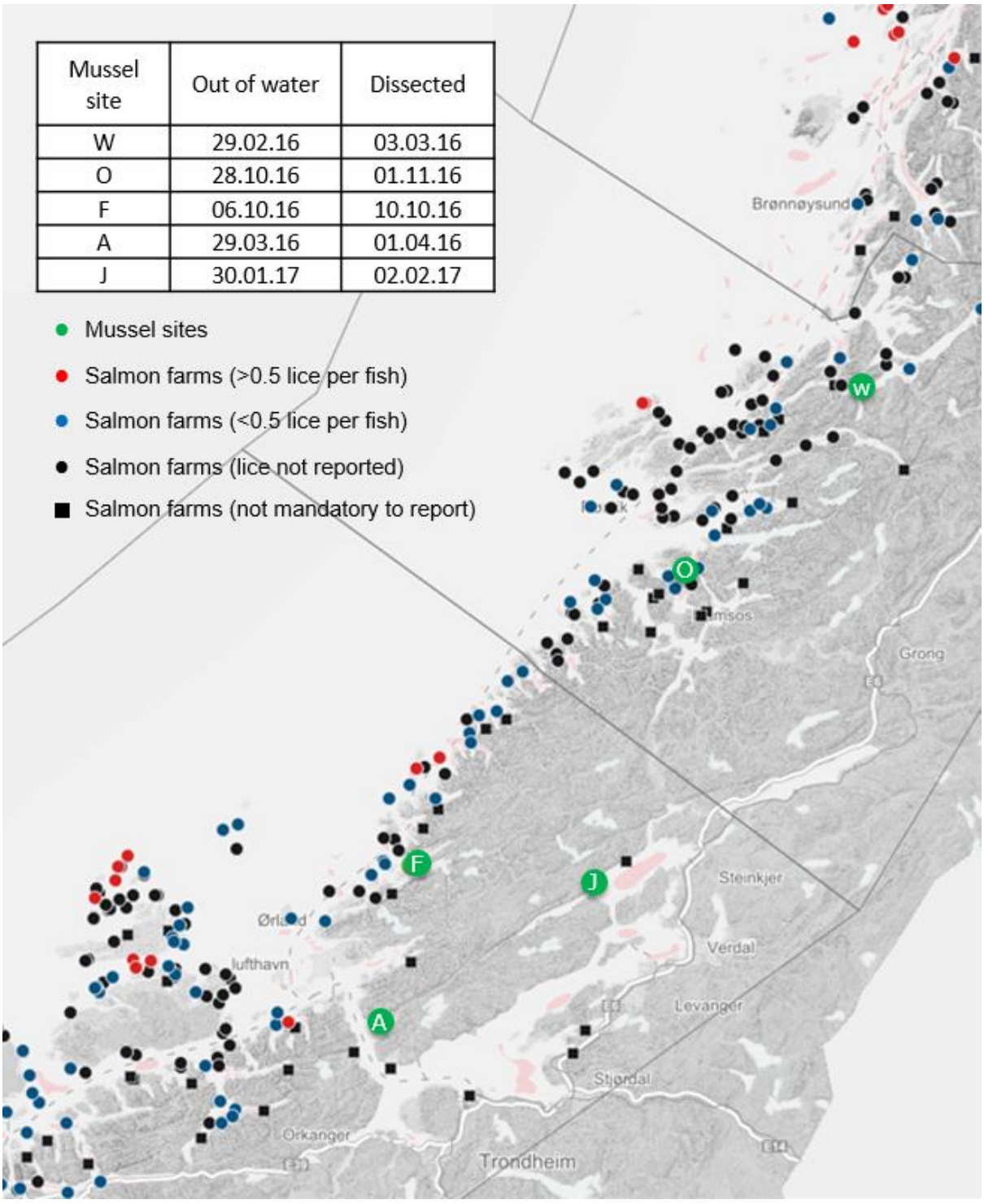


Figure 2. Location of the mussel farms sampled in relation to the position of the salmon farms. Source: https://www.barentswatch.no/en/fishhealth/2016/36, September 2016 week 36. Inserted table denotes the dates the mussels were removed from the sea and the date the mussels were dissected.

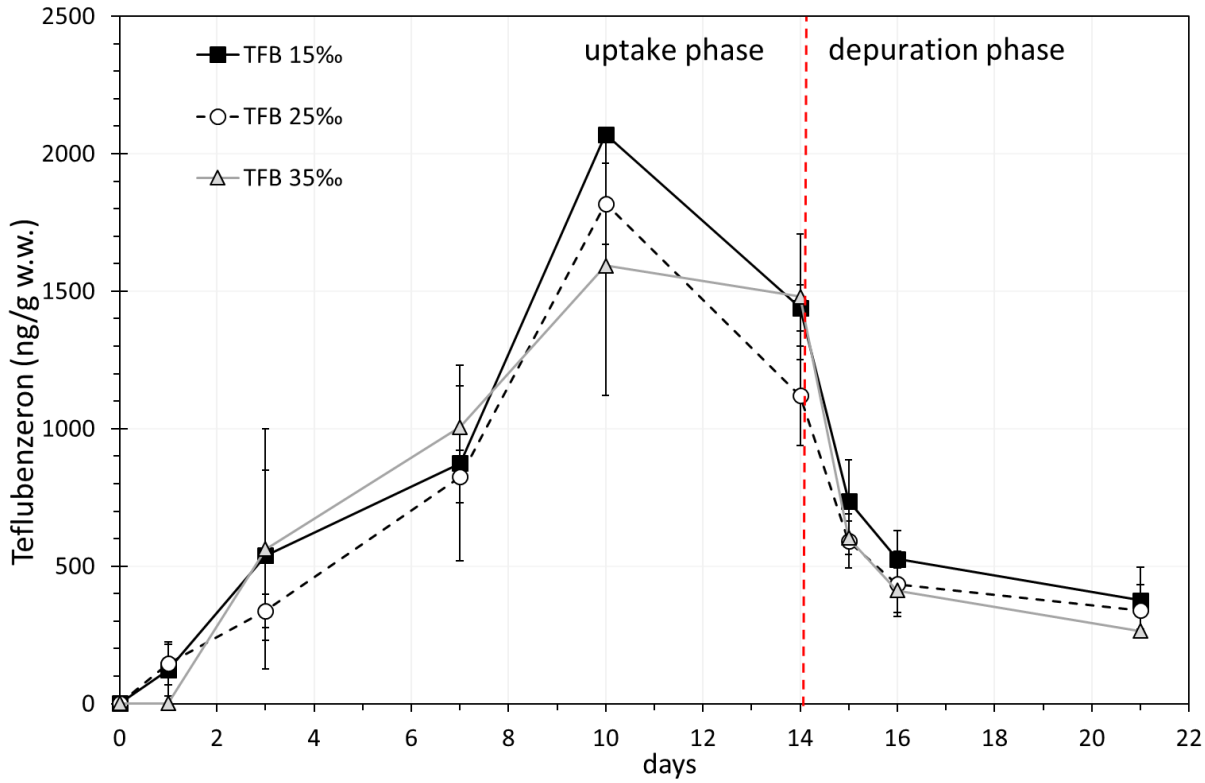


Figure 3. Salinity effects on the uptake and depuration curves for teflubenzuron in mussels. Mussels exposed to dissolved teflubenzuron (1 µg/L nominal) during the 14-day uptake phase. Mussels placed in clean flowing seawater on day 14 for the depuration phase (mean ± SD, n=3).

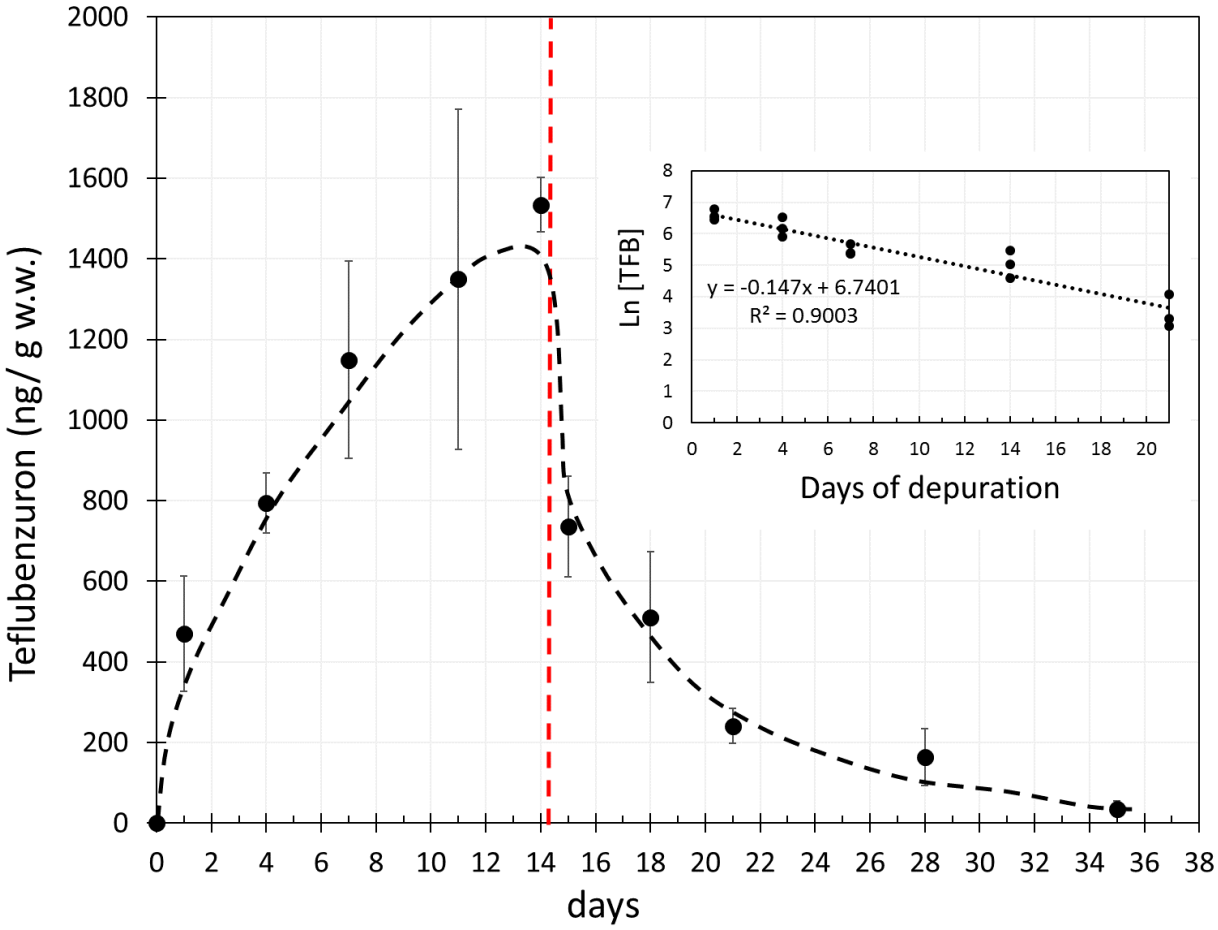
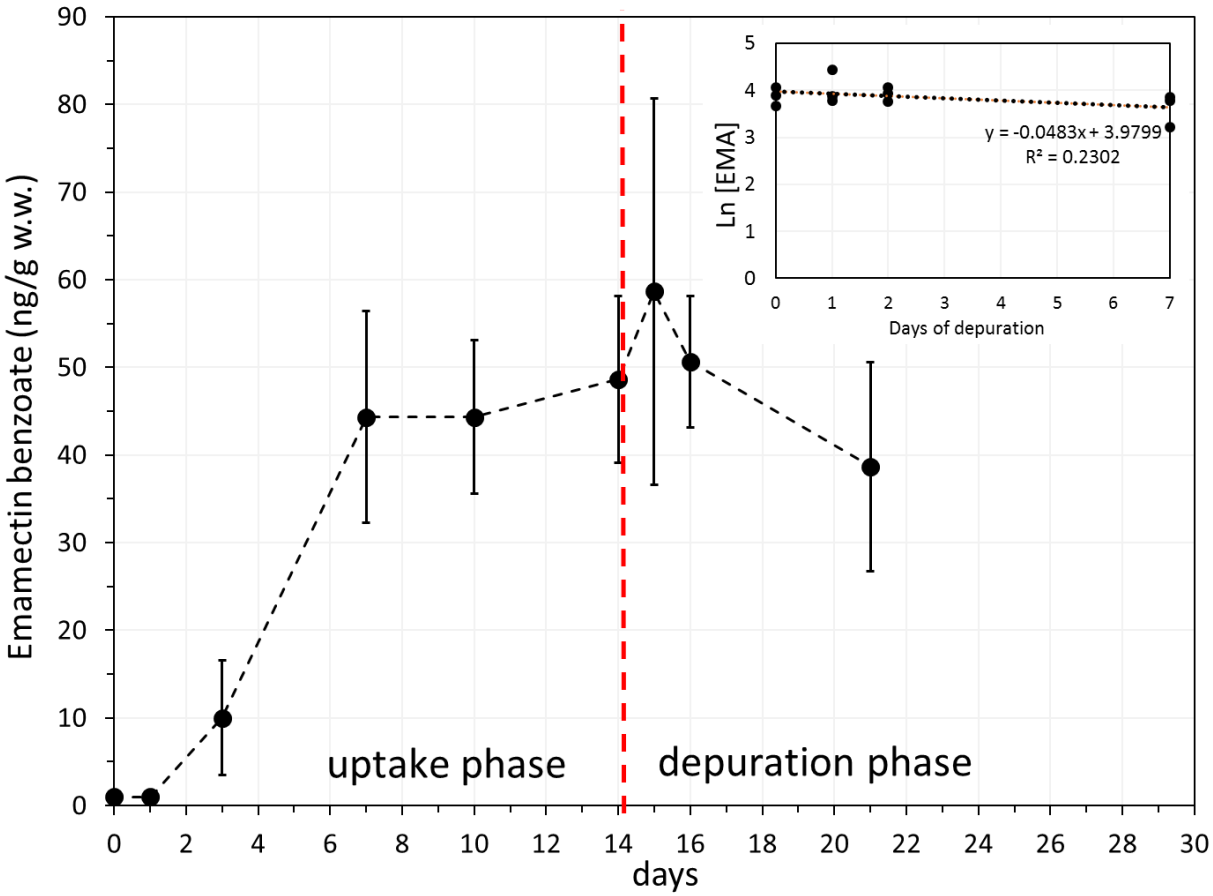


Figure 4. Uptake and depuration curve for teflubenzuron in seawater acclimated mussels. Mussels exposed to dissolved teflubenzuron (1.35 ± 0.34 µg/L time weighted mean) during the 14-day uptake phase. Mussels placed in clean flowing seawater on day 14 for the depuration phase (mean ± SD, n=3). Inserted figure is the natural log of teflubenzuron concentration in mussel tissue against time for the depuration phase, which was used for the calculation of the depuration rate constant (k_2).

673



674

675 Figure 5. Uptake and depuration curve for emamectin benzoate in seawater acclimated mussels.

676 Mussels exposed to dissolved emamectin benzoate (1 µg/L nominal) during the 14-day uptake phase.

677 Mussels placed in clean flowing seawater on day 14 for the depuration phase (mean ± SD, n=3). Inserted

678 figure is the natural log of emamectin benzoate concentration in mussel tissue against time for the

679 depuration phase, which was used for the calculation of the depuration rate constant (k_2).

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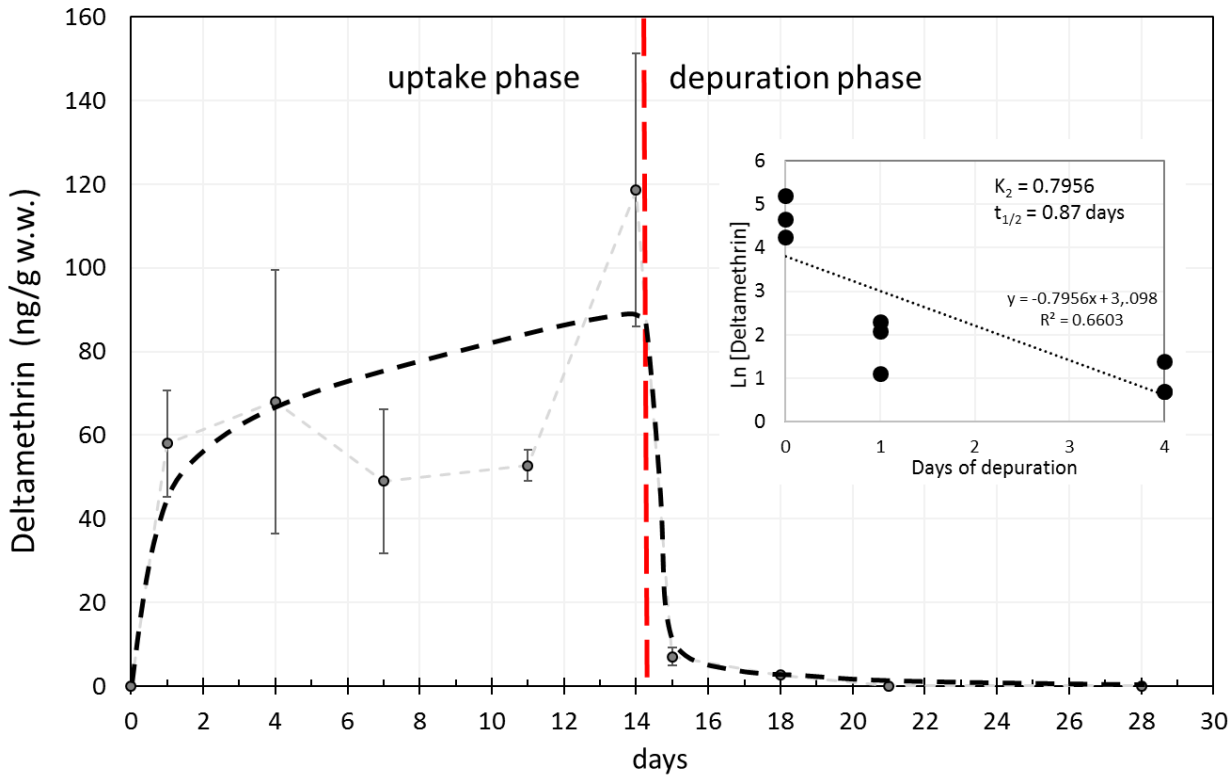

684

685 Figure 6. Uptake and depuration curve for deltamethrin in seawater acclimated mussels. Mussels
686 exposed to dissolved deltamethrin (0.043 µg/L time weighted mean) during the 14-day uptake phase.
687 Mussels placed in clean flowing seawater on day 14 for the depuration phase (mean ± SD, n=3). Inserted
688 figure is the natural log of deltamethrin concentration in mussel tissue against time for the depuration
689 phase, which was used for the calculation of the depuration rate constant (k_2).

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