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Running Head: Environmental risks of veterinary medicines in aquaculture

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Benzoylurea pesticides used as veterinary medicines in aquaculture: Risks and developmental effects on non-target crustaceans.

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Conflict of Interest Reporting

The authors have no conflict of interest to declare.

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Abstract: Diflubenzuron and teflubenzuron are benzoylureas that are used in aquaculture to control sea lice. Flubenzurons have low toxicity to many marine species such as fish and algae, but by their nature are likely to have significant adverse effects on non-target species such as crustaceans and amphipods. Although the exact mechanism of toxicity is not known, these compounds are thought to inhibit the production of the enzyme chitin synthase during moulting of immature stages of arthropods. These chitin synthesis inhibitors are effective against the larval and pre-adult life stages of sea lice. Due to their low solubility and results of recent monitoring studies conducted in Norway, the sediment compartment is considered the most likely reservoir for these compounds and possible remobilization from the sediment to benthic crustaceans could be of importance. For this reason, the epibenthic copepod, *Tisbe battagliai*, was selected for use in the investigations into acute and developmental effects of these compounds. For comparative purposes, azamethiphos was investigated to identify differences in sensitivity and act as a negative control for developmental effects at environmentally relevant concentrations. Standard acute studies with adult copepods showed little or no acute toxicity at mg/L levels with the flubenzurons, while a naupliar developmental test demonstrated that environmentally relevant concentrations (e.g. ng/L) caused a complete cessation of moulting and finally death in the exposed copepods.

Keywords: *Tisbe battagliai*, Diflubenzuron, Teflubenzuron, Azamethiphos, Naupliar development

INTRODUCTION

Aquaculture in Norway has been in existence as far back as 1850, when brown trout were first reared. The modern aquaculture industry in Norway started around 1970 after the construction of the first seawater cages [1], which proved more successful than the land based/onshore tanks that were used previously. Now the aquaculture industry in Norway is one of the most important industries in coastal areas and Norway's largest export after oil and gas, specifically commercial Atlantic salmon (*Salmo salar* L.). The latest figures from the Norwegian Seafood Council show record values for Norwegian salmon exports, with the value for the first half of 2013 totalling NOK 17.4 billion (ca. €2.1 billion) [2].

As the aquaculture industry is so important economically, possible threats, such as parasitic infections from sea lice (Copepoda, Caligidae), especially *Lepeophtheirus salmonis* and *Celigus* spp. are important to control. Medicinal products have been employed in the aquaculture industry to tackle the issue of sea lice infestation in farmed salmon since the 1980s [3]. In Norway, several products are regularly employed against sea lice. For example diflubenzuron (DIF) and teflubenzuron (TEF) which are benzoylurea medicines administered in fish food. On the Norwegian market they are sold as Releeze vet. (0.6 g/kg DIF) and Ektoban vet. (2 g/kg TEF). In the late 1990s these two products were in use, but by the end of the 1990s a voluntary ban was introduced due to the suspected adverse environmental impacts of these products [4]. However, in recent years the use of these products has again increased due to the resistance of the sea lice to the flubenzuron replacement product emamectin benzoate [5]. From 2010 to 2011 the use of flubenzurons sharply decreased, however in 2012 their usage was back up to the 2010 levels. Recently, it has also been reported that the use of TEF and DIF have doubled both from 2011 to 2012 and again from 2012 to 2013 [6]. In the past two years, there have been two different reports [4, 7] showing the negative impacts of flubenzurons on various crustaceans. Therefore the recent increase in their use in the aquaculture industry in Norway is potentially a very serious environmental situation.

Another important veterinary medicine used in salmon farming in Norway is azamethiphos (AZA) (market name Salmosan) which is a broad spectrum organophosphorus insecticide used against flies and other arthropods in agriculture as well as sea lice in salmon farming. Unlike the flubenzuron products, AZA is applied topically through bath treatments (with an application rate of 0.1 - 0.2 mg/L). The use of AZA as a potential delousing agent for salmon lice was first reported by Roth and Richards [8]. The safety of azamethiphos has previously been evaluated for the establishment of Maximum Residue Limits (MRLs) in Salmonidae and the conclusion was that no MRLs were required for the protection of consumer safety as the measured residues in salmon muscle and skin were always below the limit of detection (LOD) even one hour after treatment [9]. The inclusion of AZA in this study was based on the reported usage of the various delousing treatments used in Norway over the past few years. As the possible risks to non-target species and possible persistence of the flubenzurons became of more interest, the use of AZA was seen to increase in the period of 2008-2011, when the use of the flubenzurons decreased (Table 1). All three compounds are widely used in salmon aquaculture in Norway at present and are of fundamental importance to the Norwegian aquaculture market but also specifically to the environment.

The Ministry of Fisheries is the main authority responsible for the licensing of new farms and the control of the industry. In Norway there is mandatory sea lice monitoring, reporting and auditing. A condition for receiving a licence for aquaculture activities is that the activity does not comprise a danger for the spreading of fish disease. The management plans for the facility must be approved by the Animal Health Authority. Records must be kept of disease outbreaks, diagnoses, testing and treatment and the facility must post public notice of antibiotic use.

The licensing requirements for new medicines in Norway are very strict and comply fully with EC regulations. The pharmaceuticals for treating salmon lice are approved by the Norwegian Medicines Agency (NoMA) in accordance with the EU rules for the approval of veterinary medicine products. The Norwegian Medicines Agency is the national regulatory authority for new and existing

medicines and is responsible for all areas of medicinal products from production, trials and marketing to monitoring their use. Only approved veterinarians and fish health biologists are allowed to prescribe medicines for use on fish. In addition there are also regulations specifying the length of time necessary between treatments with veterinary products and slaughtering of the fish. The Norwegian Food Safety Authority is responsible for keeping a record of all pharmaceuticals ordered for use in the aquaculture industry in Norway.

The benzoylurea pesticides are not very toxic to many marine species such as fish and algae, but due to their specific mode of action (MOA) they are likely to have adverse effects on non-target species such as the crustaceans and amphipods in the marine environment [4]. Although the exact mechanism of toxicity is not known, these compounds are thought to inhibit the expression of the enzyme chitin synthase during moulting in immature stages of arthropod development. These chitin synthesis inhibitors are therefore effective against the larval and pre-adult life stages of sea lice. The life cycle of sea lice have been extensively researched [10] and is characterized by 3 distinct morphological life stages (naupliar, copepodid and chalimus). The number of moults per life stage can vary between species of sea lice but the free living stage is generally characterized by 2 naupliar stages and the infective copepod stages [10]. Following attachment to the host fish, the sea lice moult into a number of chalimus stages before becoming adult.

Due to the relatively low solubility of the flubenzuron compounds (9.4 and 89 µg/L for TEF and DIF respectively), they are administered to the fish via the food and consequently uneaten food and faeces are expected to be the main routes into the environment. In combination with previous monitoring studies conducted recently in Norway [4], it was concluded that the sediment is the most likely reservoir for these compounds and that possible remobilization from the sediment to benthic living crustaceans could be of ecological importance. For this reason, the epibenthic copepod *Tisbe battagliai* was selected to investigate the developmental effects of these compounds to a non-target species. *T. battagliai* is a sexually reproducing marine harpacticoid copepod commonly used in the ecotoxicological assessment of chemicals and pollutants.

133 Harpacticoids are members of the benthic fauna with a few planktonic species living in close
134 association with other organisms. They often represent the second largest meiofaunal group in
135 marine sediments after nematodes [11]. Therefore, they are highly representative of non-target
136 species that may be exposed in and around fish farms treated with veterinary medicines.
137 Development through the six naupliar stages to the copepodite stage C1 typically occurs within 96 h
138 under optimal conditions (e.g. food quality and quantity, temperature etc. [12]). At 20 °C the
139 development to adult (C6) takes approximately 10 days with the first brood appearing at
140 approximately day 14 [13]. This short development time from nauplii to adult indicate its suitability
141 for use in a short term sensitive developmental test for purported endocrine disrupting chemicals
142 that may affect normal copepod development (e.g. chitin synthesis inhibitors such as the
143 flubenzurons or moulting disruptors such as fenoxycarb). However, there are currently no existing
144 standardized regulatory test guidelines for developmental or reproductive studies with this
145 organism.
146 Harpacticoids are widespread and are an important food source for macroinvertebrates and
147 fish. *T. battagliai* is a benthic copepod and harpacticoid copepods are often one of the most
148 dominant taxa in marine sediments [14], increasing their relevance as a test species for the
149 investigation of the effects of TEF and DIF. In addition, the sea lice, like *T. battagliai*, are also
150 members of the class Maxillopoda and subclass copepod, therefore they show similarities in their
151 development and lifecycle. Based on the MOA of the TEF and DIF, they are likely to affect the non-
152 target organism *T. battagliai* in a similar fashion to the main target organisms the sea lice. Figure 1
153 and Figure 2 describe the lifecycles of *Lepeophtheirus salmonis* (a problematic target species in
154 Norwegian aquaculture) and the test species *T. battagliai,* respectively.
155 Due to the way in which these two compounds (DIF and TEF) are administered together in
156 fish farms in Norway, it is also of great importance that there is an understanding of potential
157 mixture effects in the marine environment and at the sediment water interface. For comparison
158 purposes, AZA was also assessed for acute toxicity and developmental effects with *T. battagliai*.

However, due to its mode of action, it was predicted that at sub-lethal concentrations it would be expected to have little effect on moulting and development. The selection of a developmental test for use with *T. battagliai* was based on the need to assess possible chronic or developmental effects of the chitin inhibiting flubenzurons (and the organophosphate pesticide azamethiphos) and to test the hypothesis that non-target copepods could be affected in the same manner as the target sea lice at environmentally relevant concentrations. The specific aims of the present study were (1) to look at individual and mixture toxicity of TEF and DIF and AZA, (2) to develop an early stage naupliar developmental test to investigate the sublethal toxic effects of flubenzurons and AZA on naupliar growth and ecdysis and (3) make recommendations on improvements in study design and ease of use, as well as contribute to the regulatory decision framework regarding the continued use of these pesticides.

MATERIALS AND METHODS

Test chemicals

The benzoylurea medicines, teflubenzuron (TEF) (CAS Registry No. 83121-18-0) and diflubenzuron (DIF) (CAS Registry No. 35367-38-5) were obtained from Sigma-Aldrich Norway As. The organophosphate medicine, azamethiphos (AZA) (CAS Registry No. 35575-96-3) was also obtained from Sigma-Aldrich Norway As. The organic solvent dimethyl sulphoxide (DMSO) was used as a solubilizing agent in the preparation of stock solutions of the benzoylurea compounds. Due to the higher solubility of azamethiphos no solvent was required in the preparation of the stock solutions. Chemical analysis was conducted on the DMSO stocks for both TEF and DIF using LC/MS/MS analysis to confirm the starting concentration of each test chemical [4].

Acute toxicity of veterinary medicines to T. battagliai

All testing with *T. battagliai* was conducted with in house cultures. The acute toxicity testing of all three test chemicals were conducted with slight modifications according to the ISO method [15]. Toxicity tests were conducted with copepodids 6 ± 2 days old and with nauplii ≤ 3 h old. The copepodid and naupliar tests were conducted in 12 well polystyrene plates (NUNC) and 48 well polystyrene plates (NUNC) respectively. Four replicates, each containing 5 animals, were used with a total of 20 animals per exposure concentration. All tests were incubated in a temperature controlled room at 20 ± 2 °C and with a 16:8 h light:dark photoperiod. The lethality of each test chemical at each concentration was recorded and the percentage mortality (LC50, lethal concentration of 50% of the sample population) compared to the control was calculated after 24 and 48 h. Mortality is defined as no swimming or appendage movements within an observation period of 10 seconds. At test initiation and termination dissolved oxygen (DO), salinity and pH were measured to ensure validity of the test. Measurements of these physico-chemical parameters were measured in the controls, lowest and highest test concentrations. Seawater used in all testing was approximately 34 ‰ and was obtained from the outer Oslofjord at a depth of ca.60 m.

Optimisation of naupliar development test with T. battagliai

After initial acute toxicity determination with both copepodid and naupliar stages of *T battagliai*, the optimization of a naupliar development test was conducted.

The optimization trials included the use of various plates (12, 24 and 96 well polystyrene cell culture plates), different feeding levels of the marine alga *Rhodomonas baltica*, and the use of different renewal periods during a selected 7 day exposure period. For the final study design a 7 day exposure period was selected to reflect a similar, environmentally relevant exposure scenario for the organisms, based on the method of dosing of flubenzurons in Norwegian fish farms [4]. All trials were conducted with natural filtered seawater in the absence of test chemicals at a salinity of ca. 34 ‰.

206 Under optimal conditions, *T. battagliai* should pass through 5 naupliar stages and become copepodid
207 within 4 days, therefore, this time was used as the criteria for optimization of performance of the *T.*
208 *battagliai* in the various test designs. *T. battagliai* used in the trials were of a similar age as those to
209 be used in the definitive studies, ≤ 3 h old.

210 The Optimal food levels were assessed based on the survival and time to copepodid of
211 unexposed nauplii. The suitability of test plates and exposure volumes were assessed using survival
212 and general health as endpoints as well as developmental time.

213

214 *Developmental effects of veterinary medicines to T. battagliai*

215 Based on the optimization experiments a suitable test design for a 7 day naupliar
216 development study was selected, as follows. The selected test plates were 24 well polystyrene cell
217 culture plates (NUNC) with 2 mL of medium per cell well. *T. battagliai* ≤ 3 h old were individually
218 housed in 10 replicates per test concentration. Animals of the correct age were isolate by removing
219 ten gravid females from in house cultures several days before test initiation. These gravid females
220 were housed individually in approximately 5 mL of clean seawater containing food. Observations on
221 released offspring were made at hourly intervals until sufficient nauplii ≤ 3 h old could be collected
222 for use in the tests.

223 The animals were fed only once during the experiment and there was no requirement to
224 renew the medium during the exposure period. The food was prepared from a confluent healthy
225 culture of *R. baltica* that was settled for several days prior to test initiation. Once the *R. baltica* had
226 settled, the supernatant was poured off and the remaining algae resuspended in a minimum amount
227 of filtered seawater, mixed and a cell count was made using a Neubauer Improved (Bright-light)
228 chamber. Based on the total cell count a concentration of 2 x 10^5 cells/mL was fed to all test

concentrations, controls and solvent controls. In order to reduce any dilution of the test compounds once dispensed into the exposure wells, the *R. baltica* feed was prepared and spiked directly into each of the test concentrations during preparation, prior to dispensing 2 mL per exposure well.

Observations of mortality and developmental stage were made daily during the 7 day exposure period. Time to copepodid and development rate were calculated for each individually housed organism and the total number and percentage of copepods at the end of the study were calculated per concentration and compared to the controls.

Biological data analysis

All data (individual development rate, total number of copepods after 7 days, mortality and EC values for total number of copepodids at day 7) were statistically evaluated with the commercial software programme GraphPad Prism 6 for Windows and the EC values were calculated using the Excel Macro REGtox. All calculations were performed using nominal concentrations.

Data for total number of copepodids after 7 days (numbers summed over all replicates within a treatment) were analysed using Fischer's Exact Test for 2 x 2 contingency tables. An overall significance level of $p = 0.05$ was used. Mortality data (based on survivors) was assessed in the same way for Day 7.

Individual development rates were calculated for each surviving organism. The development rate is the reciprocal of the time to copepodid (reciprocal of the day number (day on which the organism was copepodid) minus 0.5) and represents that proportion of naupliar development, which takes place per day in the exposure system.

Statistical procedures were then used to look for significant differences in individual development rates. In summary, data were tested for normality and homogeneity of variance prior

to the use of a parametric (ANOVA followed by Dunnet's post hoc test) or non-parametric (Kruskal Wallis followed by Dunn's post hoc test) procedures to identify significant differences between treatments.

RESULTS

Acute toxicity of the veterinary medicines to T. battagliai

The measured concentrations of the stocks used in all experiments were 1.5 mg/mL and 1.8 mg/mL for TEF and DIF respectively. At test termination for all acute studies, the DO concentrations were greater than 4 mg/L at all measured concentrations. Salinity and pH were within ± 2 ‰ or ± 1 unit, respectively, throughout the tests. Control mortality for all acute tests was less than 10 % and therefore all validity requirements for the standard ISO method were met.

The acute 48 h study with AZA and copepodids (as per ISO 14669) resulted in an LC10 and LC50 (and corresponding 95 % confidence intervals) of 3.6 (2.6 – 4.5) µg/L and 7.7 (6.8 – 8.5) µg/L. The same test with nauplii ≤ 3 h old at the start of the test, gave similar results of 3.4 (2.4 – 5.3) µg/L and 6.7 (5.9 – 7.3) µg/L respectively for the LC10 and LC50 values. Therefore based on these results a range for the developmental study from 0.225 to 3.6 µg/L was selected.

For the flubenzuron compounds the acute data showed no effects up to 1000 µg/L for both nauplii and copepodids with DIF. For the TEF acute studies there was no effect up to 1000 µg/L for the copepodid however, the naupliar acute study with TEF proved more sensitive resulting in 24 and 48 h LC50 values of 230 (58 – 931) µg/L and 40 (4.8 - 419) µg/L. For acute studies with TEF and DIF a limit of 1000 µg/L was used as the highest concentration. Even at these levels there is little environmental relevance or realism about the concentrations used but it was deemed unnecessary to test any higher as effects on other organisms have been observed at lower levels than those tested in our experiments [16, 17]. In addition, these compounds are highly insoluble and are unlikely

to occur in the environment in excess of these levels and are more likely to be found at ng/L levels in marine waters [18].

Optimisation of naupliar development test with T. battagliai

For the optimization of the test design for the naupliar developmental study, several approaches were used. No test chemical was used in the study design experiments. Instead filtered seawater, as would be employed as a negative control, was used for all experimental trials. The initial proposed trial was based on the acute study test design, conducted in 12 well tissue culture plates with a volume of ca. 5 mL per replicate. Ten individually housed animals per test concentration were used instead of 4 replicates containing 5 animals, this was proposed so that individual development could be tracked. After trialling a lower and higher feeding level, the final selected level for all trials was based on previously published data (2×10^5 algal cells/mL *Rhodomonas baltica*) an optimal food level for development [13, 19] of *T. battagliai*. Trials were performed for an initial 96 h, after which time at optimal conditions all nauplii should become copepodid.

Due to the small size of the animals and the large volume of solution in the wells of the 12 well plates, it was difficult to find and assess the animals during the 96 h period. Originally the test design also planned to incorporate an assessment of moulting on a daily basis. The assessment of moulting proved difficult and not all moults could be accounted for within the wells. In addition, within 96 h, under optimal conditions, they would have passed through all naupliar stages potentially moulting more than once a day, therefore this observation was removed from the study design as having limited value. In the 12 well test design with individually housed nauplii the survival was 100 % and all test organisms had developed to copepodid within the 4 days. The study was extended to 7 days to assess the need for renewal, during an extended exposure duration in the presence of a test compound. The animals survived the 7 days and it was concluded that in this case there was no need to renew the test medium. However, due to the large well size and volume of test medium (ca. 5

mL), it was time consuming to assess the animals, which were often found around the rim of the wells, where visualization with the aid of a microscope proved difficult. It was therefore concluded that the use of 12 well plates was not optimal for the assessment of naupliar development.

In the second experimental design, an assessment with 96 well plates was trialled. Due to the size of the test organisms at the start of the test (≤ 3 h) and the small well size, it was possible to assess the animals easily on a daily basis. The 96 well test design used ten individually housed animals per test concentration. Each replicate well contained 200 µL of test medium containing algal feed. This design was to incorporate a renewal period (on day 4) based on the small sample volume and the hypothesis that there would not be enough surface area for sufficient gas exchange. Daily observations were made and mortality, behaviour and any moults were observed. By day 3 the animals were recorded as being in a poor condition. As *T. battagliai* are epibenthic they are often observed on the bottom of the test wells. In the case of the 96 well test design the algae introduced to the wells had quickly settled out on the small surface area on the bottom of the wells, where the nauplii had become entangled. After 96 h there were no copepodids present, and after a further 24 h all animals were dead. During the daily observations it did not appear that the animals were grazing, instead they remained stationary, with movement only after gentle agitation. Another issue with the 96 well test design was generating sufficient volume at test termination for any required physico-chemical analysis.

The final trial involved 24 well tissue plates containing 2 mL of test solution per replicate. All other parameters were consistent with the previous two trials, feeding, medium (filtered seawater), replication etc. No renewal was used in this trial. All animals were copepodid after 96 h and there was only 10 % mortality (1 death) after 7 days. Therefore the use of the 24 well plates and all other described parameters were used for the investigation of developmental effect of veterinary pesticides to *T. battagliai*.

Developmental effects of veterinary medicines to T. battagliai

For the 7 day developmental effects investigations there were four testing scenarios using the previously described 24 well test design: teflubenzuron only, diflubenzuron only, a 2:1 mixture of teflubenzuron and diflubenzuron and azamethiphos only.

For all studies the DO, pH and salinity remained within acceptable limits and the survival in all control and solvent controls was acceptable (≤ 10%).

Mortality data

The mortality data (day 7) were assessed and the acute NOEC and LOEC for 7 day mortality for TEF were 0.0032 and 0.01 µg/L respectively. For DIF and the 2:1 TEF:DIF mixture the NOEC and LOEC were 0.01 and 0.032 µg/L respectively. After 7 days exposure to AZA there was only 10 % mortality at 3.6 µg/L, this was not statistically significant, therefore the LOEC was > 3.6 µg/L.

Total number of copepodids

The total number of copepodids on day 7 were compared to the control numbers for the four different testing scenarios as per the mortality data. For TEF, significant differences in the number of copepodids by day 7 was found between the control and 0.01 µg/L. It was not necessary to statistically assess higher concentrations as there were significant mortalities at all concentrations including 0.01 µg/L and above. Therefore, based on total number of copepods by day 7 the NOEC and LOEC were 0.0032 and 0.01 µg/L respectively. For DIF, the NOEC and LOEC were 0.01 and 0.032 µg/L respectively. For the 2:1 mixture, the NOEC and LOEC for total number of copepodids were the same as for DIF, i.e. 0.01 and 0.032 µg/L. There were no statistically significant differences found for total number of copepodids up to and including 3.6 µg/L for AZA.

Developmental rates

For individual development rates of each surviving animal (based on the day they were recorded as copepodid) the data was assessed as previously describe. There were no statistically significant differences in the individual development at any of the tested concentrations of AZA. For TEF, the development rate of the individuals that successfully became copepodid was not significantly different to the controls up to 0.0032 µg/L and at concentrations above this there were either significant effects on the number of copepodids or on the number of mortalities (as described previously). For DIF, the same was observed. At concentrations up to and including 0.032 µg/L, there were no significant differences in individual development rates, however there were significant differences in mortalities and number of copepodids by day 7 as previously described. The same effect on individual development rate was observed with the 2:1 mixture, where all animals reached copepodid at the same rate as the controls, however other endpoints such as mortality and number of animals to reach the copepodid stage showed significant differences at low concentrations (Figure 3).

Summary of NOECs/LOECs

Time to copepodid, development rate, mortality and the total number of copepodids after 7 days exposure for all test scenarios are shown in Table 2. The overall NOEC/LOEC for the developmental studies based on the most sensitive endpoints are 0.0032/0.01 µg/L (TEF, endpoints: mortality and total number of copepodids at day 7), 0.01/0.032 µg/L (DIF, endpoints: mortality and total number of copepodids at day 7), 0.01/0.032 µg/L (TEF:DIF, endpoints: mortality and total number of copepodids at day 7). There was no effect compared to the control with any concentration up to and including 3.6 µg/L for AZA with any endpoint.

DISCUSSION

The chitin synthesis inhibiting benzoylurea pesticides, teflubenzuron and diflubenzuron and the organophosphate pesticide azamethiphos were all assayed with acute and developmental

studies with the non-target marine copepod, *T. battagliai*. The benzoylureas and azamethiphos were selected due to their usage patterns in Norwegian aquaculture as well as the fact that they have different methods for treatment (to the target organism) and different modes of action. TEF and DIF are administered in fish feed, while AZA is applied as a bath treatment. TEF and DIF act by inhibiting chitin synthesis in arthropods and AZA acts by the inhibition of cholinesterase activity. Based on these points it was possible to hypothesis that TEF and DIF would be the most toxic in the developmental assay and that although AZA may be acutely toxic [20, 21], it would be unlikely to cause the same developmental effects as with the benzoylurea pesticides. This hypothesis is based on the specific MOA of the benzoylurea pesticides that are specifically targeting organisms that require chitin to develop through several stages to adult hood. Both the target organism (the sea lice) and the non-target organism (*T. battagliai*) have similar lifecycles (Figure 1 and 2) and require chitin in order to develop through several morphologically different life stages. Chitin inhibiting chemicals may affect the moulting activity of these species by halting the process completely, by retarding the process or by leaving the animals vulnerable to predation between moults. AZA on the other hand is not likely to have an increased effect on *T. battagliai* development and was included to act as a negative control (although acutely toxic) for developmental effects at environmentally relevant levels in order to highlight the risk to specific groups of organisms through the increased use of the flubenzuron pesticides.

As indicated previously, both TEF and DIF are applied in fish farms via the feed. The main challenge with the treatment methodology is that a large amount of food will remain uneaten, Chen et al. [22] estimated between 5-15 % of the administered food will be uneaten, and approx. 90% of administered DIF and TEF will be excreted in the faeces [4]. DIF and TEF have low water solubility (89 µg/L and 9.4 µg/L respectively) and are relatively hydrophobic (Log K_{ow} of 3.8 and 5.4 respectively) and may therefore bind to particles and end up in the sediment. Due to its epibenthic nature, *T. battagliai* is a relevant test species to assess the environmental hazard of these test substances within the sediment as it is present at the sediment water interface and may be exposed to both

402 water soluble and particle bound contaminants. Due to limited amounts of data on DIF, the UK
403 environment agency does not have sediment Environmental Quality Standards (EQS) for this
404 substance. The Norwegian Environment Agency, is at present evaluating proposed sediment, water
405 and biota EQS values for both TEF and DIF [23]. The proposed $EQS_{sediment}$ = 0.2 µg/kg for DIF and
406 0.0004 µg/kg for TEF. Although our study focused on the assessment of toxicological effects to non-
407 target species through water only exposure systems, it is important to consider the risk to sediment
408 dwelling arthropods that may be affected in the same way as *T. battagliai*. The report of Langford et
409 al. [18] measured sediment concentrations of TEF and DIF as high as 269.2 and 136.6 ng/kg
410 respectively in the sediments around treated fish farms. The measured levels of TEF are in excess of
411 the new proposed sediment EQS values for Norway ($EQS_{sediment}$ =0.4 ng/kg) but not for DIF ($EQS_{sediment}$
412 = 200 ng/kg). Therefore, there is a significant risk to organisms present in or on the sediment that
413 may be affected through the flubenzurons specific MOA of chitin synthesis inhibition.

414 In general, there is limited published ecotoxicity data on developmental and chronic effects
415 of benzoylurea pesticides on non-target species, especially data on marine organisms [18]. However,
416 in recent years several organizations have attempted to address the lack of data [7]. This is
417 particularly relevant in light of the recent resistance of sea lice to the commonly used emamectin
418 benzoate and the increased use of other aquaculture medicines such as the flubenzurons. Some
419 acute data for AZA exists and studies with lobster and mysid shrimp have yielded LC50 values of 1.39
420 µg/L (48 h) [24] and 0.52 µg/L (96 h) [25] while other species such as scallops and clams were
421 unaffected by AZA [26]. Therefore, from the results of the present study *T. battagliai* seems to show
422 a higher tolerance to AZA compared to other crustaceans.

423 Coppen and Jepson [27] described TEF as being more potent and toxicologically active than
424 DIF. This is apparent from the results of the present study where we have directly compared the two
425 pesticides, in both an acute and chronic test system, in which TEF consistently results as the more
426 toxic of the two. Surprisingly the toxicity observed with the 2:1 TEF:DIF mixture (reflecting treatment

with both medicines in Norwegian fish farms) elicited the same toxicological effects as DIF and not TEF despite the TEF being present at a higher concentration in the mixture. As both TEF and DIF have the same MOA, it has been suggested that the most likely description of the additive hazards from these 2 chemicals will be concentration addition. In addition it has been suggested that testing of mixtures of veterinary medicines should be conducted as though the organisms were exposed to each compound independently. This is due to the fact that the active ingredients would normally not be placed in the same product, if they compete for the same receptor target (i.e. have the same MOA). Therefore, the chances of increased sensitivity to aquatic organisms due to a mixture effect, is unlikely in the case of TEF and DIF [28]. As the mixture of the two chemicals was not more sensitive than the results of the TEF alone, an assessment factor (AF) for the risk assessment purposes could be applied to the NOEC from the TEF only developmental study. This would therefore be protective of any potential mixture effects in the environment.

Recently published monitoring data for TEF and DIF from in and around fish farms in Norway [18], treated with both TEF and DIF in combination and DIF alone, have shown elevated levels present in seawater above the UK Environmental Quality Standards (EQS). Specific UK marine water EQS values used for the assessment of the monitoring data where 5 ng/L (AA-EQS (Annual Average-EQS)) and 100 ng/L (MAC-EQS (Maximum allowable concentration-EQS)) for DIF and 6 ng/L (AA-EQS) and 30 ng/L (MAC-EQS) for TEF. The levels measured during the monitoring programme were in the range of 34.3 – 295.2 ng/L (DIF) and < 1 – 12.9 ng/L (TEF), at the site treated with both DIF and TEF, and 13.1 – 30.9 ng/L (DIF) for the site treated with DIF alone. These measured environmental levels are higher than the developmental effect levels observed in the present study. The lowest effect concentrations (LOEC) for *T. battagliai* development (number of copepodids on day 7) and mortality were 10 ng/L and 32 ng/L for TEF and DIF respectively. Therefore it can be concluded that there is a risk to non-target species in and around the areas treated with these pesticides. The recently suggested environmental quality standards for sediments, water and biota (submitted to the Norwegian Environment Agency) [23] propose $EQS_{seawater}$ values for TEF and DIF as follows. For DIF an

AA-EQS and MAC-EQS of 4 ng/L and 100 ng/L respectively have been suggested and for TEF an AA-EQS and MAC-EQS of 2.5 ng/L and 12 ng/L have been put forward. These EQS values have only been proposed to the Norwegian Environment Agency and have not officially been adopted by Norway.

With the specific MOA of flubenzurons it is important to consider the acute to chronic ratio of these substances when performing environmental risk assessments. Flubenzurons elicit their effect via chitin synthetase. Although chitin synthesis is not functionally part of the endocrine system, enzyme systems that regulate chitin synthesis are sensitive to chemical alternation by pesticides like teflubenzuron and diflubenzuron. Therefore they can be considered to be similar to endocrine disruptors which specifically affect arthropods (and possibly other chitin producing organisms). Endocrine disruptors typically have a very high acute to chronic ratio indicating that although acute effects may not be seen at relatively high levels (e.g. mg/L concentrations) sub lethal (chronic endpoints) can be observed at concentrations significantly lower (e.g. ng/L concentrations). Historically, a large number of environmental risk assessments have involved estimating chronic toxicity data from acute toxicity data using an assessment factor. Typically, the assessment factor has been 100 resulting in a predicted chronic toxicity of 100 times lower than the LC/EC50. From a more conservative point of view, some risk assessors have applied an assessment factor of 1000 which may be considered more protective for the environment for substances with non-endocrine mediated toxicity. For example, in terms of AZA which has been assessed in the present paper, the acute toxicity was calculated at a concentration of between 6.7 and 7.7 µg/L dependent on the age of the organisms. In addition, the chronic toxicity NOEC was calculated as 3.6 µg/L. This would mean that the acute to chronic ratio is only a factor of 2 and by using an assessment factor based on the acute toxicity data alone would have provided adequate protection for *T. battagliai*. However, considering the flubenzurons assessed within the present study and specifically the most toxic (TEF) the acute to chronic ratio is significantly higher than the assessment factor approach would have predicted the chronic toxicity. For example, the acute toxicity value for TEF was >1000 µg/L and the NOEC was 0.0032 µg/L which results in an acute to chronic ratio >312500.

As a preliminary risk assessment for both TEF and DIF, a PNEC has been calculated based on the NOEC values of 0.0032 and 0.01 µg/L respectively from the *T. battagliai* naupliar developmental tests. An AF of 10 was applied to both these values to derive the PNEC. Justification for the selection of this AF is based on an assessment of the sensitivity of the endpoint, existing EQS values and detection limits for DIF and TEF (1 ng/L, [4]) in water. Therefore, the $PNEC_{seawater}$ for TEF and DIF would be 0.32 and 1.0 ng/L respectively. Comparing these PNEC values with the Measured Environmental Concentration (MEC) from Langford et al., [18] to derive Risk Quotients (RQ) for TEF and DIF would indicate that an even higher number of the monitored sites would be in exceedance of the RQ of 1 than the previous conclusion [18] based on using the existing EQS values.

RECOMMENDATIONS AND CONCLUSIONS

In recent years, several naupliar developmental studies with marine copepods have been developed and now exist as ISO draft standards (ISO/DIS 16778 [29], ISO/TC 147/SC 5 N 761 [30]. These draft standards assess the developmental effects of *Acartia tonsa* and *Nitocra spinipes* from nauplii to copepodid. These studies are approximately the same length as the proposed *T. battagliai* method. However, with the latter, the controls should be copepodid by 96 h, therefore the length of time for the test could be reduced to just 4 days, compared to 6-7 days or 5-6 days for the *N. spinipes* and *A. tonsa* studies. The idea behind extending the *T. battagliai* test described in the present study, is to allow for the observation of retardation of development, where the end point may not be that the animals do not develop, rather that it happens at a slower developmental rate compared to the control. However, if the aim is to look only at number of copepodids at the point where > 80 % of the controls have developed to C1, then 96 h is an acceptable time period for the test. In addition, another benefit of the proposed *T. battagliai* test is that individual development rate can be tracked, which is not the main consideration in the aforementioned ISO standards. Possible improvements to the proposed study design are an increased number of replicates or increased number of test

concentrations (> 5) although the ease of testing with this study design and the minimal number of animals required, that still provides a reasonably robust statistical assessment, makes this method ideal for assessing chronic toxicity in copepods.

In conclusion, the results of the investigations into the acute and toxic effects of the flubenzurons (DIF and TEF) and AZA have indicated distinctly different effect patterns between the two types of veterinary medicines. Azamethiphos was acutely toxic to the test organism *T. battagliai* as has been previously described in the literature to other crustaceans. However, at lower levels, there were no observed developmental effects. In contrast, the flubenzurons, displayed little or no acute toxicity at microgram per litre levels over a period of 48 h and developmental effects were seen in the nanogram per litre range. The latter reflects the potential for adverse effects at environmentally relevant concentrations to non-target organisms within the marine environment. Taking into consideration the extremely high acute to chronic ratio of flubenzurons, underpins the importance of designing tests appropriately to the specific MOA. If the environmental risk assessment had been performed on acute toxicity data alone, there would have been a significant discrepancy in the risk quotient. This may have resulted in flubenzurons not being regulated appropriately or sufficiently enough within the aquaculture industry and other substances with similar modes of action should be considered correspondingly for environmental risk assessment purposes.

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642

643 **List of Figure legends:**

644

645 Figure 1. Lifecycle of *Lepeophtheirus salmonis* modified from Schram (1993) with adaptations based

646 on Herme (2013)

647

648 Figure 2 Lifecycle of *Tisbe battagliai* modified from Hutchinson et al (1999) with adaptations from

649 Volkmann-Rocco (1972)

650

651 Figure 3. Development rate (± standard deviation) of nauplii (N1) to copepodid (C1) for (a)

652 Diflubenzuron, (b) Teflubenzuron, (c) TEF:DIF, (d) Azamethiphos. * indicates statistical difference

653 compared to the control.

654

655

Fig 1

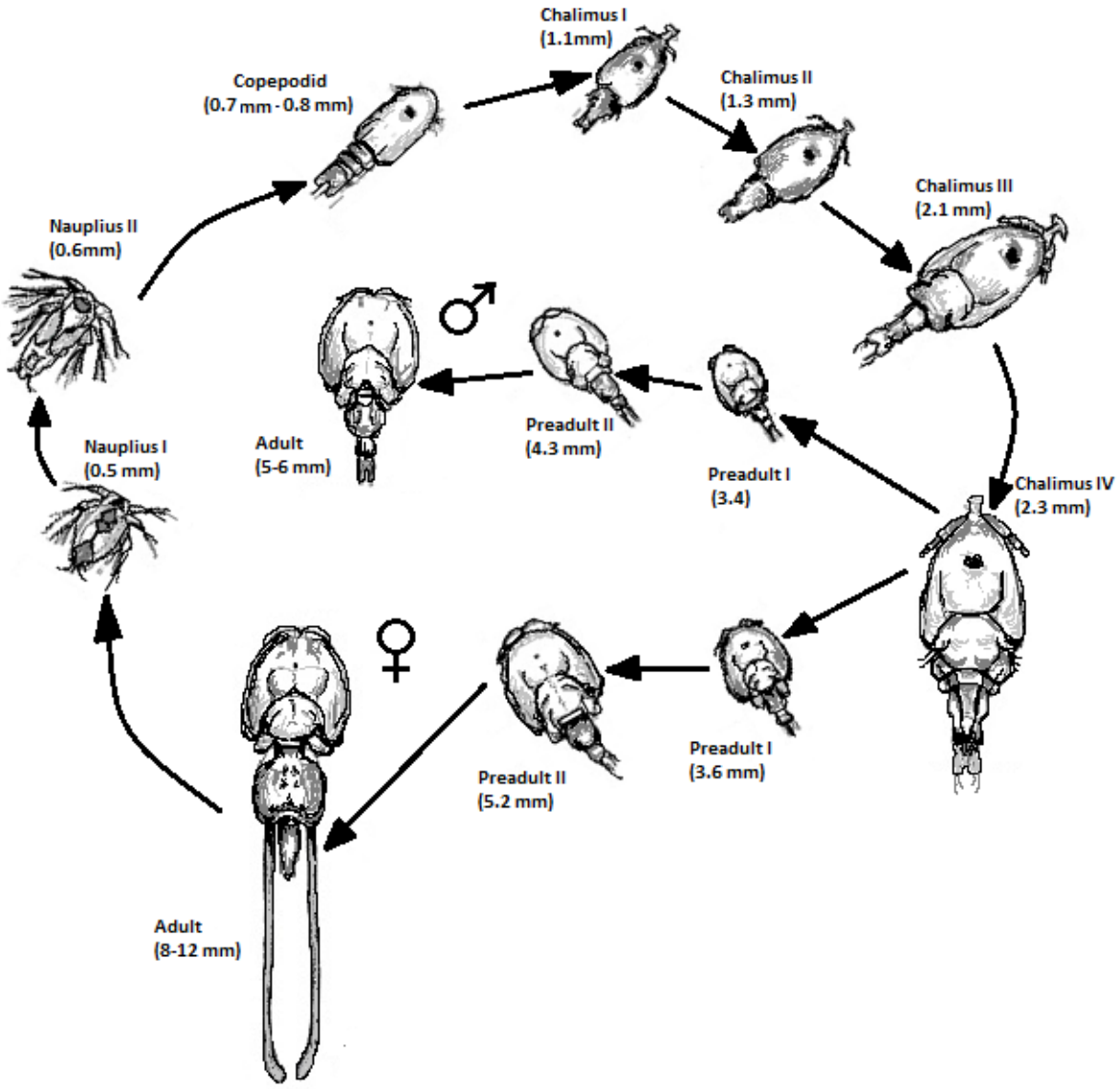
Chalimus I
(1.1mm)
Copepodid
(0.7 mm - 0.8 mm)
Chalimus II
(1.3 mm)
Chalimus III
(2.1 mm)
Nauplius II
(0.6mm)
♂
Adult
(5-6 mm)
Preadult II
(4.3 mm)
Preadult I
(3.4)
Chalimus IV
(2.3 mm)
Nauplius I
(0.5 mm)
♀
Preadult II
(5.2 mm)
Preadult I
(3.6 mm)
Adult
(8-12 mm)

Fig 2

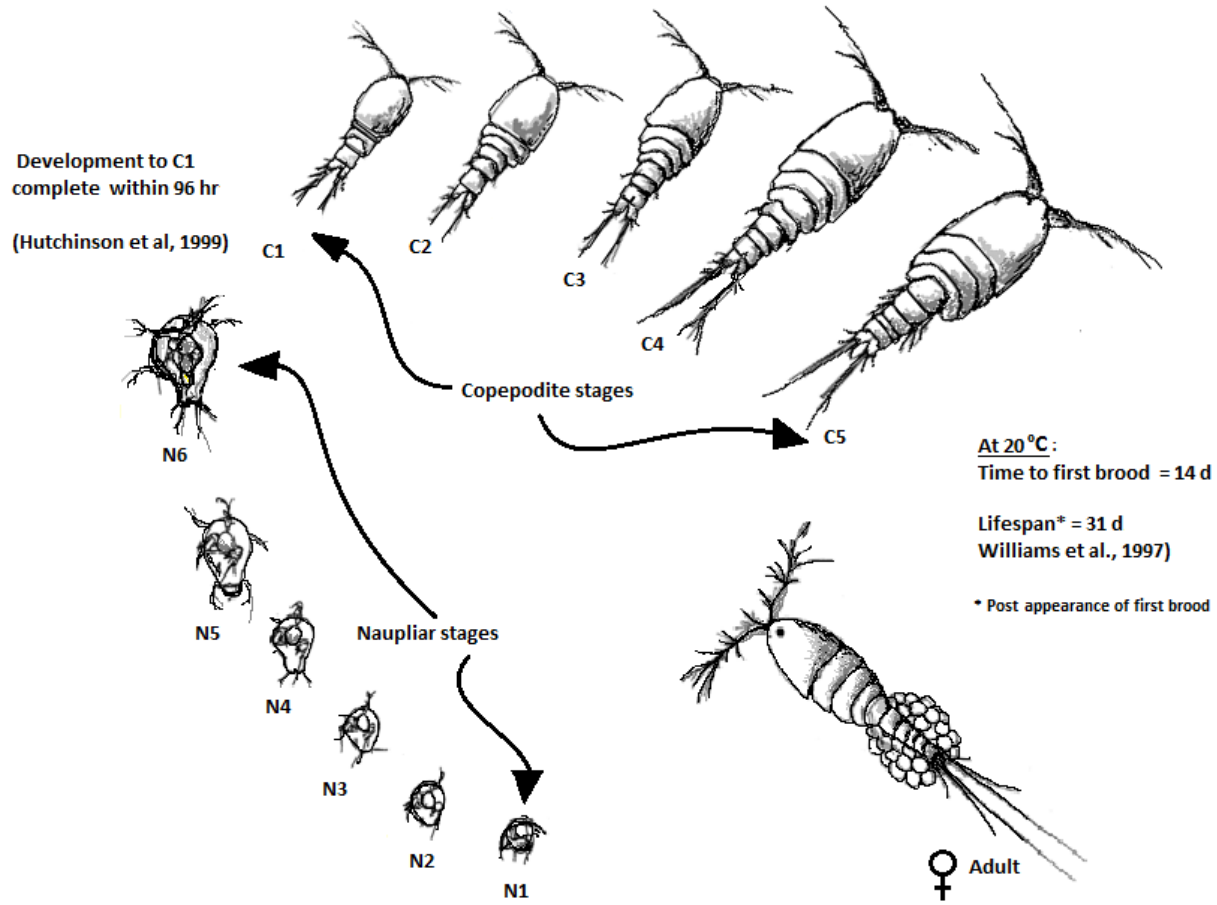
Development to C1
complete within 96 hr
(Hutchinson et al, 1999)
C1
C2
C3
C4
C5
Copepodite stages
N6
N5
N4
N3
N2
N1
Naupliar stages
At 20 °C:
Time to first brood = 14 d
Lifespan* = 31 d
Williams et al., 1997)
* Post appearance of first brood
♀ Adult

Fig 3

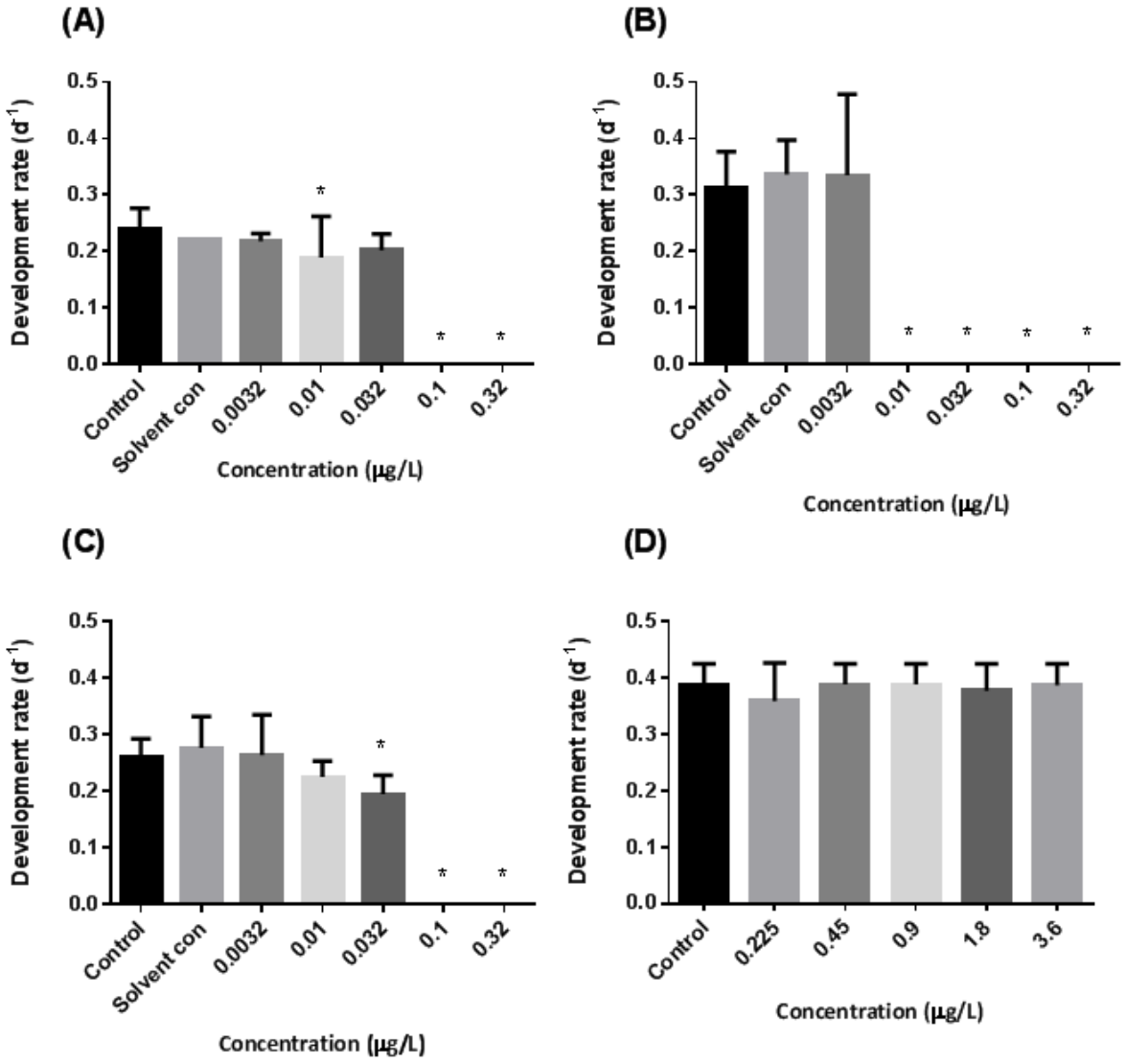
(A)
Development rate (d-1)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
Control
Solvent con
0.0032
0.01
0.032
0.1
0.32
Concentration (µg/L)
(B)
Development rate (d-1)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
*
Control
Solvent con
0.0032
0.01
0.032
0.1
0.32
Concentration (µg/L)
(C)
Development rate (d-1)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
Control
Solvent con
0.0032
0.01
0.032
0.1
0.32
Concentration (µg/L)
(D)
Development rate (d-1)
0.5
0.4
0.3
0.2
0.1
0.0
Control
0.225
0.45
0.9
1.8
3.6
Concentration (µg/L)

Table 1. Selected products used against sea lice in aquaculture in Norway. (kg of active substance).

(Source: Mattilsynet 2014)

Active substance	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Azamethiphos					66	1884	3346	2437	4059	3037
Diflubenzuron						1413	1839	704	1611	3264
Teflubanzuron						2028	1080	26	751	1704
Emamectin	32	37	60	73	81	41	22	105	36	51

Table 2 Summary of *Tisbe battagliai* development and mortality data for all test compounds

Concentration (µg/l)	Time to first copepod (days)	Mean time to first copepodid (days)	Number copepodid (day 7)[a]	Mortalities Day 7 (%)[a]	Mean development Rate ($days^{-1}$)[b]
TEFLUBENZURON					
Control	3	3.8	10	0	0.314
Solvent control	3	3.6	10	0	0.337
0.0032	3	3.9	9	10	0.335
0.01	5	5	1	80	NA
0.032	-	-	0	90	NA
0.1	-	-	0	100	NA
0.32	-	-	0	100	NA
DIFLUBENZURON					
Control	4	4.8	9	10	0.239
Solvent control	5	5.0	9	10	0.222
0.0032	5	5.1	9	10	0.218
0.01	5	5.3	8	20	0.189
0.032	5	5.5	3	70	0.202
0.1	-	-	0	90	NA
0.32	-	-	0	100	NA
2:1 Mix TEF:DIF					
Control	4	4.4	10	0	0.260
Solvent control	3	4.2	10	0	0.277
0.0032	3	4.6	9	10	0.264

0.01	4	5.1	9	10	0.225
0.032	5	5.8	4	50	0.195
0.1	-	-	0	50	NA
0.32	-	-	0	90	NA
AZAMETHIPHOS					
Control	3	3.1	10	0	0.389
0.225	3	3.4	10	0	0.359
0.45	3	3.1	10	0	0.389
0.9	3	3.1	10	0	0.389
1.8	3	3.2	10	0	0.377
3.6	3	3.1	9	10	0.387

[b] Nominally 10 animals tested per concentration.

[a] individual development rate = 1/(day number-0.5). All developmental rates are given to three significant figures.

NA = Not applicable