Master's Thesis in Chemistry

(Analytical Chemistry)

The effects of delousing chemical, imidacloprid, on the copepod *Acartia tonsa*

By

Christoffer Vesterhus Målen

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List of selected abbreviations

Animals:

Abbreviations	Latin Name	Common name
A. tonsa	*Acartia tonsa*	-
-	*Caligus curtus*	Cod louse
-	*Caligus elongatus*	The Scottish louse
-	*Gadus morhua*	Atlantic cod
-	*Lepeophtheirus salmonis*	Salmon louse
-	*Oncorhynchus mykiss*	Rainbow trout
-	*Salvelinus alpinus*	Arctic char
-	*Salmo salar*	Atlantic salmon
-	*Salmo trutta*	Brown trout
-	*Pollachius pollachius*	Pollock

Chemicals:

Abbreviations	Full Name
IMI	Imidacloprid
IMI-d4	Imidacloprid-d4
MeOH	Methanol
FA	Formic acid

Others:

Abbreviations	Full Name
$[IS]_f$	Concentration internal standard
$[X]_f$	Concentration analyte
95% CI	95% credible interval
A_{IS}	Signal to internal standard
A_X	Signal analyte
Cell Acc	Cell Accelerator Voltage
C_i	Internal concentration
CNS	Central nervous system
C_w	External concentration
D_w	Scaled damage
EC	Effective median concentration

EFSA	European Food Safety Authority
ESI	Electron spray ionization
GUTS	General Unified Threshold models of Survival
IMR	Institute of Marine Research
IS	Internal standard
IT	Individual Threshold
k_e	Elimination of toxin
k_i	Intake of toxin
LC	Liquid chromatography
LC	Lethal median concentration
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass to charge ratio
MS	Mass spectrometry
MT	Metric ton
nAChRs	Nicotinic acetylcholine receptors
q	Collision cell
QqQ	Triple quadruple
RED	Reduced
RF	Response factor
S/N	Signal to noise ratio
SD	Stochastic Death
TKTD	ToxicoKinetic-ToxicoDynamic
t_R	Retention time

Abstract

The Norwegian aquaculture is critically important to the country's industry and is the world largest exporter of Atlantic salmon (*Salmo salar*). However, the industry's scale has led to an increase of louse pressure on both farmed and wild populations of salmonid species, with salmon louse (*Lepeophtheirus salmonis*) and Scottish louse (*Caligus elongatus*) having the greatest impact. Therefore, there are strict regulations in place to prevent louse infestations. To adhere to these regulations the industry uses chemotherapeutants and non-medical treatments. The chemotherapeutant imidacloprid was first approved for use in Norwegian aquaculture in 2021. Imidacloprid has a moiety resembling nicotine, which acts as a neurotoxin selectively targeting the insect's central nervous system nicotinic acetylcholine receptors. Toxicity of imidacloprid has been extensively studied for insects and aquatic species, but little data for toxicity on marine species.

In this study the acute toxicity of imidacloprid on the marine crustacean *Acartia tonsa,* using a 96-hours constant exposure and two pulse exposures, with a 24-hours and 72-hours intermediate non-exposure periods. Additionally, the stability of imidacloprid in saltwater solution was assessed during the 96-hours exposure. Raw data was further used to calculate LC and EC and make TKTD-RED-GUTS model. Experiments showed imidacloprid to be stable during the 96-hours and *Acartia tonsa* experiencing both immobilization and mortality in the 96-hours exposure, with EC_{10} = 0.06 mg/L, EC_{50} = 1.2 mg/L and EC_{90} = 25 mg/L after 24-hours and LC_{10} = 0.0021 mg/L, LC_{50} = 0.10 mg/L and LC_{90} = 4.3 mg/L after 96-hours. Both TKTD-RED-GUTS were poor fits compared with obtained mortality. The result obtained indicates *Acartia tonsa* would experience little effects based on treated release concentration, 0.30 µg/L, from the aquaculture. However, acute exposure from untreated treatment water, 20 mg/L, of imidacloprid could potentially have a significant impact on wild *Acartia tonsa* and potentially other non-target copepods.

Table of Contents

1 Introduction

1.1 Norwegian aquaculture

Norway is the biggest producer of Atlantic salmon (*Salmo salar*) in the world (Overton et al., 2018; Taranger et al., 2015). Following Norway at 55.3%, the other top producers in 2015 were Chile with 25.4%, Scotland at 7.6%, Canada contributing 6% and the Faroe Islands at 3.3%, as reported by Iversen et al (2020). This distribution highlights Norway's substantial lead in the industry. The aquaculture industry is an important part of Norway's export sector, having seen a remarkable growth since the 1970's (Statistisk sentralbyrå, 2020a). Notably, from 1994 to 2022 the sale of Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) went from 204 685 metric ton (MT) and 14 571 MT to 1 564 948 MT and 85 223 MT, respectively (Fiskeridirektoratet, 2023). The Norwegian aquaculture includes farming several types of marine species. Including Atlantic salmon, rainbow trout, Artic char (*Salvelinus alpinus*) and Atlantic cod (*Gadus morhua*) (Statistisk sentralbyrå, 2020a; Taranger et al., 2015). In 2019 alone, Norwegian aquaculture industry sold nearly 1,36 million tons of Atlantic salmon, yielding around 67,9 billion Norwegian kroners (Statistisk sentralbyrå, 2020a). In addition to Atlantic salmon, the rainbow trout is the second largest export product (Statistisk sentralbyrå, 2020b)

Numerous Norwegian aquaculture farms are strategically located near rivers and in the fjords, which serve as a natural migration route for wild Atlantic salmon and habitants for other salmonid populations (Overton et al., 2018). However, this proximity presents several environmental challenges for the industry (Taranger et al., 2015). These challenges include the risk of farmed fish escaping into the wild and the heightened pressure from parasitic salmon louse on wild populations (Overton et al., 2018; Taranger et al., 2015). The consequences of escaped fish includes the potential risk of them migrating alongside wild populations upriver and breeding, genetically altering the wild population's gene pool (Taranger et al., 2015). Additionally, the increased pressure from louse can cause extra stress on the salmonid population, leading to harm and gateway for diseases. Salmonids with moderate to high infestation rates may suffer from physical damage, skin erosion, osmoregulatory failure, secondary infections, immunosuppression and chronic stress (Overton et al., 2018).

1.2 Ectoparasites

Along the Norwegian coastline there are several different natural occurring louse species, ranging from those specialized for specific host species to more generalist species that target a broader range of hosts (Havforskningsinstituttet, 2018c). The three most common species in Norway are the cod louse (*Caligus curtus*), Scottish louse (*Caligus elongatus*) and salmon louse (*Lepeophtheirus salmonis*). The cod louse and salmon louse mainly attaches to individuals in the *Gadidae* and *Salmonids* families. Morphologically similar, distinguishing between them based only on observations can be challenging. The most reliable method of differentiation is examining the host species or conducting genetical analysis of the louse. Scottish louse, the most prevalent species in Norway, can attach itself to a wide range of different species. All from salmon to pollock (*Pollachius pollachius*) and cod (Havforskningsinstituttet, 2018c). Unlike the other two species Scottish louse is relatively smaller in size but exhibits greater mobility, enabling it to transfer more easily from one fish to another (Havforskningsinstituttet, 2018b).

1.2.1 Lifecycle to *Lepeophtheirus salmonis*

Salmon louse has the greatest impact on Norwegian Atlantic salmon farms, with Scottish louse also reported in large numbers (Escobar-Lux, 2021). Due to the impact, and similar stages 2 nauplii stage and infective copepods stage between louse species (Hamre et al., 2013), the lifecycle of salmon louse is described in detail. Salmon louse is a natural occurring parasitic species found in the cold waters of the northern hemisphere (Irish Marine Institute, 2022). It inhabits the upper layers of the water masses in the ocean (Havforskningsinstituttet, 2018a), and is specialized in attaching itself externally to various salmonid species along the Norwegian coast, this includes Atlantic salmon, sea trout and artic char (*Salvelinus alpinus*). Additionally, the rainbow trout, an imported species, has been reported as a host as well (Havforskningsinstituttet, 2018c).

The lifecycle of salmon louse compromises 8 different stages (Escobar-Lux, 2021; Havforskningsinstituttet, 2018a) (Fig. 1). During the initial three stages, the louse is unattached to any hosts and swims freely in the ocean. The first two stages the louse is called a nauplius 1 and 2 (A.Schram, 1993; Havforskningsinstituttet, 2018a). Depending on the water temperature, nauplii stage 2 molt into stage 3, the copepodid, within approximately 2 to 14 days (Irish Marine Institute, 2022). In these stages the louse does not feed, relying instead on its yolk sac for survival (A.Schram, 1993). If the copepodite fails to find a host, it will eventually die of starvation. Its survival without a host is dependent of the water temperature, with typical survival period ranging from one week all up to a month (Havforskningsinstituttet, 2018a). Upon finding a host, the copepodite attaches itself to the outer skin using its antenna (A.Schram, 1993). As it feeds on the mucus, skin and blood of its host (Havforskningsinstituttet, 2020; Overton et al., 2018), it will molt into the first chalimus stage (Havforskningsinstituttet, 2018a; Irish Marine Institute, 2022). In both chalimus stages, it remains attached in the same location. As the chalimus 2 grows, females and males can be distinguished based on their appearance (Fig. 1). The chalimus stages typically last approximately 10 days for stage 1 and 15 days for stages 2 before molting into the preadult stage. The preadult stages 1 and 2 are mobile stages during which the louse can move freely on the host (Havforskningsinstituttet, 2018a; Irish Marine Institute, 2022). The duration of the preadult stages differs between females and males, approximately 10 to 12 days for preadult female and 8 to 9 days for preadult male, respectively at an average water temperature of 10℃ (Irish Marine Institute, 2022). Due to their faster growth, adult males are often observed attached to preadult female to ensure they are the first to mate (Havforskningsinstituttet, 2018a). Full-grown females are larger in size than the males and produce two large external egg sacs (Fig. 1), each containing roughly 150 – 400 eggs (Havforskningsinstituttet, 2018a). These eggs hatch within the sacs, releasing newborn nauplii into the ocean. The total duration of one generation varies with water temperature but typically ranges from approximately 4 to 9 weeks at temperatures ranging from 6℃ to 18℃ (Irish Marine Institute, 2022).

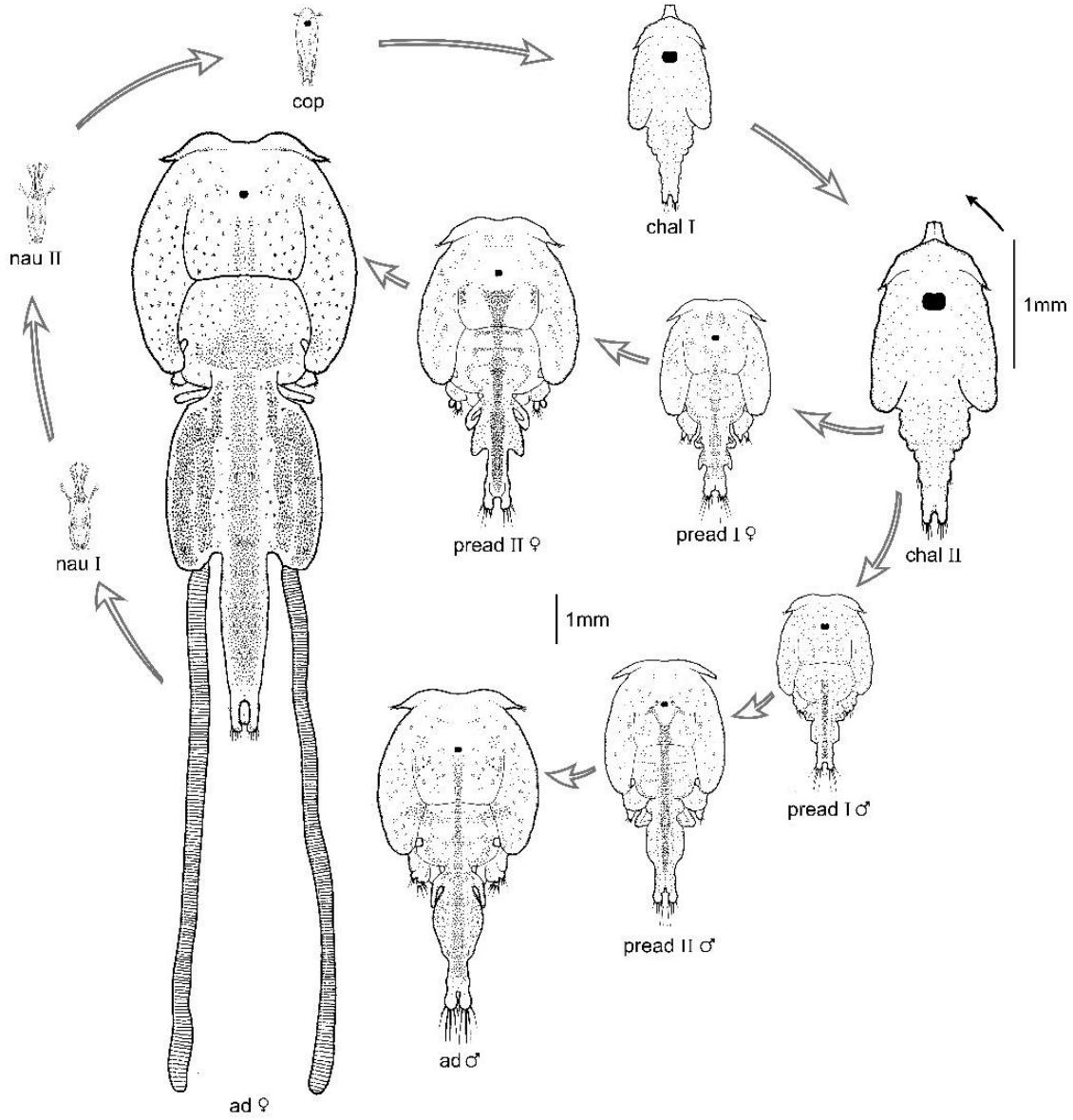


Figure 1: Life stages of Lepeophtheirus salmonis (Centre, 2020).

1.3 Delousing treatments

Salmonids grown in Norwegian farms are protected under the animal's welfare law (Dyrevelferdsloven, 2009), and must be treated accordingly by law. Regulations governing salmon louse control (lakselusbekjempelse, 2012) specify that the industry is obliged to minimize the impact and extent of damage to both farmed and wild salmonids. Therefore, farms are required to develop and implement a control plan accordingly (lakselusbekjempelse, 2012). Commonly used measurements to combat louse are divided into two main methods: chemotherapeutants and non-medicinal delousing operations (Overton et al., 2018). Chemotherapeutants, anti-parasitic agents, are often administered one of two approaches: through bath treatments and in-feed additives (Escobar-Lux et al., 2020; Overton et al., 2018; Parsons et al., 2020).

Compounds approved for the usage in bath treatments in Norway include the organophosphates azamethiphos, the pyrethroids deltamethrin, hydrogen peroxide and the neonicotinoid imidacloprid (IMI) (Escobar-Lux et al., 2020; Grefsrud et al., 2024). Bath treatments are performed by either directly administering into the cage and consequently dispersed directly into the ocean (Grefsrud et al., 2024), or reducing the water volume for the salmonids, increasing the density of animals, achieved by either lining the cage with tarpaulin or transferring the salmonids onto a well-boat (Parsons et al., 2020). The recommended treatment concentration is administered, and salmonids are immersed in the bath for the prescribed treatment duration. Following the treatment tarpaulin is removed or the animals are transferred back into the cage (Parsons et al., 2020). The treatment water is then released into the ocean while the well-boat is moving, except when treated with IMI, then treatment water is transferred into a new treatment-boat (Grefsrud et al., 2024). IMI is administered in the well-boat at a concentration of 20 mg/L with a exposure duration of 60 minutes (Grefsrud et al., 2022). Treatment water is treated in the treatment-boat to ensure that when released back in the ocean, the concentration of IMI is less than 0.30 µg/L (Grefsrud et al., 2022; Veterinærkatalogen). Approved compounds for in-feed in Norway are emamectine benzoate and teflubenzuron (Escobar-Lux et al., 2020; Grefsrud et al., 2024), which spread to the surrounding environment by feed or fecal waste, currents and size of particles determine the speed and spreading. The delousing compounds can persist for extended duration on the seafloor (Grefsrud et al., 2024). In 2022 were the total amount of prescribed medications used in Norwegian aquaculture was: 577 kg azamethiphos, 3 kg deltamethrin, 5900 kg IMI, 3900 MT hydrogen peroxide (100%), 79 kg emamectine benzoate and 732 kg teflubenzuron (Grefsrud et al., 2024). Salmon louse belongs to the taxonomical group crustaceans, therefore delousing chemicals released into the environment could affect other crustacean species referred to as non-target-species (Grefsrud et al., 2024). Non-medicinal operations include a variety of alternative methods to chemotherapeutants: depth-based preventions, cleaner fish, thermal delousing, mechanical delousing and freshwater delousing (Coates et al., 2021; Overton et al., 2018).

1.4 Imidacloprid

Imidacloprid (IMI) has the IUPAC name (NE)-N-[1-[(6-chloropyridin-3-yl)methyl]imidazolidin-2-ylidene]nitramide, the molecular formula $C_9H_{10}ClN_5O_2$ and molecular weight 255.66 g/mol (National Center for Biotechnology Information, 2024b) (Fig. 2). IMI has a reported water solubility in the range of 580 to 610mg/L at 20°C (Grefsrud et al., 2022; McHenery, 2022; Picone et al., 2022). Additionally, n-octanol-water partition coefficient (Log K_{OW}) reported in range from 0.57 to 0.63 (Frew et al., 2018; Grefsrud et al., 2022; McHenery, 2022; National Center for Biotechnology Information, 2024b). Log K_{OW} describes the ratio of concentrations of a compound in a 1-octanol phase divided by the phase water in equilibrium, with a typical range from -3 (very hydrophilic) to 10 (extremely hydrophobic) (Cumming & Rucker, 2017). Log K_{OW} is used to assess bioaccumulation and toxicity of a compound (Cumming & Rucker, 2017). Based on Log $K_{OW,}$ IMI exerts a moderate degree of hydrophobic character (Cumming & Rucker, 2017; Frew et al., 2018). Classified as a neonicotinoid insecticide, IMI finds applications ranging from agricultural to household pesticides used against piercing-sucking and chewing insects and invertebrates (Motaung, 2020; Sheets, 2010; Wang et al., 2018). IMI contains a 6-chloro-3-pyridyl moiety that resembles the compound nicotine (Motaung, 2020), and therefore acts as a neurotoxin which that selectively targets the insect's central nervous system (CNS) nicotinic acetylcholine receptors (nAChRs) (Anjos et al., 2021; Motaung, 2020; Wang et al., 2018). Studies have shown a lower toxicity of IMI towards vertebrates compared to insects (Sheets, 2010; Wang et al., 2018), due its presumed higher selectivity towards insects' nicotinic cholinergic receptors compared to vertebrate species (Wang et al., 2018). Introduced to the commercial market by Bayer CropScience in 1991, IMI has since been used globally (Motaung, 2020; Sheets, 2010). It was approved for agricultural use on seed treatment and granules by EU in 2008 (EFSA, 2016), based on a risk assessment performed by the European Food Safety Authority (EFSA) finalized on 29th of May 2008 (EFSA, 2008). In 2013, the use of IMI was restricted to limit the usage to protect bees and other pollinators, prohibiting its use on crops attractive to bees except certain applications in permanent glasshouses and for winter cereals (EFSA, 2016). IMI were first approved as delousing of salmon louse infestation treatment for commercial use in the Norwegian salmon industry in 2021 (Grefsrud et al., 2022), marketed under the commercial name Ectosan Vet (Grefsrud et al., 2022; Veterinærkatalogen).

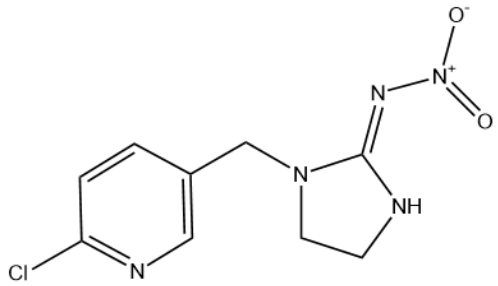


Figure 2 Structure of imidacloprid (McHenery, 2022; National Center for Biotechnology Information, 2024b)

1.5 Experiment animal – *Acartia tonsa*

Figure 3 Picture of A. tonsa *in copepod stage.*

A. tonsa (Fig. 3) is a small planktonic copepod found abundantly in coastal waters worldwide, except in polar regions (Drillet, 2010; Falkenhaug T, 2018; Hung, 2014). Its length ranges from 0.5 to 1.5 mm, depending on the life stage (Falkenhaug T, 2018). Due to the small size, *A. tonsa* and along with other zooplankton species, a natural food source for larvae and juvenile fish in the wild (Lahnsteiner et al., 2009). In aquaculture, the nauplii stages of copepods are often utilized as a primary live feed source in larvae phase and copepods stages for juvenile phase for fish (Ajiboye et al., 2010; Lahnsteiner et al., 2009). *A. tonsa* can tolerate temperatures ranging from 0℃ to 30℃, but require a minimum temperature of around 10℃ for eggs to hatch (Falkenhaug T, 2018; Hung, 2014). Unfavorable environmental conditions can lead to the dormancy of eggs, until conditions becomes more suitable (Drillet, 2010). Eggs can therefore be stored for future use at temperatures around 2℃ to 3℃ for up to 12 months (Hung, 2014).

1.6 TKTD-RED-GUTS model

Static constant concentration exposure for a finite duration reflects poorly on realistic environment exposure pattern for a toxin (Focks et al., 2018). To assess the risk a toxin poses to an aquatic or marine environment, mechanistic toxicokinetic-toxicodynamic (TKTD) models can be applied to laboratory toxicity exposure to predict the survivability and toxic effect over time (Bauer et al., 2024; Ockleford et al., 2018). Simple statistical form of TKTD models include Lethal median Concentration (LC) and Effective median Concentration (EC) models. LC and EC are relative values which describe mortality and non-lethal effects of a toxic on a species. Often represented as LC_{50} and EC_{50}, these will be the concentration of a toxic substance to be lethal or have an effect on 50 percent of the test animals experiencing exposure for the given time point.

TKTD follows the general aspect of how the internal concentration of a toxin affects the organism (Fig. 4) (Ockleford et al., 2018). Toxicokinetic (TK) generally describes the uptake and breakdown of a toxin into the organism, including absorption, distribution, metabolism and elimination (Bauer et al., 2024; Ockleford et al., 2018). For aquatic animals toxin uptake route from a water phase includes animal surface, external or internal gills or/and food intake

(Ockleford et al., 2018). The dynamics of internal concentration (C_i) can be expressed with a relation with external concentration (C_w) based on intake (k_i) and the elimination of toxin (k_e) over time (Jager et al., 2011; Ockleford et al., 2018):

$$\frac{dC_i(t)}{dt} = k_i C_w(t) - k_e C_i(t)$$

Some toxicodynamic (TD) approaches directly link the internal concentration with mortality, thereby survivability (Jager et al., 2011). Alternative approaches describe the mechanics of internal concentration with the effects on an organism, whereas toxic damage and ability to repair the damage at certain rates, which lead to mortality over time (Bauer et al., 2024; EFSA, 2016; Jager et al., 2011).

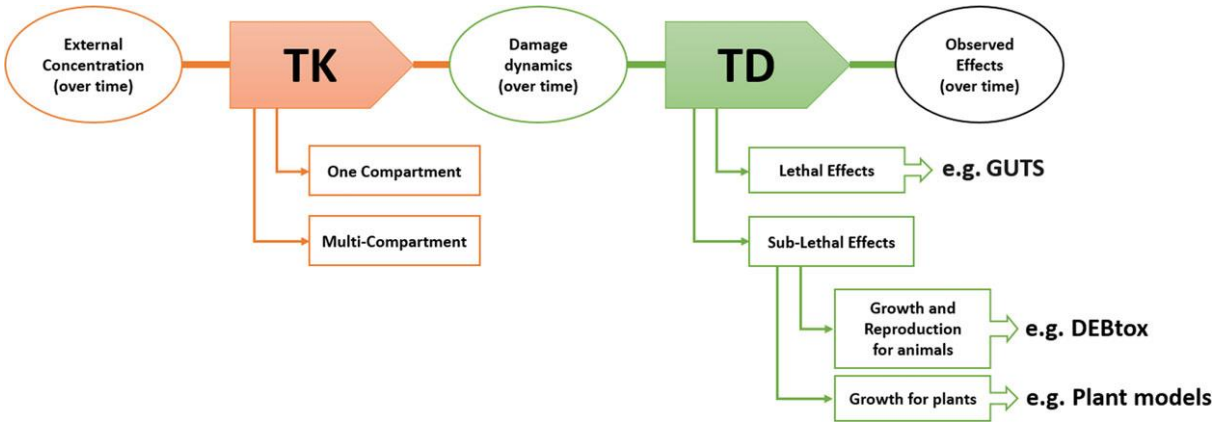


Figure 4: A schematic representation of a toxicokinetic.toxicodynamics (TKTD) model, where GUTS stands for a general unified threshold model of survival (Ockleford et al., 2018).

To further address the risk assessment on a toxin, hence survivability, the general unified threshold model of survival (GUTS), addressing damage dynamic on organism based on external concentration, can be utilized in TKTD models (Ockleford et al., 2018). The aim of TKTD-GUTS is to predict survival rate in untested exposure conditions, such as different time and concentration exposures, which can be impractical or impossible to recreate in laboratory experiments (Ockleford et al., 2018). Mortality in TKTD-GUTS is assumed to follow either two mechanisms: Stochastic Death (SD) and Individual Tolerance (IT) (Bauer et al., 2024; Jager et al., 2011; Ockleford et al., 2018). SD mortality mechanism assumes all individuals of a population have a fixed threshold for lethal effects (z_w), thereby assuming organisms have an identical probability of dying (Bauer et al., 2024; Ockleford et al., 2018). Implying mortality is random and increases as damage rises above the threshold (Ockleford et al., 2018). The IT approach implies organisms in a group have different threshold of scaled

damage for experiencing lethal effects (Ockleford et al., 2018). Thereby individuals have different sensitivity, consequently there is a 50% mortality when internal concentration reaches the median threshold distribution of the group (m_w) (Jager et al., 2011).

The simplest form of toxicokinetic model is a one-compartment model (Jager et al., 2011). A first order kinetic model is called reduced GUTS (GUTS-RED), and accounts for the uptake of chemical from the medium and links the external concentration directly to the scaled damage (Ockleford et al., 2018). The scaled damage is therefore determined without measuring the internal concentration, and directly from the observed survival data (Ockleford et al., 2018). The dynamics of scaled damage ($D_w(t)$) is expressed in relation to the dominant rate constant (k_D) and external concentration (C_w), with scaled damage expressed in same unit as concentration (Ockleford et al., 2018):

$$\frac{dD_w(t)}{dt} = k_D * (C_w(t) - D_w(t))$$

1.7 LC-QqQ-MS/MS

An LC-QqQ-MS/MS is an analytical system compromising a liquid chromatography (LC) system, an ionization source and a triple quadruple (QqQ) mass spectrometry (MS) analyzer (Harris, 2020). The primary objective of LC-QqQ-MS/MS analysis is twofold: it serves to quantify the concentration of analytes within a given sample, or to confirm the presence of specific compounds. This dual-purpose approach ensures both the accurate measurement and the precise identification of substances in the sample.

1.7.1 Reverse phase LC

LC is a separation technic, in which an eluent solution is pushed through an open or packed column, and compounds in the matrix are separated according to the distribution coefficient between the mobile and stationary phase (Harris, 2020; Pang et al., 2016). Reverse phase LC is a separation technique where the stationary phase compromises a nonpolar or weakly polar component, while the mobile phase a more polar solvent (Harris, 2020; Zuvela et al., 2019). As the mobile phase travels through the column, the time a compound uses to travel through from the time of injection is referred to as retention time (t_R) and determined by the flow rate and time a compound spend in the mobile phase (Harris, 2020).

1.7.2 Tandem mass spectrometry

Mass spectrometry is a technic that measures the mass to charge ratio (*m/z*) of different atoms or molecular ions, with the resulting spectrum showcasing the intensity for each *m/z* (Harris, 2020). This is achieved by accelerating gaseous ions through an electric field and separating them based on their mass to charge ratio. In MS, three essential components are involved; an ion source, a mass/charge separator and a detector (Harris, 2020). Electrospray ionization (ESI) is a widely used ionization source for polar analytes in liquid samples (Harris, 2020; Wilm, 2011). An ESI setup consists of a nebulizer, spray chamber, a vacuum pump, and glass capillary (Harris, 2020) Liquid enters the spray chamber through the steel nebulizer. Within the spray chamber, an electric field is established. Additionally, a constant flow of drying gas, typically N_2-gas, is directed along the nebulizer towards the glass capillary by the vacuum pump, reducing the pressure (Harris, 2020). As a result, the liquid leaving the nebulizer forming fine aerosols of charged particles (Harris, 2020).Tandem mass spectrometry is an MS technic where ions undergo separation by passing through two or more stages of analysis (IUPAC, 2019). For the QqQ MS/MS utilized, two *m/z* separators are connected in series and separated by a collision cell (Agilent Technologies, 2012; Harris, 2020). Both *m/z* separators are referred to as Q1 and Q2, while the collision cell is referred to as q (Agilent Technologies, 2012). The Q represents quadruple and is composed of four parallel metal rods, either hyperbolic or cylindrically shaped (Harris, 2020).

Ions migrating from the ionization chamber to Q1, are referred to as precursor ions (Agilent Technologies, 2012; Harris, 2020). A constant voltage and a radio-frequency oscillating voltage is applied, creating an electric field. The electric field deflects ions, allowing only ions with target *m/z* ratio to pass through Q1. Other ions, referred to as nonresonant ions, collide with the rods and are lost before entering the quadruple ion guide (Harris, 2020). Precursor ions migrating from Q1 pass through the quadruple ion guide and entrance lens. These consist of short quadrupole hyperbolic rods, only exerting an electric field to optimize transmission. Precursor ions collide with an low-reactive gas, often Ar, He or N_2, creating fragments referred to as product ions (Harris, 2020). The hexapole collision cell consist of six rods, to generate a potential difference for ions to be transmitted from the Q1 to Q2 (Agilent Technologies, 2012). Product ions traverse the collision cell and through an exit lens and a quadruple ion guide. The product ions enter Q2, allowing only product ions with target *m/z* ratio to enter the detector (Agilent Technologies, 2012; Harris, 2020). The most sensitive scan for a QqQ-MS/MS is for both Q1 and Q2 to monitor for a specific precursor ion and specific

product ion, referred to as selected reaction monitoring (SRM), where normal operation include running multiple SRM based on the same precursor ion, referred to as multiple reaction monitoring (MRM) (Agilent Technologies, 2012). This is performed by allowing multiple scan on different precursor ions in Q2, allowing to select scan according to expected retention time of the analyte and continuously throughout the chromatogram (Agilent Technologies, 2012).

The detector employed is a high energy dynode detector (Agilent Technologies, 2012). It compromises two dynodes, a conversion and a continuous electron multiplier dynode (Agilent Technologies, 2012; Harris, 2020). The conversion dynode has a set potential to either a negative or positive voltage, attracting cations or anions, respectively (Harris, 2020). When ions strike the conversion dynode an electron eject and migrate toward the electron multiplier. Both dynodes are oriented orthogonally to the ion beam, reducing neutral compounds impacting the dynodes (Agilent Technologies, 2012). Conversion dynode ejects same amount of electron for all ions. When electrons strike the wall, they liberate several electrons and are accelerated toward the more positive end of the horn. These electrons strike the wall several times, enough to make an detectable electrical signal to measure the intensity of the set *m/z* ratio (Harris, 2020).

1.7.3 Internal standard

An internal standard (IS) is a known amount of a compound that is added to the unknown sample, calibration standards, and blanks in quantitative analysis (Harris, 2020). The internal standard should be chemically stable and unreactive to any compounds either in the matrix or with the analyte. The use of stable isotopes, deuterated compounds of the analyte, are particularly useful as these experience similar matrix effects and losses, but has different masses detected by an MS detector (Harris, 2020). Matrix effect is a change in analytical sensitivity due to compounds other than the analyte (Harris, 2020). To calculate the amount of analyte based on IS in chromatography analysis is first done by calculating the response factor (RF) (Harris, 2020). RF is a relative value determined by the relation between the IS and the analyte detected by the detector. This is achieved by preparing a calibration sample with known amount of both IS and the analyte.

1.7.4 LOD and LOQ

Limit of detection (LOD) is the smallest quantity of an analyte that can be measured with a significant difference from a blank sample (Harris, 2020). One aspect to determine LOD and LOQ is based on signal to noise ratio (S/N). If a signal is three times greater than the noise it can be detected (> 3) it is regarded as above LOD. A signal to noise ratio greater than ten (> 10) is the smallest quantity that can be quantified, referred to limit of quantification (LOQ) (Harris, 2020).

1.8 Aim of the study

The objective of this study is to evaluate acute toxicity posed by IMI to the copepod species *A. tonsa*, through static exposures conducted under various concentrations, exposure periods, and repeated exposures. Additionally, to examine marine solutions with IMI for degradation. Data obtained from these experiments is used to calculate LC- and EC-values and make TKTD-RED-GUTS models to further assess how different exposure profile effects *A. tonsa*. The findings from this study will contribute to assessing how the use of IMI in the Norwegian salmon industry may have an impact on the Norwegian marine ecosystem and other non-target invertebrate species.

2 Materials and methods

The methodical description is organized into five sections. The first part details the chemicals and general equipment used. The second part explains the husbandry of the copepod *A. tonsa*. The third part outlines the toxicology experiments conducted at Austevoll on the IMR research facilities. These experiments include a preliminary study, a 96-hour static constant exposure and two pulse exposures with a 24-hour and a 72-hour intermediate period. The fourth part explains the LC-QqQ-MS/MS analysis of water samples from exposures solutions, carried out at Nordnes, Bergen. Finally, the last section describes the statistical analysis and modeling conducted. The flow of the process is shown in Fig. 5.

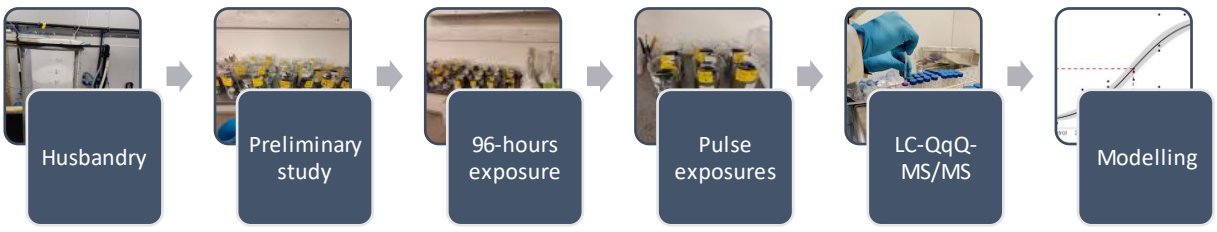


Figure 5: Flowchart of the methodical process.

2.1 General reagents and equipment

Chemicals used in exposure solutions and during LC-MS analysis is listed in Table 1. Equipment used in husbandry and both preparation and during exposure experiments are listed in Table 2 and Table 3, respectively. The equipment used for LC-MS analysis is listed in Table 4. To draw the molecular structures of IMI and IMI-d4 was utilized by ChemDraw (v. 20.1.1.125/ Perkin Elmer, America).

Table 1 *Chemicals utilized for exposure experiments and water sample analysis.*

Chemicals	**CAS Number**	**ATC Number**	**Supplier**	**Purity**
Acetonitrile	75-05-8	-	Honeywell	> 99.90%
Ectosan® Vet	-	QP53A X17	Benchmark Animal Health	1000 mg/g
Formic acid	64-18-6	-	Merck	≥ 98%
Imidacloprid	138261-41-3	-	Sigma Aldrich	≥ 98%
Imidacloprid-d4	1015855-75-0	-	Merck	≥ 99.0%
Methanol	67-56-1	-	Honeywell	≥ 99.9%

Table 2 *Equipment and supplies for husbandry of A. tonsa.*

Equipment/supply	**Description**
O_2 Plate	Ensuring high O_2-saturation
Acartia tonsa eggs	Supplier: CFEED
Airflow source	Ensured constant movement of water
Deep seawater	Deep sea water from facility Filters: 10 µm and 5 µm
Hatching container	See Fig. 7
Heater rod	Fixed temperature ranged from 25 to 26°C
Sensor	To monitor oxygen saturation and water temperature.
Sieve	Mesh: 60 µm, Fig. 6B

Table 3 *Equipment for exposure experiments.*

Equipment	**Description**
Analytic weight	± 0.1 mg
Beaker	1000 mL, Glass
	2000 mL, Glass
Counter	Counting animals
Deep seawater	Deep sea water from facility
	Filters: 10 µm and 5µm
Dissecting microscope	Monitoring and counting animals
Measuring cylinder	100 mL, Glass, ± 0.5 mL at 20℃
	250 mL, Glass, ± 1.0 mL at 20℃
	2000 mL, Plastic, ± 10 mL at 20℃
Needle	Monitor copepods state
Pasteur pipette	5 mL, Plastic
PVC pipe	Size: 7.5 cm in diameter and approximately 10 cm to 12 cm tall
	Mesh: Nitex, 50 µm
Volumetric flask	500 mL, Glass, ± 0.25 mL at 20℃
	2000 mL, Glass, ± 0.6 mL at 20℃
Zooplankton counting chamber	Grooves to place treatment water with copepods in, for monitoring and counting.

Table 4 *Equipment for LC-QqQ-MS/MS analysis.*

Equipment	**Description**
Analytic weight	± 0.1 mg
	± 0.01 mg
Auto pipette	10 – 200 µl
	200 – 1000 µl
Centrifugal sample glass with Teflon cap	10 mL
	25 mL
Dark vials with tread caps	1.5 mL
Pasteur pipette	1 mL, Plastic
	5 mL, Plastic
Syringe with filter	Nylon 13mm, Non-sterile, Size: 0.45 µm
	RephiLe Bioscience
Volumetric flask	10 mL, Glass, ± 0.04 mL at 20℃
Whirl mixer	Mixing solutions to homogenous solutions

2.2 Husbandry of *Acartia tonsa*

The eggs of *A. tonsa* were purchased from the Norwegian company CFEED (Fig. 6A). The husbandry procedures outlined by CFEED (Appendix B) were strictly adhered to throughout the experiment. The eggs were carefully transported in a glass container, as depicted in Fig. 6A, where they were intermingled with clay to ensure their preservation. This method of packaging is designed to maintain the integrity and viability of the eggs during transit. These glass containers were promptly stored in a refrigerator at a temperature around 1 - 4°C, for maintaining optimal conditions until the exposure experiments. The eggs could be safely stored at this temperature for up to a two weeks period for optimal hatching success (CFEED). Given the multiple exposures, set ups conducted, approximately half of the content from each container was utilized for each hatching batch. To prepare the eggs for hatching, they were carefully separated from the clay by passing the mixture through a sieve and thoroughly rinsing with filtered seawater (Fig. 6B). The used sieve had a mesh size of 60 µm, ensuring that the clay particles passed through while retaining the eggs in the sieve (Fig. 6C).

Figure 6: Transferring and preparations of the eggs in preparation of husbandry. A): Eggs of A. tonsa *in glass container from distributor. B): Sieve with a 60 µm mesh. C): Rinsing of* A. tonsa *eggs. Eggs in sieve and clay rinsed off in the bucket.*

Next, the eggs were transferred to the hatching tank (Fig. 7). Prior to the transfer, the tank was filled with approximately 20 L of filtered seawater. To ensure optimal conditions for hatching, continuous aeration was maintained via a hose positioned at the tank's bottom. Not only did this serve to maintain oxygen levels but also to prevent the eggs from settling to the tank's bottom or sticking on its walls. To monitor oxygen saturation and water temperature, a sensor was submerged in the tank. The temperature was held constant around 25.5 ± 0.5℃ with the use of a heater rod and the oxygen saturation held around 95 – 100% with the use of a O_2 gas in the aeration.

Figure 7: The hatching tank for A. tonsa, *with airflow and sensor attached.*

The hatching period for each batch typically ranged between 24-hours and 48-hours from the moment the eggs were placed in the tank, depending on the batch size. Upon hatching, nauplii were transferred to a holding tank (Fig. 8) using buckets. This was performed by emptying the hatching tank by opening a valve in the bottom, ensure collection of all nauplii. The animals were held in the holding tank for further development and easy access for the exposure experiments. The holding tanks were situated in a room where the air temperature was maintained at the range from 15℃ to 17℃. To ensure stable and optimal water conditions by having a constant supply of clean seawater, the tanks were equipped with a flowthrough system, with a replacement flow set to approximately 30 L/hour. To help reduce temperature loss the container was insulated (Fig. 8).

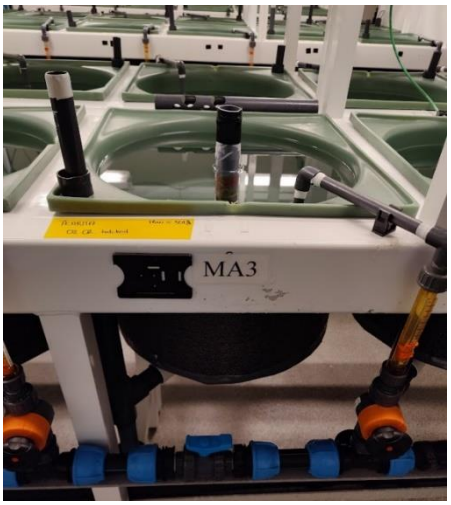


Figure 8: The holding tank. with flowthrough system on the right side and insulation

The copepods were fed daily, once in the morning and once in the afternoon with the red algae from the genus *Rhodomona*. For the morning feeding the waterflow was halted for 2-3 hours, while feeding occurred to prevent a loss of algae through the flowthrough system. The second feeding was administered with continuous water flow.

2.3 Exposure of IMI experiments

2.3.1 Preparation of holding chambers

To achieve an effective transfer and ensure more efficient control over the copepods, holding chambers were utilized. Copepods were placed in these chambers in the exposure experiments preparations, and in the initiation for exposure were the chambers moved into the water bath. To build the holding chambers, PVC pipes were used, with one side closed with a 50 µm Nitex mesh fixated with the use of a glue gun (Fig. 9). The mesh was securely glued all around to prevent potential escape of copepods. The mesh size was chosen for its suitable size in comparison to the copepods size, and therefore ability to prevent escapes while allowing efficient water flow. All chambers underwent a thorough cleaning process and left to airdry. Furthermore, before their initial use and subsequent uses, pipes were rinsed with freshwater.

Figure 9: Holding chambers made out of PVC pipes with 50µm Nitex mesh.

2.3.2 Preparation of Replicates

The copepods were collected from the holding tank and with the use of a dissecting microscope the copepods were randomly picked with a pipette and placed in a holding chamber and kept in tempered (~14°C - 16°C) filtered seawater (Fig. 10). Their life stage and condition were monitored, and only animals in the adult stage were selected. This procedure was repeated for each replicate, until each reached the target number of individuals.

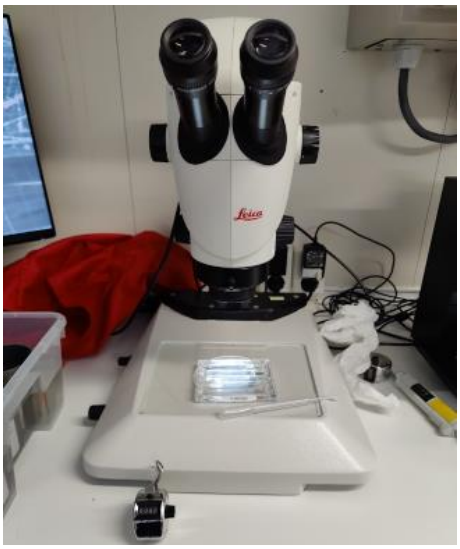


Figure 10: Counting and separating A. tonsa *on a zooplankton counting chamber with a pipette under a magnifying glass.*

2.3.3 Preparation of solutions

From previous studies conducted on the seawater parameters used at IMR research center at Austevoll, were measured salinity at 34.7 ppt and measured mean pH at 7.9. The amount of Ectosan® Vet used for stock solution is summarized in Table 5. For each replicate the necessary volume to submerge the holding chamber in the exposure beakers was approximately 0.4 L of the IMI solution. Additionally, this ensured enough solution for water sampling, approximately 40 mL for each replicate for further analysis. The control group compromised filtered seawater without Ectosan® Vet. Throughout the solution preparation process, each volumetric flask was meticulously wrapped in aluminum foil to ensure the stability of Ectosan® Vet and no light induced degradation occurred (Fig. 11).

Figure 11: The volumetric flask wrapped in aluminum foil.

Table 5: *The Amount of Ectosan® Vet utilized for the preparation of stock solutions*.

Experiment	Amount Ectosan® Vet (mg)
Preliminary	40.0
96-hour	100.5
24-hour Pulse-A First exposure	100*
72-hour Pulse-A First exposure	99.9
24-hour Pulse-B Second exposure	100*
72-hour Pulse-B Second exposure	100.1

** Amount of Ectosan® Vet was not recorded, but in the range of 100.0mg ± 0.5mg.*

2.3.4 Setups of exposure experiments

To determinate the concentrations to use in the experiments, a preliminary study was performed, followed by a 96-hour exposure, a 24-hours pulse exposure and a 72-hours pulse exposure. The reasons for the preliminary were a combination of testing at which concentration had which effect on the animals, but also to evaluate out what procedure for making the different solutions was the better option. The other exposures were designed to examine how different time factors and durations of IMI exposure affected *A. tonsa*.

2.3.4.1 Preliminary study, 1-hour exposure

For the preliminary study 6 different concentrations were used: 20 mg/L, 2 mg/L, 0.2 mg/L, 0.02 mg/L, 0.002 mg/L, 0.0002 mg/L IMI and a control group. The 20 mg/L and 2 mg/L were prepared in the laboratory. Initially, the highest concentration (Table 6, Nr. 1) was prepared by measuring 40.0 mg of Ectosan® Vet on a measuring scale. This amount was then carefully transferred into a 2000.0 mL volumetric flask, which was sealed with an aluminum foil after adding a magnetic rod. The flask underwent stirring on a magnetic stirrer for 30 minutes to dissolve all of the Ectosan® Vet. 200.0 mL of this solution was extracted and transferred to a new 2000.0 mL volumetric flask where it was then diluted with filtered seawater to the target volume. The remaining solutions were prepared by extracting 200 mL into a bucket and diluting them with 1800 mL filtered seawater (Table 6, Nr. 3 - 6). The solutions were thoroughly stirred to ensure a homogenous mixture before repeating the process for the rest of the solutions.

For this study, animals from batch Nr. 1 were used (Appendix A), with two replicates allocated for each concentration and control group. The decision to have only 2 replicates was made to minimize waste, as the primary aim was to evaluate the procedure and the chosen range of concentrations. Upon commencement of the exposure, each holding chamber was placed in their respective beakers and the *A. tonsa* were exposed for 1 hour. At the end of the exposure period, each holding chamber was transferred to a new beaker with fresh filtered seawater. The first two rows (Fig. 12) were the beakers for each concentration containing Ectosan® Vet, while the next two rows contained only filtered seawater. Afterwards all animals within each replicate were observed and recounted in an orderly manner, following the same procedure employed during preparation stage.

Figure 12: Experimental set up for the preliminary exposure. The first two rows contain the PVC-pipes submerged in their target concentration of Ectosan® Vet. The last two rows contain only filtered sea water.

Table 6. *Plan for making solutions and dilution series of Ectosan® Vet to make the different target concentration for the preliminary study.*

Solution number	Target concentration (mg/L)	Amount Ectosan (mg)	Dissolved in seawater (L)	Dilution from solution number	Volume from previous solution (ml)	Diluted with seawater (mL)	Total volume of solution (mL)
1	20	40	2000	-	-	-	2000
2	2	-	-	1	200	1800	2000
3	0.2	-	-	2	200	1800	2000
4	0.02	-	-	3	200	1800	2000
5	0.002	-	-	4	200	1800	2000
6	0.0002	-	-	5	200	1800	2000

2.3.4.2 96-hours exposure

For the 96-hour exposure, preparation began with the making of a stock solution (Table 7, Stock), with a target concentration of 200 mg/L IMI in the laboratory. This stock solution was meticulously made by measuring 100.0 mg of Ectosan® Vet on a measuring scale and transferring it into a 500.0 mL volumetric flask, which was then filled with filtered seawater. After wrapping the flask in aluminum foil and adding a magnetic rod, it was placed on an automatic stirrer and stirred for 30 minutes without heating. Following this solution 1 (Table 7, Nr. 1) was prepared by extracting 200.0 mL from the stock solution and transferring it into a new 2000.0 mL volumetric flask and diluting it with filtered seawater till the target volume of 2000.0 mL was reached. This process was repeated for each concentration.

The design of the 96-hours exposure experiment involved the utilization of copepods from batch Nr. 1 (Appendix A). Copepods were exposed to IMI continuously over the 96-hours period. The copepods were placed in the holding chamber, with approximately 20 animals in each chamber. The experimental setup included a control group and six different concentrations of IMI ranging from 0.0002 mg/L to 20 mg/L, each with 4 replicates (Fig. 13A). At the start of the exposure each chamber was immersed in their respective beaker filled with the corresponding concentration. The static exposure had a constant temperature (13-15°C), with a progressive decrease in the O_2-saturation after 96-hours. The O_2 levels were monitored not to drop below an 80% saturation. Both parameters were monitored every 24-hours.

Every 24-hours, each replicate was assessed, both immobilization and mortality were monitored. This involved removing the animals from the holding tanks using a pipette and placing them on the counting chamber while ensuring they remained in their respective solution to prevent interruption of the exposure. After the counting, all the animals returned to their holding chamber, and the counting chamber was washed and dried. This step was repeated for every replicate. Upon completion of exposure duration, all replicates were transferred to a new beaker with clean seawater (Fig. 13B), to ensure simultaneously end of exposure.

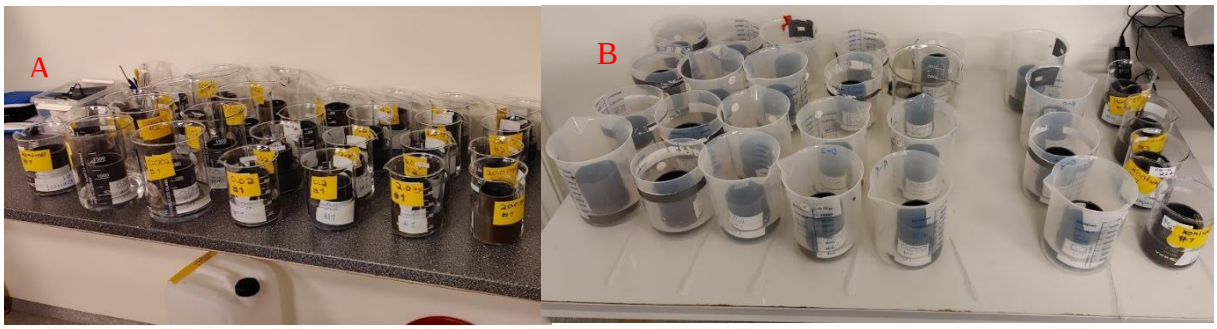


Figure 13: Experimental set up for the 96-hours exposure. A) Every replicate of each concentration of Ectosan® Vet and control group. Control group to the left and nominal concentration 20mg/L the right. B) Beakers with clean filtered seawater. Control group to the left and nominal concentration 20mg/L to the right.

Table 7. *Plan for making solutions and dilution of Ectosan® Vet to make the different target concentration for the 96-hours exposure experiments, and for 24-hours and 72-hours pulses.*

Solution number	Target concentration (mg/L)	Amount Ectosan (mg)	Dissolved in seawater (mL)	Dilution from solution number	Volume form previous solution (ml)	Diluted with seawater (mL)	Total volume of solution (mL)
Stock	200	100.0	500.0	-	-	-	500.0
1	20	-	-	Stock	200.0	1800.0	2000.0
2	2	-	-	1	200.0	1800.0	2000.0
3	0.2	-	-	2	200.0	1800.0	2000.0
4	0.02	-	-	3	200.0	1800.0	2000.0
5	0.002	-	-	4	200.0	1800.0	2000.0
6	0.0002	-	-	5	200.0	1800.0	2000.0

2.3.4.3 3-hours pulse exposures

The experiment compromised two sets of pulse like exposures, each involving a post-exposure intermediate phase and a subsequent exposure. Both sets were prepared by first making a stock solution (Table 7, Stock), and concentrations of 20 mg/L and 2 mg/L were achieved by doing a series dilution (Table 7, Nr.1 – Nr. 2). Two replicates were conducted for each concentration (Fig. 14).

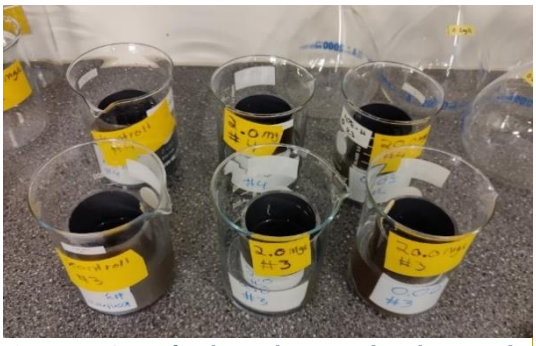


Figure 14: Setup for the 24-hours and 72-hours pulse exposures. To the left is the control group and to the right is the 20 mg/L.

As these exposures included an intermediate phase with new seawater, one for each group. The solutions were prepared for each corresponding time, once for each exposure during both pulses. Copepods from batch Nr. 3 (Appendix A), were used for this experiment. Each pulse included concentrations 20 mg/L and 2 mg/L along with a control group, with two replicates each. In 24-hours pulse copepods were first exposed to IMI for 3 hours. After this period, all replicates were transferred to clean filtered seawater, and kept there for a 24-hours period. Subsequently, they were exposed again for 3 hours to newly prepared solutions with their target concentrations. In 72-hours pulse, the setup was identical, except for a longer 72-hours period in clean seawater before the second exposure.

2.3.5 Observation of mortality and immobilization

To observe the mortality in all animals both during and after exposure, they were placed on a counting chamber under a magnifying glass, as previously described. Determining if an animal was deceased involved initially checking for any movements. If no movements were detected, a gentle poke with a needle was performed. Subsequently, closer observation was conducted to detect any twitches or movements around the mouth area. If no movements were observed, the copepod would be considered dead. Additionally, dead copepods exhibited a color change from transparent to a lighter yellow shade, then finally a dark orange. Occasionally, dead copepods were noted to have their antennas more parallel to their bodies rather than perpendicular, as observed on healthy *A. tonsa*.

To assess immobilization, a similar method as described in the previous. If a copepod remained motionless upon initial observation, a gentle stimulus was performed by prodding it with a needle. If the copepod responded to the stimulus, the nature of its response was noted. Healthy individuals in the control group typically responded with vigorous leaps or jumps away from the needle. Therefore, copepods that remained still or unmoved by the stimulus were considered immobilized. In cases where there was no initial response observed, twitching was monitored. If twitching was observed, the copepod was classified as immobilized, with behaviors ranging from uncontrolled muscle contractions to flickering around the mouth area.

2.3.6 Water sampling of exposure solutions

To determine the measured concentrations compared to the nominal concentrations of IMI in all solutions and control groups, water samples were collected from each solution, including all replicates. Two samples were collected per replicate. For the 96-hours exposure one sample before exposure started and another immediately after exposure ended, while for pulse exposures samples were collected before initiation of each exposure. These samples were collected in 50 mL vials (Fig. 15), ensuring each vial was filled to approximately 40 mL, as indicated right below max freeze volume mark (Fig. 15). Following collection, all samples were stored in a freezer room at -20°C until completion of all exposures. Subsequently, all samples were transferred to the IMR laboratory in Nordnes, Bergen for further analysis.

Figure 15: 50mL vails used for water sampling.

2.4 LC-MS analysis

2.4.1 Sample preparation

Water samples from all replicates underwent analysis with LC-QqQ-MS/MS to verify the concentrations of IMI in the different solutions and examine if there was degradation of IMI over time. The samples were prepared in three series. Series 1 contained samples from all replicates in both pulse A and pulse B in pulse exposures. Series 2 contained samples of replicates with nominal concentrations ranging from 20 mg/L to 0.2 mg/L and series 3 contained samples from replicates with nominal concentrations ranging from 0.02 mg/L to 0.0002 mg/L in the 96-hours constant exposure. Each series compromised one blank without matrix, one blank with matrix, a calibration curve, three controls and samples from respective experiment (Fig. 16). For preparation of the LC-QqQ-MS/MS were standard solutions with IMI and an IS solution, IMI-d4, prepared. The standard solution was prepared in different concentrations for the three series. All solutions were first prepared by extracting 100 µL of 1 mg/mL IMI and diluting till 10 mL with acetonitril, achieving solution 1 with 10 µg/mL IMI. Both series 1 and series 2 included solution 2 (1 µg/mL), was achieved by extracting 1mL of solution 1 and diluting till 10 mL with acetonitril (Appendix C, Appendix D). Further dilution was necessary for samples in range from 0.02 mg/L to 0.0002 mg/L. Series 2 included solution 3 (100 ng/mL), achieved by extracting 100 µL of solution 2 and diluting till 1 mL with acetonitril. Solution 2 and solution 3 were added to the corresponding samples. For series 3 solution 2 was prepared by extracting 100 µL of solution 1 and diluting till 10 mL with acetonitril (Appendix E). Solution 3 was achieved by extracting 100 µL of solution 1 and diluting till 1 mL acetonitril. IS solution followed the same procedure for all three series. 50 µL of a 0.2 mg/mL IMI-d4 stock solution was extracted and diluted till 10 mL with acetonitril in a 10 mL volumetric flask. All series were further analyzed in the LC-QqQ-MS/MS equipment.

Figure 16: One series with dark vial for each sample, calibration curve, blank without matrix, blank with matrix and controls for one series.

Sample processing for each series involved preparing a blank without matrix, a blank with matrix, calibration curve, controls and sample from exposure solutions (Appendix C, Appendix D, Appendix E). All samples comprised of identical volume of IS and different amount of acetonitrile to reach target a total volume of 1 mL. Blank without matrix comprised only IS and acetonitrile, while blank with matrix included 100 µl of matrix. Samples in calibration curve comprised 100 µl of matrix and added standard solution of IMI in increasing order with target concentration. Controls consisted of 100 µl of matrix and added volume of standard solution IMI for concertation in the lower, middle and higher levels. Samples from exposure in series 1 and series 2 were diluted a tenfold before sample preparation, thereafter 100µl of the diluted sample were added in the analysis samples.

Imidacloprid-d4 (IMI-d4) has nearly the same molecular formula as IMI, $C_9D_4H_6ClN_5O_2$ except that four hydrogens atoms in the middle pentagon are replaced by deuterium, an isotope of hydrogen (National Center for Biotechnology Information, 2024a) (Fig. 17). Due to the extra neutron on each hydrogen atom, the molecular weight of IMI-d4 is 259.66 g/mol (Merck, 2024; National Center for Biotechnology Information, 2024b). Chemically similar to IMI, IMI-d4 is suitable as IS for LC-analysis on IMI.

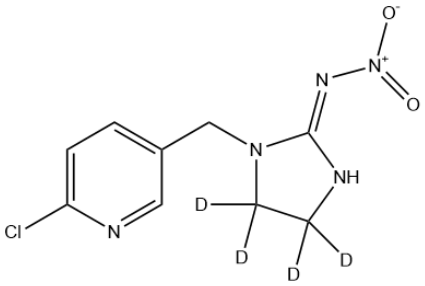


Figure 17 Structure of imidacloprid-d4 (National Center for Biotechnology Information, 2024a).

The seawater sourced from Nordnes did not undergo an initial filtration process as the deep seawater at the Austevoll research station. Deep seawater was collected from the fjord outside Bergen city and stored in a holding tank for sediments settling. Therefore, seawater was filtered with syringe filters (0.45 µm) (Fig. 18). The filters were close to filter size used at Austevoll, resulting in similar matrixes.

Figure 18: Filtering seawater with a syringe filter (0.45 µm).

2.4.2 LC-QqQ-MS/MS

Figure 19 Agilent technologies LC-QqQ-MS/MS system.

Analysis was performed accordingly to an established method for IMI by the IMR. After sample preparation, samples, blank without matrix, blank with matrix, standard calibration curve and three controls were analyzed with an LC-QqQ-MS/MS (Fig. 19) with each respective series. Series 1 and 2 were conducted at the same time, and series 3 at separate later point. Analyzing sample was performed in an orderly manner (Appendix F, Appendix G, Appendix H). The LC-QqQ-MS/MS system utilized comprised: an Agilent 1260 LC system, analytic column, Agilent Jet Stream Technology electrospray ionization source, Agilent 6460 Triple Quadrupole Mass spectrometer and Agilent MassHunter Workstation Software. The Agilent 1260 LC system included: autosampler, autosampler cooler, a binary pump and a thermal column compartment, while the column had the following parameters: SB C18, RRHD (1200 bar), column size 21 x 50 mm and particle size of 1.8 µm. Collison gas utilized was N_2, with purity ≥ 99.995%. The established method from IMR included analytical setup for the Agilent 1290 LC with a mobile phase consisting of a mixture of methanol (MeOH) and formic acid (FA) (Table 8), parameters for Agilent 6460 QqQ-MS/MS (Table 9) and parameters for precursor ions and product ions (Table 10). For qualifier, quantifier and IS were fragmentations series 256.06 → 208.9, 256.06 → 175 and 260.1→ 212.9 used, respectively. To determine LOD and LOQ, S/N- ratio of fragmentation series of precursor ion to product ion (256.06 → 208.9) of IMI were utilized. MRM scan was utilized in MS/MS. Between day imprecision for the method was < 10% and recovery 90 – 110 %. RF is calculated with the signal from analyte (A_X) and the signal from the IS (A_{IS}), and both known concentrations of the analyte ($[X]_f$) and IS ($[IS]_f$) in the calibration curve with the formula (Harris, 2020):

$$F = \frac{A_X * [IS]_f}{A_{IS} * [X]_f}$$

Table 8: *Analytical setup Agilent 1290 LC*

Injection volume		**2 µL**
Run time		**5 min**
Column temperature		**25°C**
Flow		**0.5 mL/min**
Mobile phase		A: MeOH[1] B: 0.1% FA[2] in Milli Q water
Analytic gradient	Time (min)	Mixture percentage of B
	0	90
	0.2	90
	3.3	10
	3.5	10
	3.6	90
	5.00	90

1 Methanol
2 Formic acid

Table 9: *Agilent 6460 QqQ parameters*

Ionization mode	ESI positive with Agilent Jet Stream
Scan type	MRM
Gas temperature	200°C
Gas flow	6 L/min
Nebulizer	35 psi
Sheath gas heater	400°C
Sheath gas flow	12 L/min
Capillary voltage	Positive: 4000V Negative: 2500V
Nozzle voltage	1000V

Table 10: *Scan parameters for Agilent 6460 QqQ-MS/MS*

Compound	Precursor ion	Product ion	Collison energy	Quantifier/ qualifier/IS	Dwell	Fragmentor	Cell Acc[1]	Polarity
IMI-d4[2]	260.1	212.9	5	IS	70	110	7	Positive
IMI[3]	256.06	208.9	13	Qualifier	70	110	7	Positive
IMI[3]	256.06	175	13	Quantifier	70	110	7	Positive

1 Cell Accelerator Voltage
2 Imidacloprid - d4
3 Imidacloprid

2.5 Statistical models

Statistical analysis was conducted using RCode software (v. 4.3.3) for LC- and EC-values, and TKTD models. To account for mortality in control groups, Schneider-Orelli's formula for corrected mortality was used in LC, EC and TKTD models (Appendix I, Appendix J, Appendix K). The LC- and EC-values with respective 95% confidence intervals (95% CI) for IMI were calculated at different time points using $\log_{10}$ model from *ggplot2* R package with binomial error, using data obtained in 96-hour constant exposure (Appendix I, Appendix J). Data from both 96-hours static exposure and both pulse exposures were utilized in TKTD-RED-GUTS modeling, using the *morse* R package (Baudrot et al., 2022), compromised three steps: calibration, validation and prediction (Appendix K, Appendix L, Appendix M). To assess the quality of TKTD models, a visual check against observed mortality and PPC plots were conducted. The PPC plot highlights the observed number of survivors, x-value of black dot, with predicted number of survivors marked, y-value, as colored line as the 95% credible interval. Green line cross the line y = x, indicating a good fit, while red lines does not overlap and indicate a poor fit (Ockleford et al., 2018). Further was results from pulse exposure used in validation to models obtained in calibration. To calculate the appropriate intermediate non-exposure period for pulse exposure, DRT_{95} is calculated following formula (Ockleford et al., 2018):

$$DRT_{95} = -\frac{\ln(0.05)}{k_D}$$

3 Results

3.1 Analysis of water samples

The aim of the analysis was to measure the concentration of IMI in exposure solutions and to examine if IMI were degraded during the exposure period of 96-hours. The calibration curves from series 1 to series 3 were linear ($R^2 > 5$). The calibration curve from series 1 is shown in Fig. 20. A chromatogram of quantifier, qualifier and IS from nominal concentration 20 mg/L R1 T1 for 24-hours pulse are shown in Fig. 21. Quantifier, qualifier and IS have t_R close in value. Nominal concentrations, measured concentrations and the mean measured concentration with standard deviation for the given nominal concentration are presented in Table 11 and 12. All results from the analyses are presented in (Appendix F, Appendix G, Appendix H).

Analyzed replicates from series 1 were all replicates observed to have measured concentrations with small deviations, except replicates at nominal concentration 2 mg/L and 20 mg/L after 72-hours in pulse B (Table 11). The analyzed replicates from the 96-hours exposure, series 2 and series 3, were the majority of replicates observed to have measured concentrations lower than nominal concentration, additionally variation between replicates within same nominal concentration is reflected in the standard deviations (Table 12). The replicates observed to have the largest deviation were reanalyzed and the new measured concentration were observed close in value to previously obtained concertation (Table 12).

Based on S/N of 3 and 10 for LOD and LOQ, respectively, LOD was determined to be 0.001 mg/L and LOQ to be 0.003 mg/L. The results from series 3 showed that concentration 0.0002 mg/L to be under the LOD, based on the signal to noise ratio being below 3 for all replicates (Appendix H). Furthermore, concentration 0.002 mg/L was observed to be above LOD and but S/N fluctuating around 10, resulting in replicates being above and below LOQ (Appendix H). In nominal concentration from 0.02 mg/l to 20 mg/L is little deviation observed between mean concentrations in 0-hours and 96-hours. Therefore, for replicates in the 96-hours exposure is nominal concentration 0.0002 mg/L and 0.002 mg/L used, while mean measured concentration between sampling is used for analysis and discussion. For pulse exposure is measured concentration used.

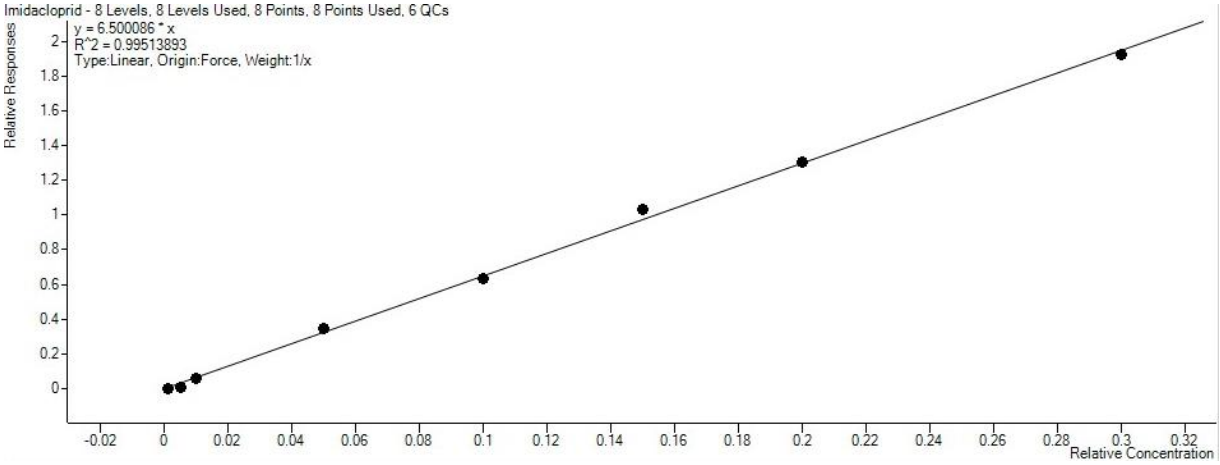


Figure 20: Standard curve from series 1, with black dots being samples in calibration curve with a linearity R^2 at 0.9995. Response on the y-axis and relative concentration on the x-axis.

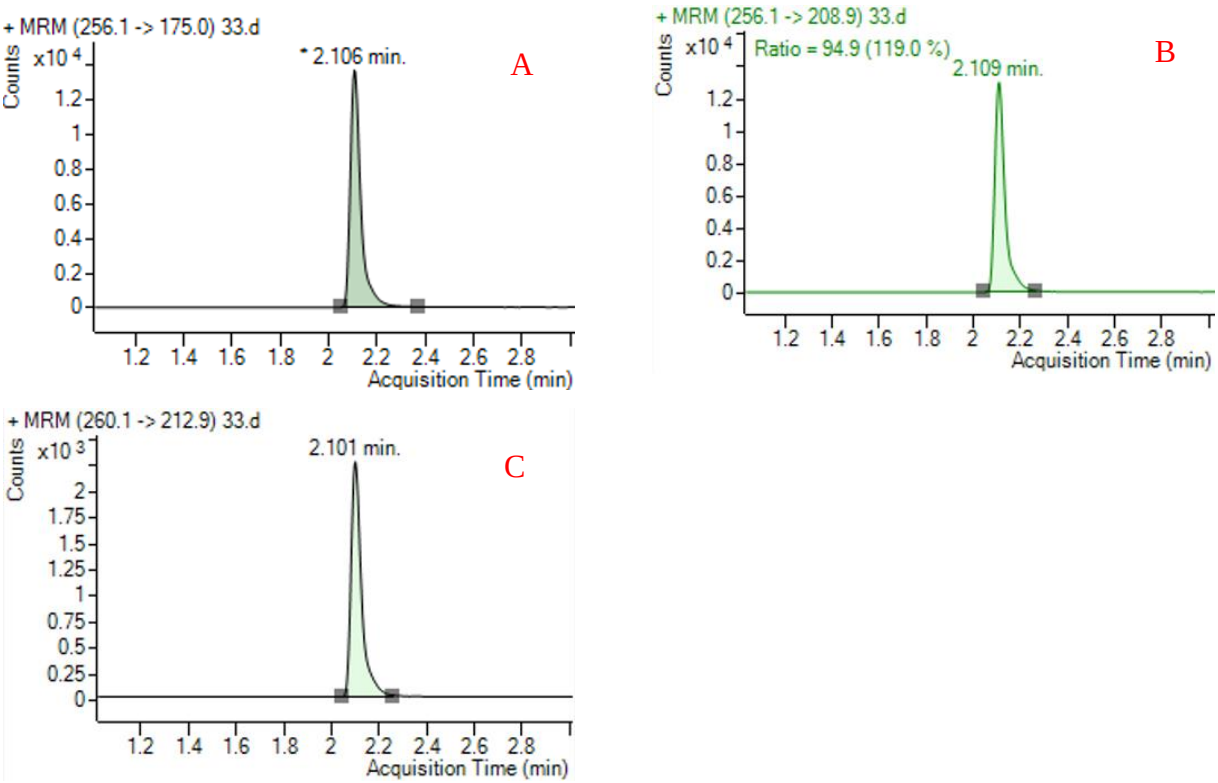


Figure 21: Chromatogram from replicate 1 in nominal concentration 20mg/L at 0-hours in 24-hours pulse from series 1. (A) Chromatogram of quantifier, (B) chromatogram of qualifier, (C) chromatogram of IS.

Table 11: *Analyzed replicates from 24-hours and 72-hours pulse exposure in series 1 with nominal concentrations, which exposure, measured concentration and mean concentration with standard deviation.*

Pulse	Replicate	Nominal concentration (mg/L)	Exposure number	Measured concentration sample (ng/mL)	Measured concentration solution (mg/L)	Mean concentration (mg/L)
24-hours	1	2	1	16.2	1.62	1.64
	2			16.6	1.66	(±0.03)
	1	2	2	17.4	1.74	1.74
	2			17.4	1.74	(±0.00)
	1	20	1	174	17.4	17.6
	2			178	17.8	(±0.3)
	1	20	2	173	17.3	17.1
	2			169	16.9	(±0.3)
72-hours	1	2	1	16.0	1.60	1.63
	2			16.6	1.66	(±0.04)
	1	2	2	19.9	1.99	1.95
	2			19.0	1.90	(±0.06)
	1	20	1	169	16.9	16.4
	2			158	15.8	(±0.8)
	1	20	2	197	19.7	19.8
	2			199	19.9	(±0.1)

Table 12: *Analyzed replicates from series 2 and 3 with nominal concentrations, time point, measured concentration and mean measured concentration with respective standard deviation.*

Exposure experiment	Replicate	Nominal concentration (mg/L)	Time of exposure (hours)	Measured concentration sample (ng/mL)	Measured concentration solution (mg/L)	Mean concentration (mg/L)	Mean concentration between sampling (mg/L)
96-hour	1	0.0002	0	0.0200	0.000200		
	2			0.0162	0.000162		
	3			0.0157	0.000157	0.00020	
	4			0.0295	0.000295	(±0.00006)	
	1	0.0002	96	0.0199	0.000199		
	2			0.0082	0.000082		
	3			0.0215	0.000215	0.00019	0.00020
	4			0.0275	0.000275	(±0.00008)	(±0.00007)
	1	0.002	0	0.163	0.00163		
	2			0.186	0.00186		
	3			0.111	0.00111	0.0016	
	4			0.163	0.00163	(±0.0003)	
	1	0.002	96	0.146	0.00146		
	2			0.149	0.00149		
	3			0.195	0.00195	0.0017	0.0016
	4			0.178	0.00178	(±0.0002)	(±0.0003)
	1	0.02	0	1.82	0.0182		
	2			1.52	0.0152		
	3			1.37	0.0137	0.015	
	4			1.10	0.0110	(±0.003)	
	1	0.02	96	0.89	0.0089		
	2			1.10	0.0110		
	3			1.35	0.0135	0.011	0.013
	4			1.07	0.0107	(±0.002)	(±0.003)
	1	0.2	0	1.60	0.160		
	2			1.20	0.120		
	3			1.24	0.124	0.13	
	4			1.09	0.109	(±0.02)	
	1	0.2	96	1.09	0.109		
	2			1.60	0.160		
	3			1.40	0.140	0.13	0.13
	4			1.12	0.112	(±0.02)	(±0.02)
	1	2	0	15.8	1.58		
	2			10.2	1.02		
	3			7.06	0.71[1]	1.2	
	4			15.2	1.52	(±0.4)	
	1	2	96	13.4	1.34		
	2			9.84	0.98		
	3			14.8	1.48	1.3	1.3
	4			15.7	1.57	(±0.3)	(±0.3)
	1	20	0	156	15.6		
	2			160	16.0		
	3			90.7	9.1[2]	14	
	4			161	16.1	(±3)	
	1	20	96	162	16.2		
	2			156	15.6		
	3			161	16.1	15	15
	4			132	13.2	(±1)	(±3)

1. Reanalyzed with measured concentration 0.99 mg/L
2. Reanalyzed with measured concentration 10.2mg/L

3.2 96-hours exposure

3.2.1 Result raw data

The total number of copepods, mortality and immobilization for all replicates from the 96-hours exposure are summarized in Table 13. For all nominal concentrations mortality was observed to increase over time and increase with increasing concentration of IMI. Immobilization was observed to increase with increasing concentration after 24-hours but fluctuating over time. Immobilization was observed before mortality (Table 13). Mortality and immobilization of copepods are illustrated with replicates combined for each nominal concentration during the exposure period shown in Fig. 22 and Fig. 23, respectively. For all concentrations, a low mortality was observed on the initial monitor (Table 13, Fig. 22), and mortality was first observed at different time points for the different concentrations. A high mortality rate was observed for concentrations 0.13 mg/L – 15 mg/L in time points 72-hour and 96-hour. For 0.013 mg/L mortality was first observed at 72-hours, while for nominal concentrations 0.002 mg/L and 0.0002 mg/L mortality was first observed at 96-hours. All nominal concentration experienced immobilization in the initial monitor, with 15 mg/L and 1.3 mg/L experiencing a high initial number of immobilized copepods (Table 13, Fig. 23). In control were no immobilized copepods observed throughout the exposure. For 15 mg/L were majority of copepods observed to be immobilized in the initial monitor, with the number decreasing as mortality increased. In 1.3 mg/L and 0.13 mg/L was the same trend observed, as for 15 mg/L, however at a slower rate. For concentrations ranging from 0.013 mg/L to 0.00020 mg/L a small number in immobilized copepods was observed till 48 hours observed and observed increasing at 96-hours, except 0.013 mg/L increasing at 72-hours. In control R2 a considerable number of mortality was observed. For R1 in both nominal concentration 0.0002 mg/L and concentration 0.013 mg/L, a decrease in mortality were observed over time (Table 13). These replicates were discarded for further analysis. A difference in the total number of animals in each replicate was observed and summarized in Table 14. The difference is calculated and shown in absolute number. The lowest count in total number of animals was observed in the 72-hours monitor for the majority of replicates. In the majority of replicates, the total number was observed to increase in the 96-hour monitor compared to the 72-hour monitor. Other replicates are observed decreasing at 48-hours, 72-hours and 96-hours, and not increasing back to initial count. For some replicates, the total number was observed to increase beyond the initial count, occurring in R2 0.13 mg/L and R2 1.3 mg/L. Therefore, an inconsistency in the total number of animals were observed in all replicates, except replicates R2 0.013 mg/L and R4 15 mg/L.

Table 13: *Raw data for the 96-hours constant exposure experiment, total number of copepods, number of dead, number of immobilized in each replicate for each time point.*

TIME POINT		**24-HOURS**			**48-HOURS**			**72-HOURS**			**96-HOURS**		
TREATMENT (MG/L)	Replicate	Tot[1]	Dead	Imm[2]	Tot[1]	Dead	Imm[2]	Tot[1]	Dead	Imm[2]	Tot[1]	Dead	Imm[2]
CONTROL	R1	18	0	0	18	0	0	16	0	0	18	1	0
	R2*	19	0	0	19	0	0	12	1	0	19	4	0
	R3	22	0	0	22	0	0	16	1	0	21	2	0
	R4	22	0	0	21	0	0	18	0	0	16	1	0
0.0002	R1*	20	0	0	20	1	2	19	0	0	16	3	4
	R2	20	0	1	20	0	1	19	0	0	20	4	2
	R3	23	0	1	22	0	1	21	0	1	20	3	4
	R4	17	0	0	17	0	1	12	0	0	17	6	2
0.002	R1	16	0	0	16	0	1	14	0	1	16	3	5
	R2	24	0	1	23	0	1	19	0	2	20	5	4
	R3	19	0	0	18	0	0	12	0	3	16	4	4
	R4	18	0	0	18	0	2	16	0	3	17	2	2
0.013	R1*	17	0	1	17	0	2	15	2	7	16	1	6
	R2	18	0	0	18	0	1	18	0	5	18	1	8
	R3	23	0	2	23	0	2	21	3	1	23	4	9
	R4	20	0	0	20	0	2	19	1	7	19	1	9
0.13	R1	20	0	4	17	0	5	16	5	3	17	5	8
	R2	18	0	4	20	0	3	14	5	5	19	11	8
	R3	20	0	2	20	1	3	23	3	11	20	8	4
	R4	22	0	3	22	0	4	23	2	8	22	15	5
1.3	R1	20	0	9	20	1	8	20	13	7	19	16	3
	R2	16	0	8	21	0	9	16	3	13	20	15	5
	R3	19	0	6	19	1	7	15	9	5	18	18	0
	R4	17	0	5	17	0	5	19	13	6	16	16	0
15	R1	18	1	17	18	1	17	14	12	2	14	14	0
	R2	19	0	19	19	4	15	14	14	0	15	15	0
	R3	20	0	18	20	4	16	20	19	1	22	22	0
	R4	15	0	15	15	1	14	15	15	0	15	15	0

** Replicates discarded for further analysis due to high mortality or deviation in observed mortality.*
1 Total number of animals
2 The number of immobilized animals

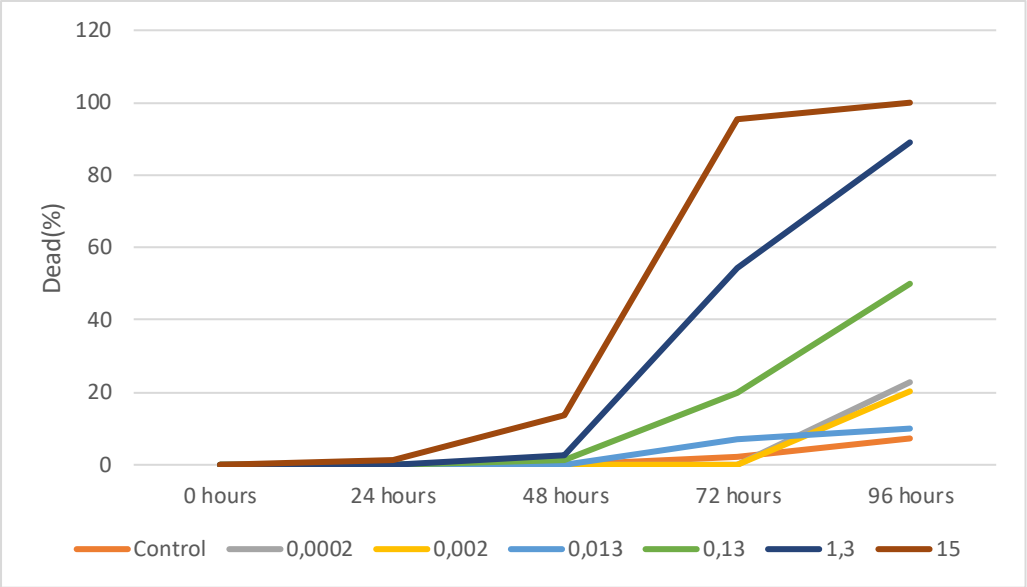


Figure 22: Mortality represented in percentage of total number of animals with combined replicates in each measured concentration for the 96-hours exposure.

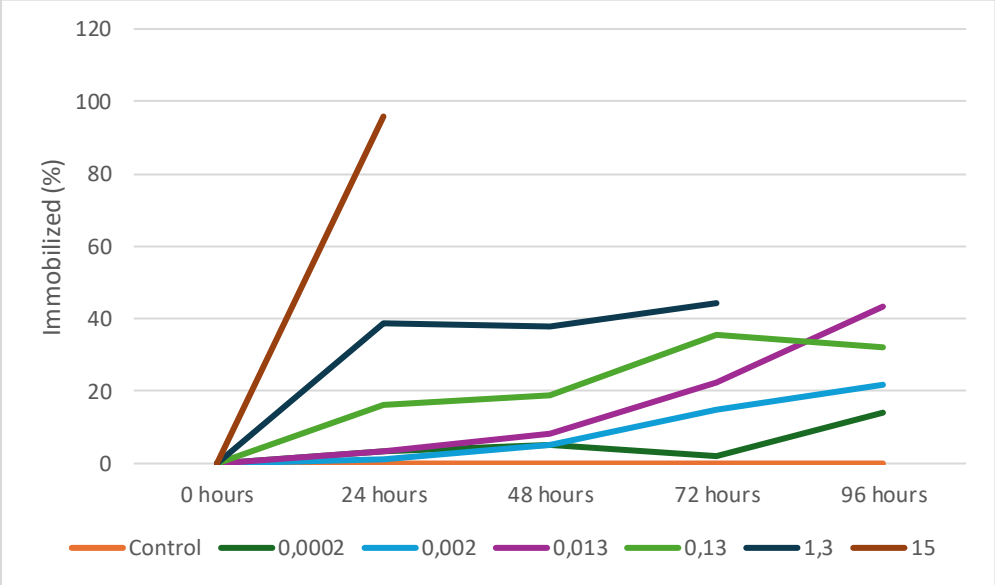


Figure 23: Immobilization represented in percentage of total number of animals with combined replicates in each measured concentration for the 96-hours exposure. For measured concentrations 15 mg/L and 1.3 mg/L immobilization was observed to decrease after respective 24-hours and 72-hours due to immobilized animals dying.

Table 14: *Total number of monitored copepods in each replicate during the 96-hour exposure, with a maximum, minimum, mean value and total difference in absolute number.*

Treatment	Replicate	0	24	48	72	96	Min	Max	Mean	Difference
Control	R1	18	18	18	16	18	16	18	18	2
	R2	19	19	19	12	19	12	19	17	7
	R3	22	22	22	16	21	16	22	20	6
	R4	22	22	21	18	16	16	22	19	6
0.0002	R1	20	20	20	19	16	16	20	19	4
	R2	20	20	20	19	20	19	20	20	1
	R3	23	23	22	21	20	20	23	22	3
	R4	17	17	17	12	17	12	17	16	5
0.002	R1	16	16	16	14	16	14	16	16	2
	R2	24	24	23	19	20	19	24	22	5
	R3	19	19	18	12	16	12	19	16	7
	R4	18	18	18	16	17	16	18	17	2
0.013	R1	17	17	17	15	16	15	17	16	2
	R2	18	18	18	18	18	18	18	18	0
	R3	23	23	23	21	23	21	23	23	2
	R4	20	20	20	19	19	19	20	20	1
0.13	R1	20	20	17	16	17	16	20	18	4
	R2	18	18	20	14	19	14	20	18	6
	R3	20	20	20	23	20	20	23	21	3
	R4	22	22	22	23	22	22	23	22	1
1.3	R1	20	20	20	20	19	19	20	20	1
	R2	16	16	21	16	20	16	21	18	5
	R3	19	19	19	15	18	15	19	18	4
	R4	17	17	17	19	16	16	19	17	3
15	R1	18	18	18	14	14	14	18	16	4
	R2	19	19	19	14	15	14	19	17	5
	R3	20	20	20	20	22	20	22	21	2
	R4	15	15	15	15	15	15	15	15	0

3.2.3 Calculated LC and EC values form the 96-hours exposure

LC- and EC- values from the 96-hours exposure were calculated (Appendix I, Appendix J) and summarized in Table 15. Selected values of LC and EC models are illustrated with respective value and corresponding concentration highlighted with a red line (Fig. 24-30). The LC values for 24-hours and EC-values for 96-hours were not calculated. Only LC_{10} was observed within experiment boundaries, while for 72-hours were both LC_{10} and LC_{50} observed within region of exposure boundaries with LC_{90} slightly above. At 96-hours of exposure were all LC-values within exposure boundaries, with LC_{10} (95% CI) overlapping the release concentration in the Norwegian aquaculture, ≤ 0.30µg/L (Grefsrud et al., 2024), also at 96-hours exposure were all calculated LC-values below treatment concentration Norwegian aquaculture, 20 mg/L (Grefsrud et al., 2024). For EC-values were EC_{10} within the concentration range, EC_{50} after 24-hours and 48-hours were within the concentration range, while EC_{90} had values larger than 20 mg/L.

Table 15: *Calculated acute LC- and EC-values with respective mean concentration of IMI and 95% credible interval (95% CI) after each monitor from the 96-hours exposure.*

Time point	***LC_{10} (mg/L)***	***LC_{50} (mg/L)***	***LC_{90} (mg/L)***	***EC_{10} (mg/L)***	***EC_{50} (mg/L)***	***EC_{90} (mg/L)***
24-hours	-	-	-	0.06 (0.02-0.1)	1.2 (0.7-1.7)	25 (5-45)
48-hours	8.5 (0.8-16)	256 (OR-816)	7706 (OR*-39545)	0.018 (0.003-0.033)	1.4 (0.6-2.2)	111 (OR*-240)
72-hours	0.052 (0.016-0.088)	1.3 (0.7-1.8)	31 (4.7-56)	0.0005 (OR*-0.0017)	3029 (OR*-1420)	$1.9*10^{10}$ (OR*-$1.9*10^{11}$)
96-hours	0.0021 (0.0005-0.0038)	0.10 (0.05-0.14)	4.3 (0.8-7.9)	-	-	-

** OR = out of range, calculates below 0 mg/L.*

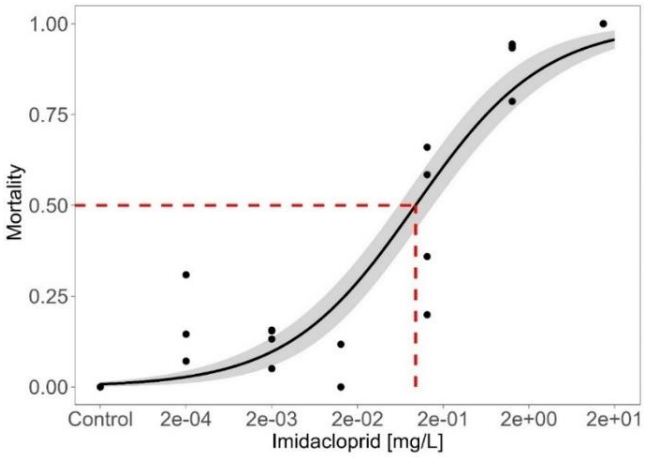


Figure 24: Mortality plotted against concentration from the 96-hours constant exposure. Calculated mortality based on a log regression analysis of observed mortality. Calculated LC_{50}, shown in red line, for time point 96-hours.

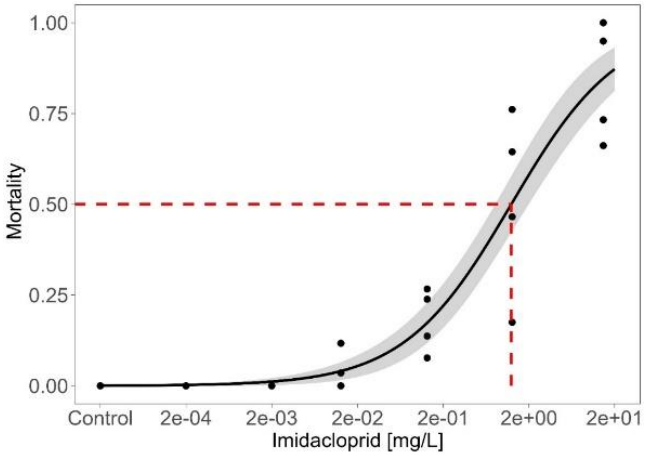


Figure 25: Mortality plotted against concentration from the 96-hours constant exposure. Calculated mortality based on a log regression analysis of observed mortality. Calculated LC_{50}, shown in red line, for time point 72-hours.

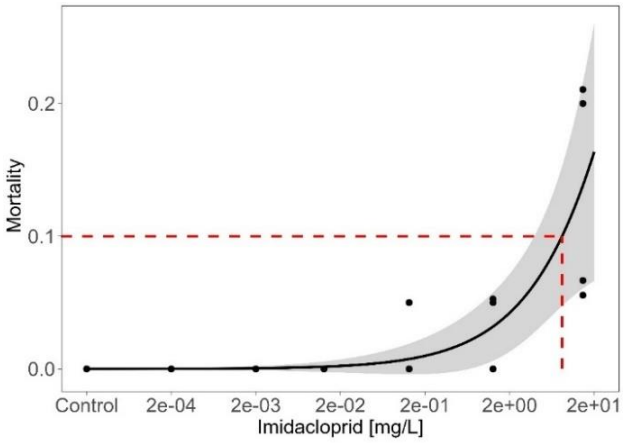


Figure 26: Mortality plotted against concentration from the 96-hours constant exposure. Calculated mortality based on a log regression analysis of observed mortality. Calculated LC_{10}, shown in red line, for time point 48-hours.

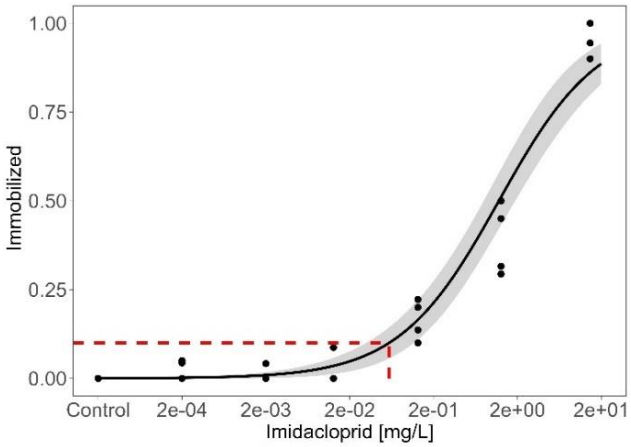


Figure 27: Immobilization plotted against concentration from the 96-hours constant exposure. Calculated immobilization based on a log regression analysis of observed immobilization. Calculated EC_{10}, shown in red line, for time point 24-hours.

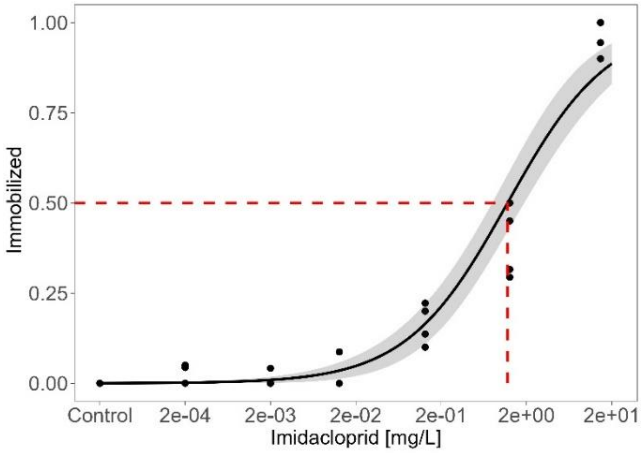


Figure 28: Immobilization plotted against concentration from the 96-hours constant exposure. Calculated immobilization based on a log regression analysis of observed immobilization. Calculated EC_{50}, shown in red line, for time point 24-hours.

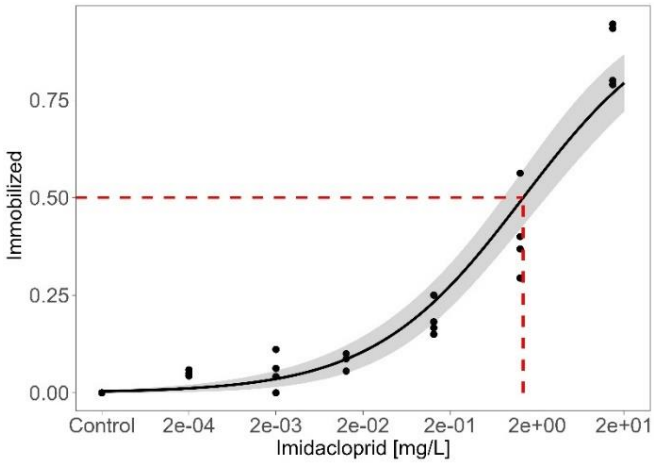


Figure 29: Immobilization plotted against concentration from the 96-hours constant exposure. Calculated immobilization based on a log regression analysis of observed immobilization Calculated EC_{50},shown in red line, for time point 48-hours.

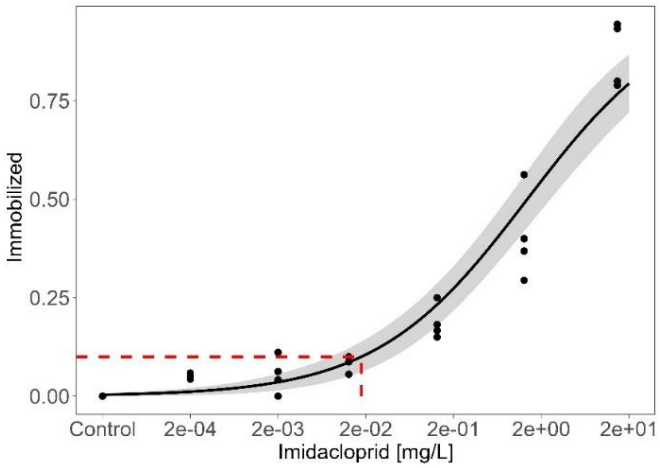


Figure 30: Immobilization plotted against concentration from the 96-hours constant exposure. Calculated immobilization based on a log regression analysis of observed immobilization. Calculated EC_{10}, shown in red line, for time point 48-hours.

3.3 24-hours and 72-hours pulse exposures

Number of dead, immobilized and total number of copepods in each replicate for 24-hours pulse and 72-hours pulse are summarized in Table 16 and Table 17, respectively. There was no mortality in any replicates for 24-hours pulse for both counts. For replicates in nominal concentration 2 mg/L no copepods were observed to be immobilized after the initial exposure. Monitoring the second exposure, one copepod was observed to be immobilized in each replicate. For replicates in nominal concentration 20 mg/L were some immobilized copepods observed after the initial exposure, and a slight increase after second exposure. In pulse B no mortality or immobilization was observed in control. For nominal concentration 2 mg/L were only one copepod observed to be immobilized in the initial count, while both a small number of dead and immobilized after second count. Replicates with nominal concentration 20 mg/L had a higher number of mortality and immobilization observed in both monitors, compared to nominal concentration 2 mg/L. In the initial count only immobilized animals were observed, while both dead and immobilized were observed in the second count.

In both pulses were animals in nauplii stage observed. Nauplii were not included in monitoring, thereby excluded in total number of animals. After comparing raw data from the initial and secondary monitor for all replicates, an increase in total number and fewer animals in nauplii stage were observed. The increase is due to animals in nauplii stage reaching copepod stage, due long intermediate periods. The final result is thereby affected due to this consequence. For further use in modeling is the total number of copepods in initial count utilized for the second monitor.

Table 16: *Raw data for 24-hours pulse with total number of copepods, number of observed dead and immobilized after each exposure*.

First exposure					Second exposure			
Treatment	**Replicate**	**Total**	**Dead**	**Immobilized**	**Treatment**	**Total**	**Dead**	**Immobilized**
Control	R1	11	0	0	**Control**	11	0	0
	R2	14	0	0		14	0	0
1.64 mg/L	R1	9	0	0	**1.74 mg/L**	9	0	1
	R2	12	0	0		12	0	1
17.6 mg/L	R1	16	0	3	**17.1 mg/L**	16	0	6
	R2	9	0	3		13*	0	4

** Observed increase in total number of copepods, due nauplii molting and reaching copepods stage between exposures.*

Table 17: *Raw data for 72-hours pulse with total number of copepods, number of observed dead and immobilized after each exposure*.

First exposure					Second exposure			
Treatment	**Replicate**	**Total**	**Dead**	**Immobilized**	**Treatment**	**Total**	**Dead**	**Immobilized**
Control	R3	13	0	0	**Control**	13	0	0
	R4	13	0	0		15*	0	0
1.63 mg/L	R3	10	0	0	**1.95 mg/L**	10	1	2
	R4	13	0	1		15*	2	1
16.4 mg/L	R3	12	0	7	**19.8 mg/L**	12	3	5
	R4	10	0	6		12*	2	6

** Observed increase in total number of copepods, due nauplii molting and reaching copepods stage between exposures.*

3.4 TKTD-RED-GUTS Models

3.4.1 Calibration

The number of survivors over time for each replicate are illustrated in Fig. 31a and Fig. 31b, which Fig. 31a show the mortality based on the raw data (Table 13) and Fig. 31b is survival rate after corrected mortality (Appendix K). Further showcase Fig. 32a and Fig. 32b TKTD-RED-GUTS models, respectively SD and IT, based on corrected number of survivors for each nominal concentration (Appendix K). The mean number of observed survivors are illustrated as black dots with line variations between replicates, yellow line is the predicted survivability based on its respective model and grey band is the calculated confidence interval in the model (Ockleford et al., 2018). Both models is visually poor fits based on observed survivors. The SD model seems to be an acceptable fit for concentrations ranging from control to 0.013 mg/L, while the IT model is visually a good fit only for concentration 15 mg/L. For both model the number of not intersecting uncertainty predictions are above 50%, indicating poor result (Fig. 31a, Fig. 31b). In total both models indicate poor fit based on visual observation and PPC check. Parameters calculated for both models summarized in Table 18. Based on calculated DRT_{95}, both pulse profiles should have respective shorter and longer intermediate period than 217 days and 546 days, based on dominant rate constant (K_D) for respective SD and IT model (Table 18).

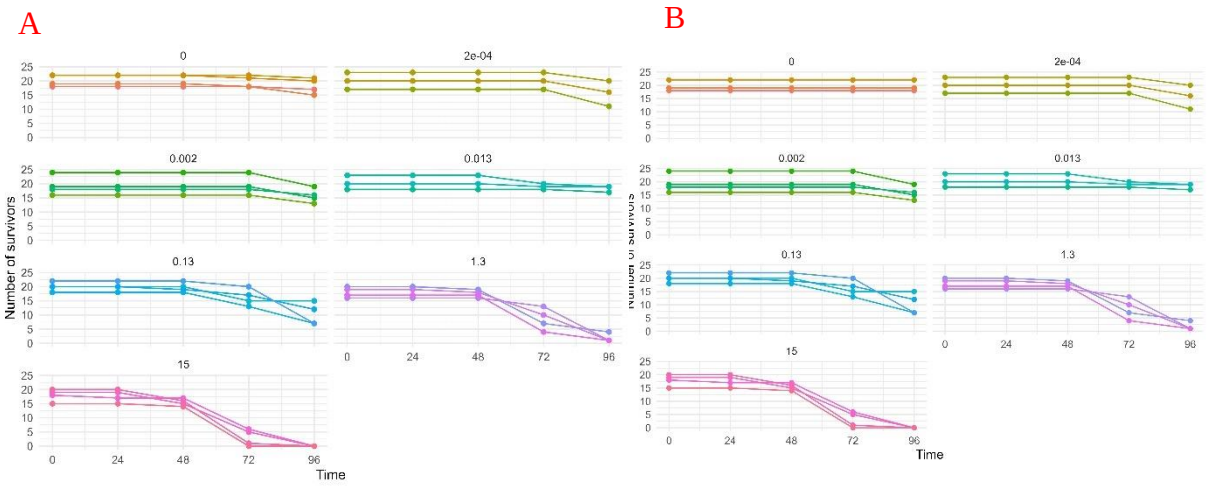
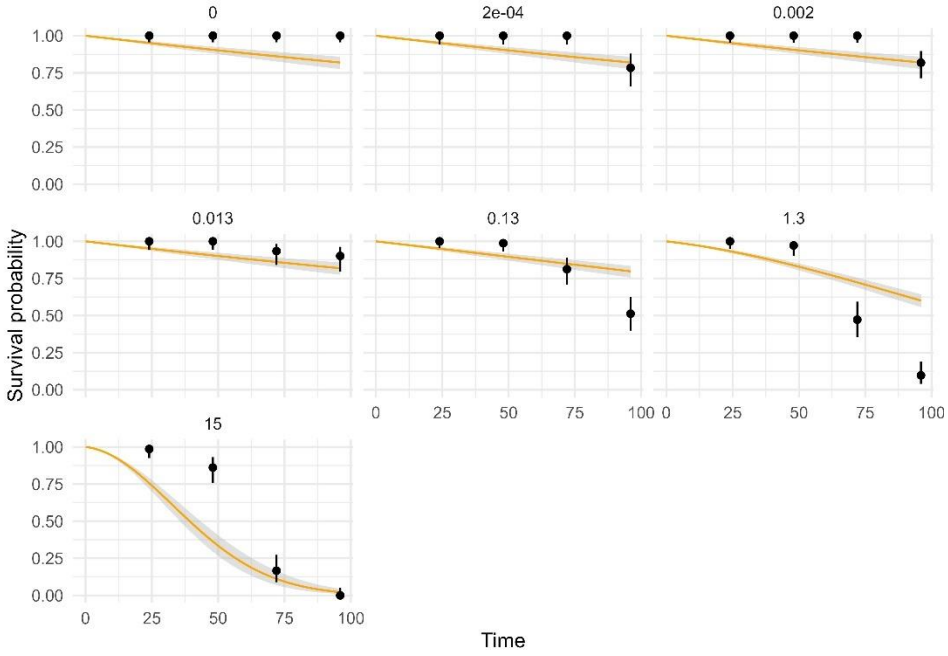


Figure 31: Acute mortality for each replicate in each nominal concentration for the 96-hours exposure. (A) is the raw mortality and (B) is the corrected mortality.

A



B

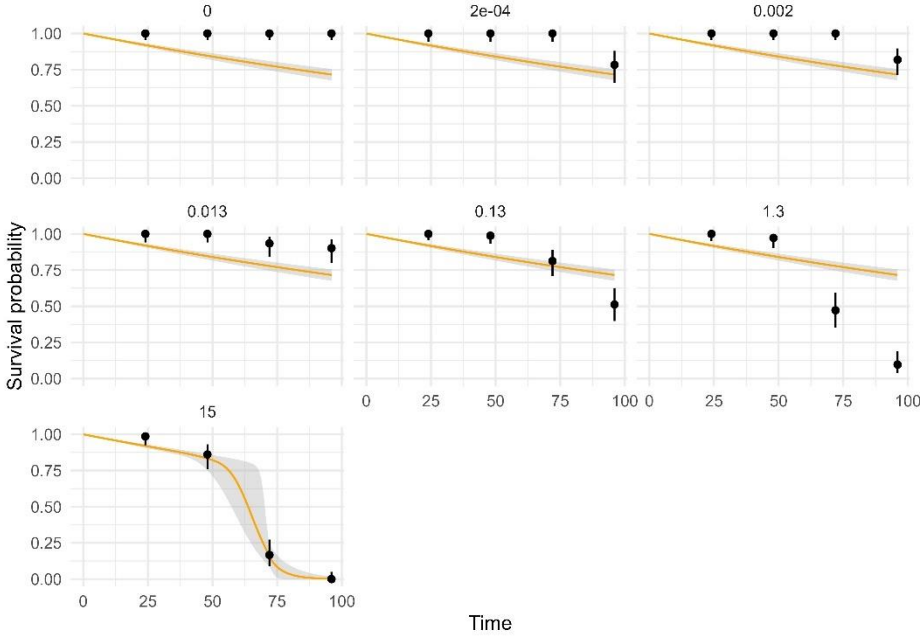
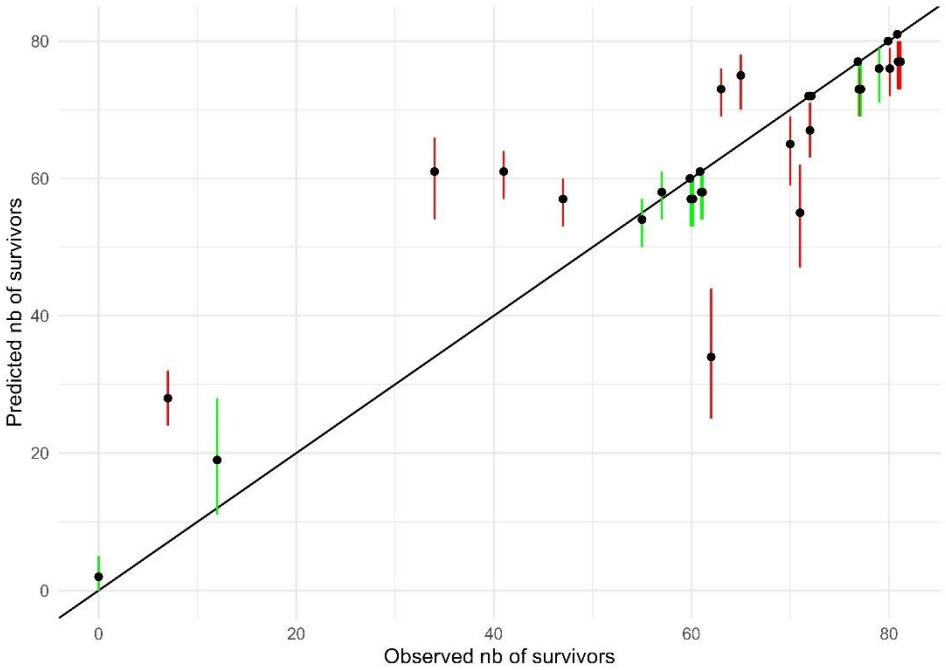


Figure 32: TKTD-RED-GUTS model for acute mortality for the 96-hour exposure. (A) SD and (B) IT. Black dots represent the number of survivors divided by initial number, and black line is the between replicate variability. Orange line is the median curve, and grey band is the 95% credibility interval.

Table 18: *Parameter estimates for TKTD-GUTS-RED-SD and TKTD-GUTS-RED-IT for 96-hours constant exposure, with median and 95% credible intervals. Range between 2.5% and 97.5% quantiles of marginal posterior distributions.*

GUTS-RED model	Parameters	Symbol	Median	2.5% quantile	97.5% quantile	Unit
SD	Dominant rate constant	K_D	5.745e-04	4.817e-05	2.845e-03	$Hour^{-1}$
	Background mortality	h_b	2.075e-03	1.587e-03	2.650e-03	$Hour^{-1}$
	Concentration threshold	z_w	2.994e-04	9.592e-06	3.959e-03	mg/L
	Killing rate	b_w	9.279e-02	1.931e-02	1.098e+00	mg/L per hour
	DRT_{95}	5215/217	-	-	-	Hours/days
IT	Dominant rate constant	K_D	2.285e-04	6.023e-06	1.053e-02	$Hour^{-1}$
	Background mortality	h_b	3.478e-03	2.934e-03	4.079e-03	$Hour^{-1}$
	Median of the threshold distribution	m_w	2.245e-01	5.974e-03	7.572e+00	mg/L
	Width of the threshold distribution	β	1.579e+01	8.806e+00	7.921e+01	-
	DRT_{95}	13110/546	-	-	-	Hours/Days

A



B

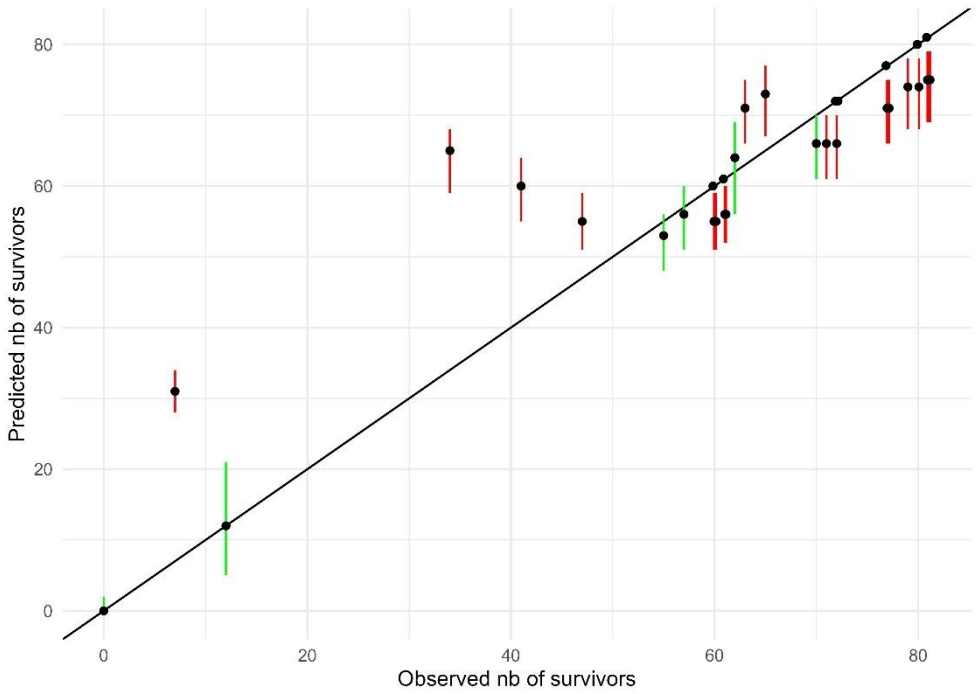


Figure 33: Posterior Predictive Check (PPC) plot for TKTD-RED-GUTS models. (A) SD and (B) IT. x-coordinate for black dot is observed number of survivors and while y-coordinate is predicted median number of survivors, with a 95% credible value for predicted numbers. Green lines overlap with the line x = y.

3.4.2 Validation

Both pulse exposures profiles are illustrated in Fig. 34, where each combined nominal concentration highlight the number of survivors plotted against time from initiation of first exposure. Calculated parameters for TKTD-RED-GUTS models, both SD and IT model, based on pulse exposures are summarized in Table 19. SD and IT models are plotted with observed mortality in Fig. 35 and Fig. 36, respectively. Both models is visually a good fit with observed mortality, and both have a high number of intersecting uncertainty predictions with the y = x line in PPC plot (Fig. 37, Fig. 38). The observed mortality in pulse exposures with predicted survivability based on models obtained in calibration, is illustrated in Fig. 39 and Fig. 40. Predicted survivability is observed to be substantially lower than observed mortality. The parameters PPC, NRMSE and SPPE are listed in Table 20, with PPC plots illustrated in Fig. 41 and Fig. 42 (Appendix L).

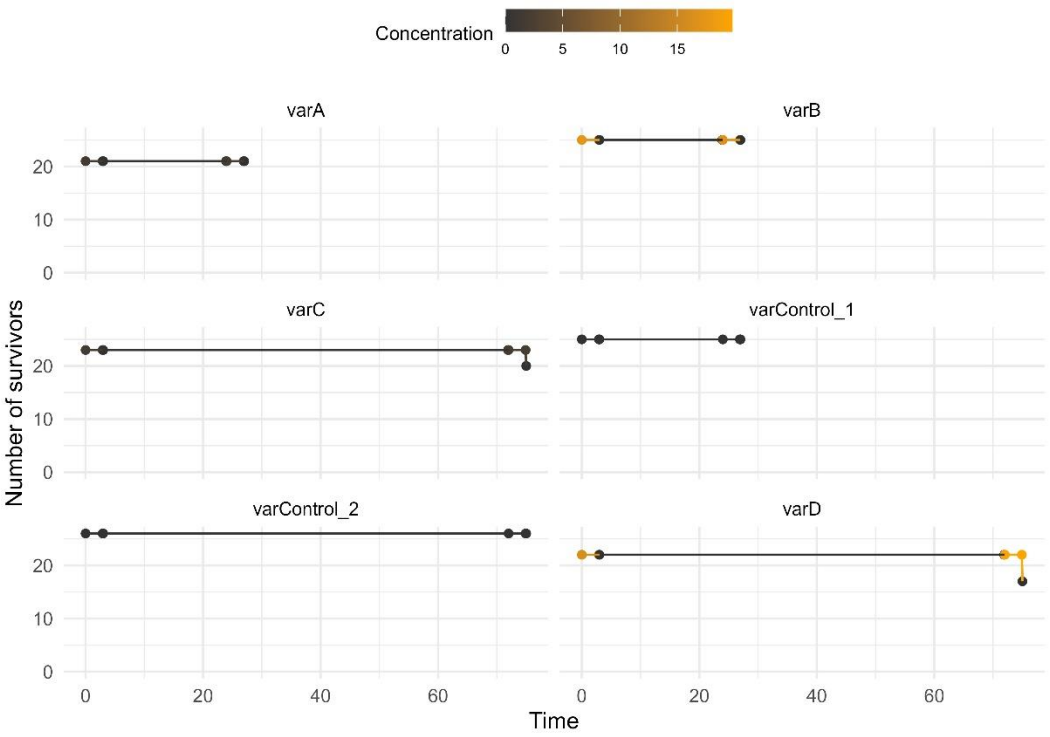


Figure 34: Plotted observed number of survivors based on time of monitoring. VarA and VarB represents replicates for from 24-hours pulse, respectively nominal concentrations 2 mg/L and 20 mg/L. VarC and VarD represents replicates from 72-hours pulse with respectively nominal concentrations 2 mg/L and 20 mg/L. VarControl_1 and VarControl_2 represents control group in respectively 24-hours pulse and 72-hours pulse.

Table 19: *Parameter estimates for TKTD-GUTS-RED-SD and TKTD-GUTS-RED-IT for pulse exposure, with median and 95% credible intervals. Range between 2.5% and 97.5% quantiles of marginal posterior distributions.*

GUTS-RED model	Parameters	Symbol	Median	2.5% quantile	97.5% quantile	Unit
SD	Dominant rate constant	K_D	1.218e-01	7.998e-02	2.105e-01	$Hour^{-1}$
	Background mortality	h_b	4.028e-04	1.088e-04	1.032e-03	$Hour^{-1}$
	Concentration threshold	z_w	5.982e+00	4.187e+00	8.943e+00	mg/L
	Killing rate	b_w	1.592e+02	5.212e-01	1.421e+03	mg/L per hour
IT	Dominant rate constant	K_D	9.963e-02	8.088e-02	1.496e-01	$Hour^{-1}$
	Background mortality	h_b	4.068e-04	1.083e-04	1.041e-03	$Hour^{-1}$
	Median of the threshold distribution	m_w	5.205e+00	4.361e+00	7.264e+00	mg/L
	Width of the threshold distribution	β	8.232e+01	3.957e+01	9.934e+01	-

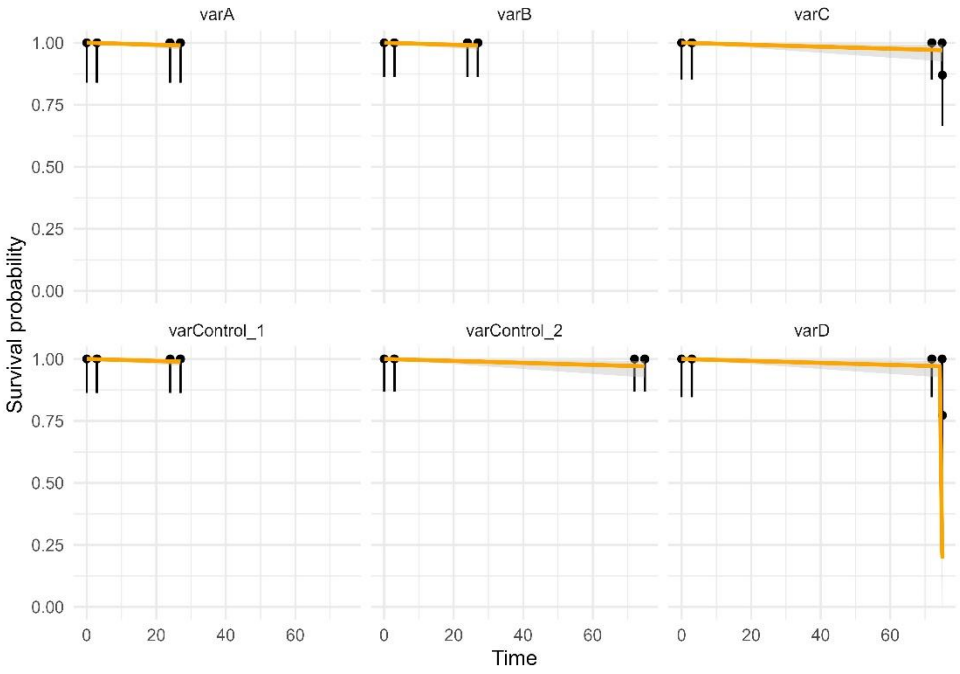


Figure 35: Plotted observed survivability with predicted numbers in TKTD-RED-GUTS-SD model based on pulsed exposure. Black dots represent the number of survivors divided by initial number, and black line is the between replicate variability. Orange line is the median curve, and grey band is the 95% credibility interval.

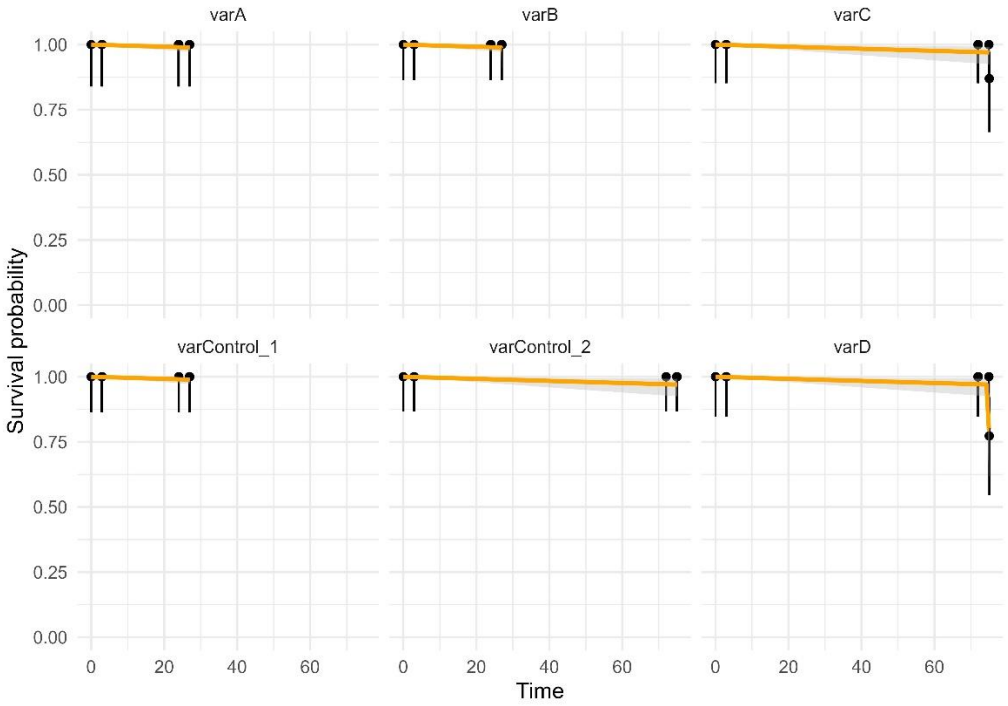


Figure 36: Plotted observed survivability with predicted numbers in TKTD-RED-GUTS-IT model based on pulsed exposure. Black dots represent the number of survivors divided by initial number, and black line is the between replicate variability. Orange line is the median curve, and grey band is the 95% credibility interval.

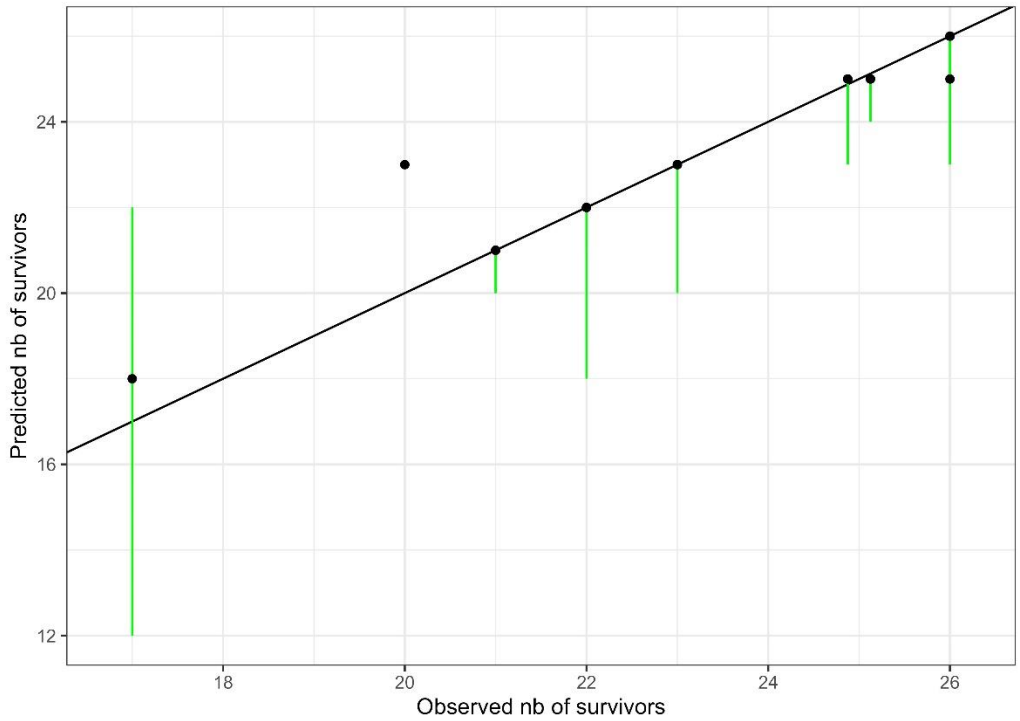


Figure 37: PPC plot for TKTD-RED-GUTS-SD model of predicted number of survivors vs observed number of survivors based on pulsed exposure. x-coordinate for black dot is observed number of survivors and while y-coordinate is predicted median number of survivors, with a 95% credible value for predicted numbers. Green lines overlap with the line x = y.

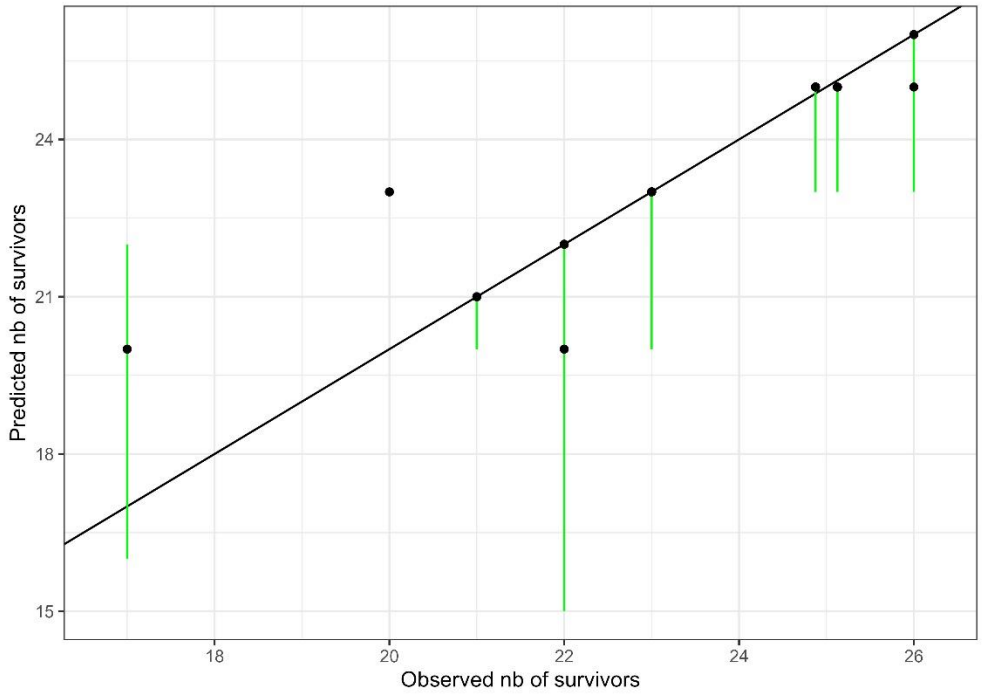


Figure 38: PPC plot for TKTD-RED-GUTS-IT model of predicted number of survivors vs observed number of survivors based on pulsed exposure. x-coordinate for black dot is observed number of survivors and while y-coordinate is predicted median number of survivors, with a 95% credible value for predicted numbers. Green lines overlap with the line x = y.

Table 20: *The three quantitative criteria, PPC, NRMSE and SPPE, for validation of pulse data on models, SD and IT, obtained during calibration.*

Model	Pulse exposure	Nominal concentration	Name in figure	PPC (%)	NRMSE (%)	SPPE (%)
SD	24-hours	2 mg/L	varA	100	3.6	4.8
	24-hours	20 mg/L	varB	100	7.7	12.0
	72-hours	2 mg/L	varC	86	9.9	4.3
	72-hours	20 mg/L	varD	57	18	4.5
	24-hours	-	varControl_1	100	2.8	4.0
	72-hours	-	varControl_2	50	10.0	15.4
IT	24-hours	2 mg/L	varA	100	7.2	9.5
	24-hours	20 mg/L	varB	100	6.0	8.0
	72-hours	2 mg/L	varC	57	14.9	8.7
	72-hours	20 mg/L	varD	57	15.4	0.0
	24-hours	-	varControl_1	100	5.7	8.0
	72-hours	-	varControl_2	50	16.3	23.1

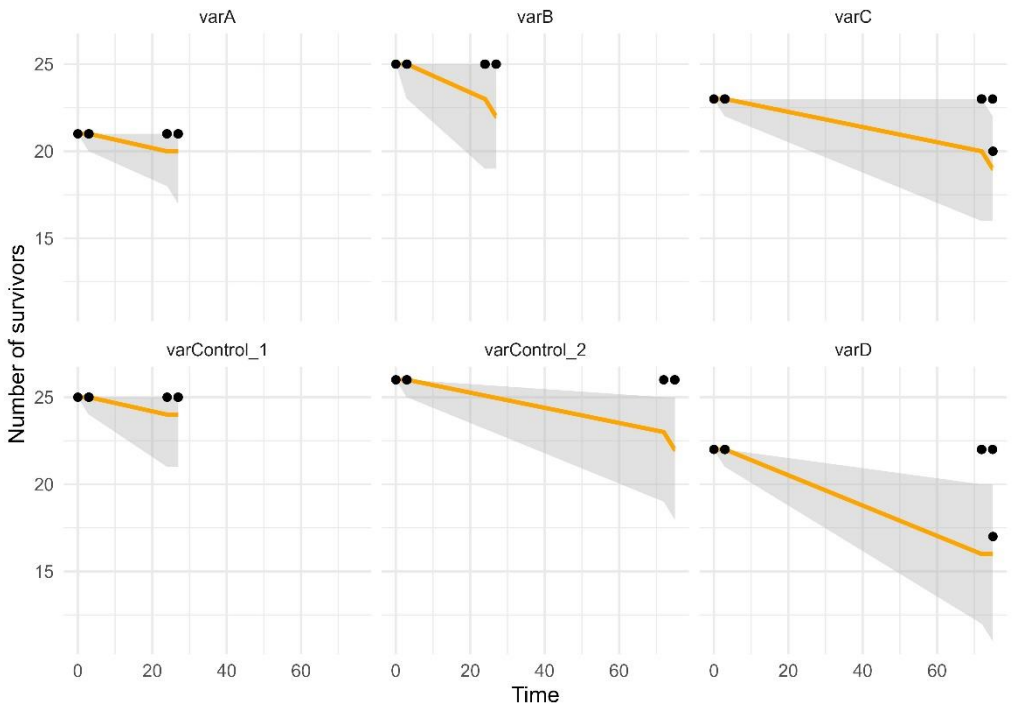


Figure 39: Plotted observed survivability from pulse exposures with predicted number of survivors from the TKTD-RED-GUTS-SD model. Black dots represent the number of survivors divided by initial number, and black line is the between replicate variability. Orange line is the median curve, and grey band is the 95% credibility interval.

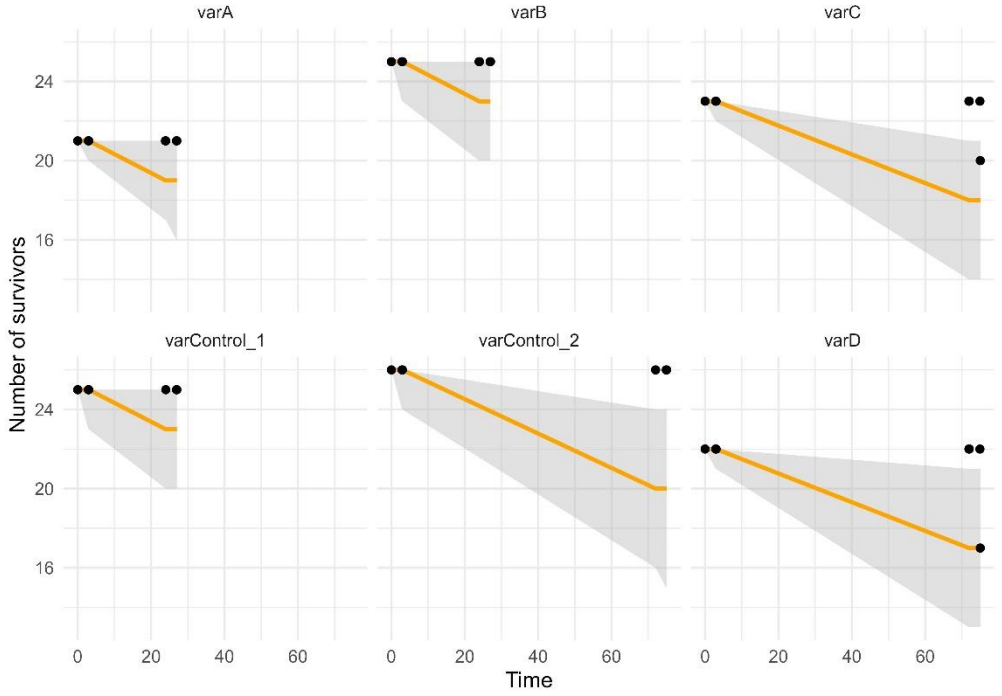


Figure 40: Plotted observed survivability from pulse exposures with predicted number of survivors from the TKTD-RED-GUTS-IT model. Black dots represent the number of survivors divided by initial number, and black line is the between replicate variability. Orange line is the median curve, and grey band is the 95% credibility interval.

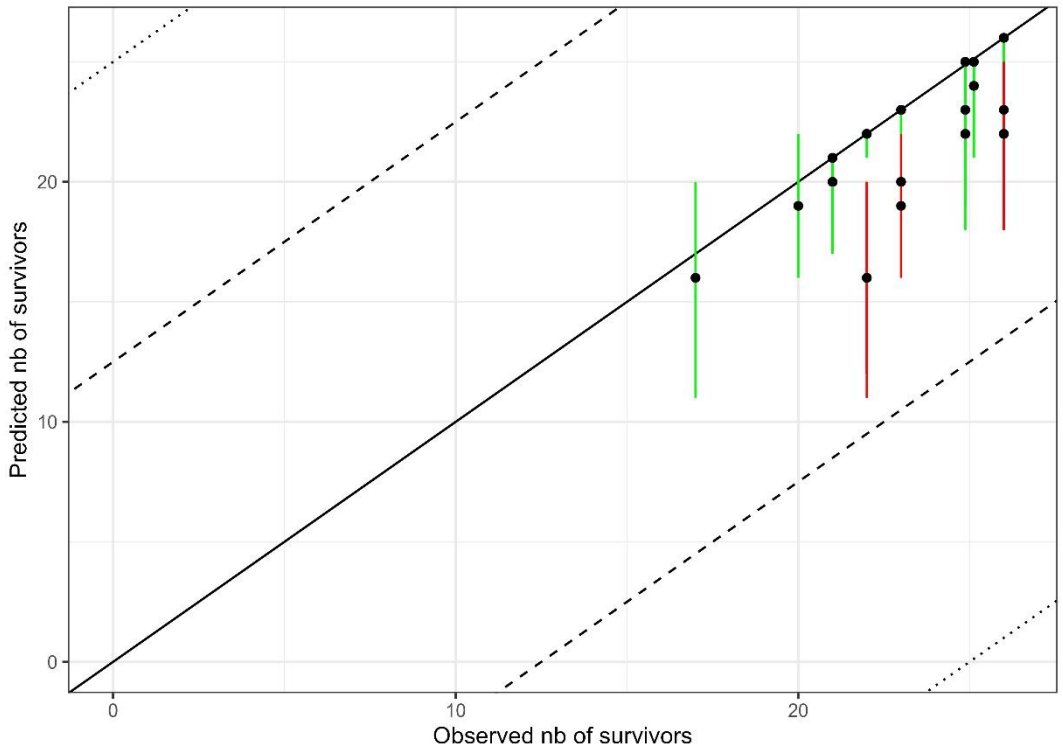


Figure 41: PPC plot for TKTD-RED-GUTS-SD model with predicted number of survivors based on pulsed exposure. x-coordinate for black dot is observed number of survivors and while y-coordinate is predicted median number of survivors, with a 95% credible value for predicted numbers. Green lines overlap with the line x = y.

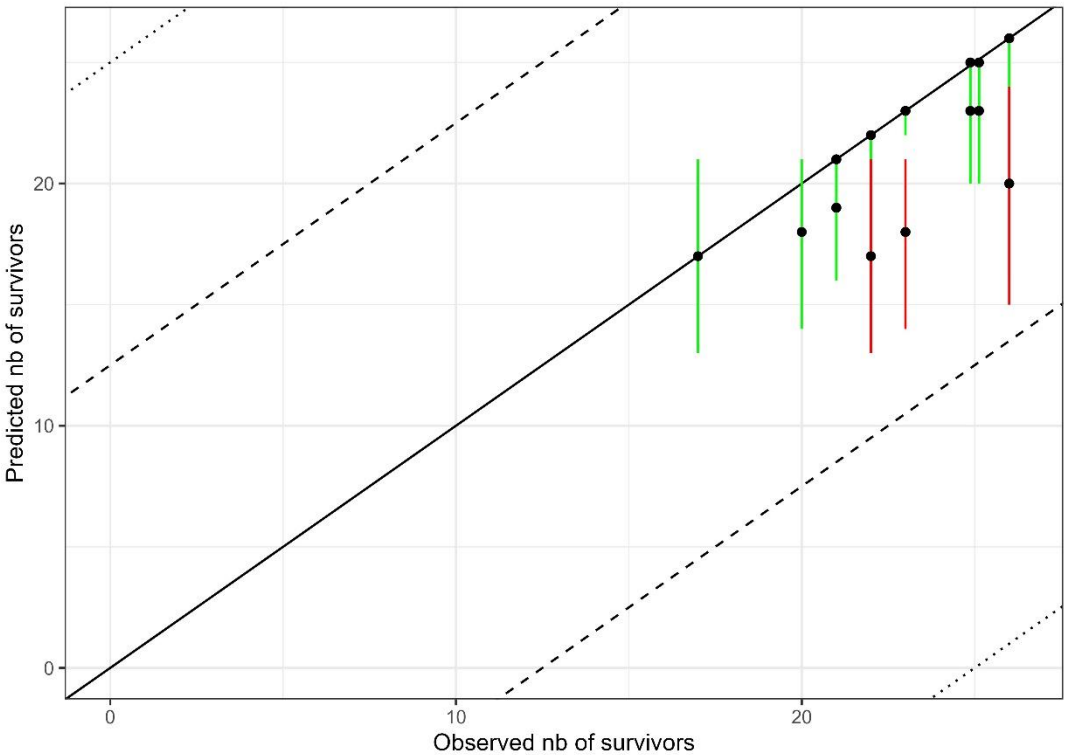


Figure 42: PPC plot for TKTD-RED-GUTS-IT model with predicted number of survivors based on pulsed exposure. x-coordinate for black dot is observed number of survivors and while y-coordinate is predicted median number of survivors, with a 95% credible value for predicted numbers. Green lines overlap with the line x = y.

4 Discussion

4.1 Imidacloprid concentrations and stability

From LC-MS/MS analysis (Appendix F, Appendix G, Appendix H), based on obtained LOD and LOQ, only presence of IMI in nominal concentration 0.002 mg/L could be confirmed and samples in nominal concentrations 0.0002 mg/L could not be confirmed. To quantify samples below 0.02 mg/L or qualify samples below 0.002 mg/L utilizing the same analytical setup, a new sample preparation would be necessary. For example, reducing the volume from samples initially below LOQ, resulting in increasing the concentration above LOQ. Based on the purity of Ectosan® Vet (Table 1), the measured concentrations of IMI in the solutions used were expected to be close to nominal concentrations. For the 96-hours exposure (Table 5, Table 12), all measured concentrations were below expected levels. The deviation in the LC-MS method is < 10%, and not sufficient to alone explain the deviations. This discrepancy may result from solutions preparation or sample preparation, such as unsolved IMI or lost Ectosan® Vet during transferring to volumetric flask, or sample preparation during LC-MS analysis. Based on concentrations followed a serial dilution pattern from a stock solution, the error most probably lies in the stock solution. Undissolved clumps of Ectosan® Vet were observed in the flask after stirring, which if they consisted of IMI, would explain similar deviations in all used concentrations. For the solutions used in the pulse exposures, fewer or no clumps were observed. Resulting in higher concentrations, which were closer to the nominal values based on used amount of Ectosan® Vet (Table 5, Table 11). Undissolved clumps of Ectosan® Vet could be a result of poorly mixing but based on solubility (580 to 610 mg/L at 20°C) expected to dissolve completely. Another possibility is degradation of IMI from solution preparation to initiation exposure. However unlikely, due to the short duration and minimal light exposure. This is supported by a study indicating that IMI showed little absorption under wavelengths of natural light (> 300 nm), and maximum absorption in range of wavelengths 211 nm to 268 nm (Liu et al., 2006). From sample analysis (Table 12) IMI was found to be stable in seawater solutions for the 96-hours duration, with deviations between sampling attributable to uncertainty in the LC-QqQ-MS/MS method.

A study assessing the stability of IMI in aquatic solutions over 22 days reported similar finds (Tišler et al., 2009). They noted degradation only at higher IMI concentrations, specifically 105 mg/L and 140 mg/L, whereas no degradation was observed at concentrations 17.5 mg/L and 8.75 mg/L, consistent with the observations in this thesis. Another study examined the

stability of IMI under different pH-levels at a concentration of 20 mg/L (Liu et al., 2006), with increased degradation under alkaline conditions. This finding is supported by another study, which reported a higher degradation rate of IMI in alkaline solutions over a 30-day period (Mahapatra et al., 2017). In contrast, one study reported a higher degradation rate of IMI in acidic solutions compared to alkaline solutions, with the half-life of IMI ranging from approximately 31 to 46 days, depending on pH and initial concentration (Sarkar et al., 1999). Based on the stability and slightly alkaline pH-level in the filtered saltwater utilized (7.9), is compiling with studies reporting stability around neutral and alkaline pH-levels for a period of 96-hours. However, a longer duration is necessary to compare with the reported in previous study long-term stability.

In selected replicates, significant deviations in concentration were observed. For instance, replicate 1 at a concentration of 0.013 mg/L showed a 51% decrease (Table 12), which was notable since other replicates at the same nominal concentration came from the same solution, and all beakers were treated identically. One measurement could have been to conduct some replicates in the dark to examine for light degradation, or measure pH in all replicates to examine for changes in pH-levels. In other replicates, the final concentration increased compared to the initial measured concentration, which was unusual as the other replicates at the same nominal concentration were stable or showed a slight decrease. This occurred in replicate 3 in both nominal concentrations of 2 mg/L and 20 mg/L (Table 12). To confirm these observations, the replicates were reanalyzed (Table 12), and concentrations again in nominal concentration 20 mg/L appeared low compared to other replicates, indicating low initial concentration and significantly higher final concentrations. Replicate 3 in nominal concentration 2 mg/L appear closer to other replicates, however lower in level. A similar trend was observed in unpublished research performed by the IMR, which analyzed different saltwater solutions containing IMI under both light and dark conditions. The setup included three parallels for each condition, each divided into two replicates. Samples were taken from replicates after 0, 2, 4, 8 and 24-hours, and analyzed with LC-QqQ-MS/MS. Two parallels under both conditions experienced minor change in concentration of IMI, while one parallel showed an increase in concentration over time. Since all replicates originated from the same solution and were treated identically, this warrants further research to understand the underlying causes. Based on the result in this thesis and previously published studies, indicating IMI having a high stability given the right parameters, thereby could persist for lengthy periods in the wild given the right conditions.

4.2 Exposure to IMI

Comparing mortality and immobilization for concentration 15 mg/L during the 96-hour exposure, it is evident that nearly all copepods were immobilized after 24-hours before mortality increased at subsequent time points (Table 13). Specifically, copepods in each replicate were observed to be dead after 48-hours, with the majority of animals in all replicates dead after 72-hours. This pattern was not evident at other nominal concentrations, although in all replicates, immobilization of copepods was observed before mortality at later time points. It is difficult to determine whether mortality affected previously immobilized copepods or healthy copepods, due to the challenge of distinguishing between animals. However, the observations at 15 mg/L IMI exposure suggest that copepods experience immobilization effects before lethal effects. When comparing mortality and immobilization rates (Fig. 22, Fig. 23), a clear trend in mortality is evident, whereas no obvious trend is seen for immobilization due immobilization fluctuated over time. Comparing all obtained LC- and EC-values, some values were calculated to be within the concentration range of the experiment while other were had values extremely out of range (Table 15), additionally higher than the solubility of IMI (580 to 610 mg/L at 20°C). Therefore, extreme values was discarded. A low mortality was observed after 48-hours of exposure, making LC_{10} the most reliable value as indicated in LC_{90} after 48-hours, while longer exposure LC values have a higher certainty due to higher numbers in mortality and in all concentrations. The EC-values were observed to be within the range after 24-hours exposure and for EC_{10} throughout exposure, while EC_{90} having values higher or extremely high. One explanation could be utilized concentration were too high to assess immobilization for acute exposures. Additionally, the number of immobilization was observed fluctuating over time, resulting in the animals becoming immobilized and dying after being classified as immobilized. If only sub-lethal concentrations were used, EC-values would be more accurate as the number of immobilization would likely fluctuate less.

One study followed a similar method for acute mortality and constant exposure for 96-hours (Stoughton et al., 2008). The LC_{50} values obtained for the aquatic species *Chironomus tentans* and *Hyalella azteca* were 5.75 µg/L and 65.43 µg/L, respectively, for technical IMI. They concluded that, based on available toxicity data, *Chironomus tentans* and *Hyalella azteca* are more acutely resilient to IMI. Comparing the obtained LC_{50}-values (Table 15), suggest that *A. tonsa* has a higher resilience to IMI after 96-hours exposure. Another study conducted toxicity tests on several different species, including two freshwater and to saltwater species, of

analytical grade IMI in aquatic solutions, including the aquatic crustacean water flea *Daphnia magna* (Song et al., 2009). This study has higher concentrations of IMI than used in this thesis, but the lower concentrations overlapped with the range used here. The reported LC_{50}-values were 10.44 mg/L at 27°C and 17.36 mg/L at 20°C after 48-hours exposure, and LC_{90}-values were 263.61 mg/L and 85.19 mg/L. They observed no temperature-dependent tolerance differences for IMI. Comparing the obtained LC_{50}-values with those of this study suggest that *A. tonsa* has lower resilience to IMI after 48-hours exposure than *Daphnia magna*. Based on the observed mortality (Table 13), copepods were first observed dead after 48-hours, with mortality drastically increasing after 96-hours exposure. For a shorter constant exposure period, the mortality indicates that *A. tonsa* to be less tolerant to IMI. Another study reported EC_{10} and EC_{50} for *Daphnia magna* of 36.8 mg/L and 97.9 mg/L, respectively, after 24-hours exposure, and 22.5 mg/L and 56.6 mg/L after 48-hours exposure (Tišler et al., 2009). Comparing the obtained EC_{10}- and EC_{50}-values after 48-hours exposure in this study (Table 15), indicates that *A. tonsa* is overall more sensitive to IMI than *Daphnia magna*. This could indicate different mechanisms in either or both toxicokinetic and toxicodynamic for *A. tonsa* compared to other freshwater species. One study conducted a similar setup on acute exposure looking at mortality on 27 different freshwater zooplankton species, with constant IMI exposure at concentrations: 0 µg/L, 0.5 µg/L, 5 µg/L, 50 µg/L, 100 µg/L and 500 µg/L (Suzuki et al., 2024). The aim was to broaden the understanding of zooplankton toxicity and move away from a *Daphnia*-centric approach. The study used species from three crustaceous orders: cladocerans, calanoids and cyclopoid. It reported that two out of three calanoid species were not affected by IMI, and *A. tonsa* belongs to the calanoid order (Boxshall, 2004), showed higher resilience to IMI compared to some freshwater zooplankton species, but was more sensitive compared to *Daphnia magna*. Given that previous studies assessed toxicity primarily on freshwater zooplankton, more data on marine species is needed to better assess the resilience compared to freshwater species and species within same crustaceous order. One study assessed the toxicity of acetamiprid, clothianidin, IMI, thiacloprid and thiamethoxam on their ability to inhibit the larval development of *A. tonsa* (Picone et al., 2022). Reported EC_{10}-values were 0.05 µg/L, 0.30 µg/L, 0.50 µg/L, 0.53 µg/L and 0.06 µg/L, and EC_{50}-values were 0.73 µg/L, 1.9 µg/L, 8.84 µg/L, 2.34 µg/L and 1.71 µg/L, respectively, following 5 days exposure, indicating that IMI had the least effect on *A. tonsa*. They used a concentrations: 0.02 µg/L, 0.14 µg/L, 1.01 µg/L and 10.1 µg/L, with concentrations 1.01 µg/L and 10.1 µg/L overlapping with concentrations in this study. Only obtained EC_{10} (0.0005 mg/L) after 72-

hours is comparable with this study, which concentrations is close in level given a 2 days exposure difference. Given this study used concentration where high mortality rates were observed, could affect the EC-values given the number of immobilized copepods fluctuated over time. However, the obtained result could indicate similar result in EC for *A. tonsa* in copepod stage compared to larval stage.

Comparing the obtained results from the 96-hours exposure (Table 13) and previous studies with the release concentration in Norwegian aquaculture (Grefsrud et al., 2022; Veterinærkatalogen), it appear that for short exposure periods minimal acute effects take place and longer exposure periods is needed for chronic effects to take place. However, this study and previous studies performed shorter constant exposure periods and pulsed exposure, which poorly reflects a realistic exposure profile in the wild, where IMI disperse affecting concentration and duration of exposure. Based on the stability of IMI, as previously discussed, low concentrations released in the wild could affect *A. tonsa* in other ways than mortality. This can include sublethal effects like larval development (Picone et al., 2022), immobilization or reproduction. There were no pulse profiles conducted in the lower concentration range, but comparing with obtained results from both profiles (Table 16, Table 17), suggest low to no mortality in acute exposure from released treatment water from aquaculture (Grefsrud et al., 2022; Veterinærkatalogen). There was no observed mortality and few animals experiencing immobilization in nominal concentrations 2 mg/L. Further, studies examining chronic exposure could provide more knowledge, with more experiments using sublethal concentrations to examine for instance respiration, reproduction and gracing. If there was a problem with the procedure for reducing the treatment concentration of IMI and treatment water were released directly into the ocean, the results from this study indicates that *A. tonsa* would experience mortality and immobilization. As indicated by calculated LC- and EC-values (Table 15), which is substantially lower concentrations than 20 mg/L. This could indicate other zooplankton species would experience similar effects. However, further research is needed to assess the toxicity of IMI on other marine zooplankton species.

4.3 TKTD-RED-GUTS models

Comparing both TKTD-RED-GUTS-SD (Fig. 32a) and TKTD-RED-GUTS-IT (Fig. 32b) models obtained from the 96-hours exposure in calibration (Appendix K), both visually appear to be a poor fit compared to observed mortality. However, the TKTD-RED-GUTS-IT model (Fig. 32b) was a good fit for the observation at a concentration of 15 mg/L IMI. From PPC plots (Fig. 33a, Fig. 33b), the numbers of data points intersecting with the y = x line is low for both models, further confirming their poor quality. Comparing calculated parameters estimates for both SD and IT models (Table 18), the dominant rate constant and concentration threshold for both SD, as well as the median of threshold distribution, are higher for both parameters in the IT model. The IT model assumes a higher increase in scaled damage based on external concentration, but also a higher threshold before an animal experience hazard effects. Thus, both models predicts mortality differently, but neither aligns well with the observed data. The dose response in the 96-hours exposure set up had a wide range, with a tenfold increase between doses. For concentrations ranging from 0.0002 mg/L to 0.013 mg/L, negligible effect was observed. Clear effects were seen at concentrations from 0.13 mg/L to 15 mg/L, but 100% mortality was only observed at 15 mg/L following 96-hours exposure. For a good GUTS model, the calibration data should span treatments level from no effect to large effect (Ockleford et al., 2018). Increasing concentrations beyond 15 mg/L, thereby expecting more extreme observations, might improve the performance of the TKTD-RED-GUTS models for *A. tonsa*. The chosen concentration range, was based on interest in effects for release concentration and effects for concentration used in bath treatment, and therefore environmentally relevant for the Norwegian ecosystem (Grefsrud et al., 2022; Veterinærkatalogen).

In order for the validation data to be considered of good quality, it should fulfill four requirements: at least two exposure profiles with two pulses separated by a no exposure period of different lengths; mortality and immobilization monitored at least for seven time points; DRT_{95} calculated with one pulse profile having longer duration and the other profile a shorter duration of non-exposure; exposure dose response tested at a minimum three concentration levels (Ockleford et al., 2018). The performed validation experiments only fulfill one criterion. Both pulse exposure profiles should include longer intermediate periods and monitored at more time points, to have a clearer view of observed effects over time. However, calculated DRT_{95} is significantly large, making impossible to fulfill for *A. tonsa*. When comparing parameter estimates for both models from pulse exposures (Table 19), the

concentration threshold for SD and median of threshold distribution for IT appear similar, however IT have higher dominant rate constant than SD, indicating higher hourly scaled damage. The comparison with the models from calibration parameters is substantially different. Therefore, all models predict the number of survivors differently. Despite models being a visually good fit (Fig. 35, Fig. 36), and all 95% CI predictions for both models observed intersecting the y = x line int PPC plots (Fig. 37, Fig. 38), validation data is still considered to be of poor quality due not fulfilling initial requirements. Additionally, to the requirements three statistical criteria, PPC, NRMSE and SPPE, needs to be assessed. PPC should be above 50% and NRMSE should not exceed 50%. NRMSE is based on predicted and observed number of survivors matches a 1:1 line on a scatter plot, while SPPE is the probability to survive from the beginning to the end based on both observed and predicted number of survivors (Ockleford et al., 2018). Based on a visual check from validation, observed number of survivors is higher than predicted survivors in both SD and IT models (Fig. 39, Fig. 40). PPC-values (Table 20, Fig. 41, Fig. 42) is observed to have high value for concentration in 24-hours pulse, while having lower for concentration in 72-hours pulse. The NRMSE-values are low, therefore within requirement. Calculated SPPE for all concentration are low positive values, confirming the models to predict slightly lower compared to observed numbers. Despite criteria's not exceeding suggested values, the validation models have a poor quality, due to calibration model already being of poor quality. In conclusion, models obtain in calibration and validation represents poor models to assess mortality from IMI on *A. tonsa*. This study need to be repeated, with new concentrations and fulfill validation requirements and statistical criteria, to better make predictions on untested exposure profiles.

4.4 Challenges handling *Acartia tonsa*

Utilizing *A. tonsa* as subject-animal presented posed multiple challenges in handling. Animals were lost during handling and transferring, and nauplii reached the copepod stage during the exposure period. Further, multiple replicates were observed to have a deviation in total number of animals between monitoring time points (Table 14). Consequently, missing animals were considered alive for the lower concentrations and either dead or immobilized for the higher concentrations, depending on the time point. This is because live animals were the most difficult to transfer, as they were the most active, while immobilized or dead animals tended to remain still or sank to the bottom of the holding chamber. Monitoring all replicates became time consuming. To avoid delaying the process and prolonging exposure, monitoring

started 2-3 hours before scheduled time and continued for a similar duration after. Resulting in approximately half of all replicates monitored before and after the time point.

Monitoring was conducted by two individuals, the master student and the supervisor, as a practical measurement. The supervisor performed monitoring for time points 0-hours to 48-hours, while student performed monitoring for the 72-hours and 96-hour time points. At the time point 72-hour mark (Table 14), a lower total number of animals in a considerable number of replicates was observed. However, by the 96-hour time point, the total number of copepods monitored was closer to the totals counted at the 24-hours and 48-hour time points. This discrepancy is likely due to the student's previous lack of experience in handling copepods and inefficiency in the performed method. By the 96-hour time point, handling was more efficient and precise, due to increased experience. Another consequence of multiple people perform the monitoring is the potential for inconsistent classification. Immobilized copepods may have been classified differently. Ideally, the same person should conduct monitoring to minimize differences in classification and ensure consistency.

5 Conclusion

This research determined the acute toxicity of the neonicotinoid IMI on *A. tonsa*, with the following lethal concentrations after 96-hours exposure: LC_{10} = 0.0021 mg/L, LC_{50} = 0.10 mg/L and LC_{90} = 4.3mg/L. Additionally, effective concentrations after 24-hours exposure were: EC_{10} = 0.06 mg/L, EC_{50} = 1.2 mg/L and EC_{90} = 25 mg/L. When these findings are compared with the release concentration from the Norwegian aquaculture, 0.30 µg/L, they suggest minimal acute effects. However, they also suggest acute exposure from untreated treatment water in Norwegian aquaculture, 20 mg/L IMI, could potentially have a significant impact on wild *A. tonsa* and potentially other non-target copepods. Further, the stability of IMI in filtered marine solutions was observed to be stable for a duration of 96-hours in all concentration under the temperature range from 13℃ to 15℃ and a pH-level at 7.9. The TKTD-RED-GUTS models, including both SD and IT, were visually and statistically poor fits for observed mortality from the 96-hours exposure and both pulse exposures, indicating their unsuitability for predicting acute effects on *A. tonsa* across varying exposure profiles.

6 Further research

To better understand sub-lethal effects on *A. tonsa* on exposure from IMI, more research with sub-lethal concentrations is needed. Furthermore, other ways to address sub-lethal effects than immobilization needs to be examined, like respiration, behavioral pattern and grazing. To make better TKTD-RED-GUTS models the experimental setup needs to be repeated, with modifications to concentrations of IMI. One possibility could be testing higher concentration than 20 mg/L. Additionally, repeat pulse exposure to validate the new experimental setup. Also, examine the stability of IMI in saltwater for a longer duration should be conducted, to better compare differences with available data for degradation in freshwater. To further assess the risk IMI poses to marine ecosystems, more toxicity data on more marine species is needed and making models for prediction the dispersion of IMI. To further address the stability in the ocean, more degradation experiments in marine solutions could be of interest. Additionally, sediment experiments to study the stability and accumulation of IMI in sediments.

References

A.Schram, T. (1993). Supplementary descriptions of the developmental stages of Lepeoptheirus salmonis (Krøyer, 1837) (Copepoda: Caligidae). In G. A.Boxshall & D. Defaye (Eds.), *PATHOGENS OF WILD AND FARMED FISH: sea lice* (pp. 30-50). CRC Press.

Agilent Technologies. (2012). *Agilent 6400 Series Triple Quadrupole LC/MS System* (G3335-90135). https://www.agilent.com/Library/usermanuals/Public/G3335-90135_QQQ_Concepts.pdf

Ajiboye, O. O., Yakubu, A. F., Adams, T. E., Olaji, E. D., & Nwogu, N. A. (2010). A review of the use of copepods in marine fish larviculture. *Reviews in Fish Biology and Fisheries*, *21*(2), 225-246. https://doi.org/10.1007/s11160-010-9169-3

Anjos, C. S., Lima, R. N., & Porto, A. L. M. (2021). An overview of neonicotinoids: biotransformation and biodegradation by microbiological processes. *Environ Sci Pollut Res Int*, *28*(28), 37082-37109. https://doi.org/10.1007/s11356-021-13531-3

Baudrot, V., Charles, S., Delignette-Muller, M. L., Duchemin, W., Goussen, B., Kehrein, N., Kon-Kam-King, G., Lopes, C., Ruiz, P., Singer, A., & Veber, P. (2022, 28.10). *morse: Modelling Reproduction and Survival Data in Ecotoxicology*. https://cran.r-project.org/package=morse

Bauer, B., Singer, A., Gao, Z., Jakoby, O., Witt, J., Preuss, T., & Gergs, A. (2024). A Toxicokinetic-Toxicodynamic Modeling Workflow Assessing the Quality of Input Mortality Data. *Environ Toxicol Chem*, *43*(1), 197-210. https://doi.org/10.1002/etc.5761

Boxshall, G. (2004, 27.06.2008). *Acartia tonsa Dana, 1849*. World Register of Marine Species. Retrieved 21.05 from https://www.marinespecies.org/aphia.php?p=taxdetails&id=104262

Centre, S. L. R. (2020). *LSa_LC_short.tif* Version V1) [Illustration]. DataverseNO. https://doi.org/doi/10.18710/GQTYYL/QG1SDA

CFEED. *How to hatch and harvest your copepods*. https://cfeed.no/procedure

Coates, A., Phillips, B. L., Bui, S., Oppedal, F., Robinson, N. A., & Dempster, T. (2021). Evolution of salmon lice in response to management strategies: a review. *Reviews in Aquaculture*, *13*(3), 1397-1422. https://doi.org/10.1111/raq.12528

Cumming, H., & Rucker, C. (2017). Octanol-Water Partition Coefficient Measurement by a Simple (1)H NMR Method. *ACS Omega*, *2*(9), 6244-6249. https://doi.org/10.1021/acsomega.7b01102

Drillet, G. (2010). *Copepods and their resting eggs. a potential source of nauplii for aquaculture* [Ph.D, Roskilde University].

Dyrevelferdsloven, (2009). https://lovdata.no/dokument/NL/lov/2009-06-19-97

EFSA. (2008). Conclusion regarding the peer review of the pesticide risk assessment of the active substance imidacloprid. *EFSA J*, *6*(7), 148r. https://doi.org/10.2903/j.efsa.2008.148r

EFSA. (2016). Peer review of the pesticide risk assessment for the active substance imidacloprid in light of confirmatory data submitted. *EFSA Journal*, *14*(11), e04607. https://doi.org/https://doi.org/10.2903/j.efsa.2016.4607

Escobar-Lux, R. H. (2021). *The effects of the chemotherapeutants hydrogen peroxide, deltamethrin and azamethiphos on non-target crustaceans* [Doctral dissertation, Department of Biological Sciences, Bergen Open Research Archive. https://bora.uib.no/bora-xmlui/handle/11250/2774014

Escobar-Lux, R. H., Parsons, A. E., Samuelsen, O. B., & Agnalt, A. L. (2020). Short-term exposure to hydrogen peroxide induces mortality and alters exploratory behaviour of European lobster (Homarus gammarus). *Ecotoxicol Environ Saf*, *204*, 111111. https://doi.org/10.1016/j.ecoenv.2020.111111

Falkenhaug T, G. B., Jelmert A, Oug E, Sundet J. (2018, 05.06). *Acartia tonsa, vurdering av økologisk risiko. Fremmedartslista 2018*. Artsdatabanken. Retrieved 17.01.2024 from https://artsdatabanken.no/Fab2018/S/104

Fiskeridirektoratet. (2023). *Salg av laks og regnbueørret*. Fiskeridirektoratet. Retrieved 17.03 from https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Laks-regnbueoerret-og-oerret/salg-av-laks-og-regnbueorret

Focks, A., Belgers, D., Boerwinkel, M. C., Buijse, L., Roessink, I., & Van den Brink, P. J. (2018). Calibration and validation of toxicokinetic-toxicodynamic models for three neonicotinoids and some aquatic macroinvertebrates. *Ecotoxicology*, *27*(7), 992-1007. https://doi.org/10.1007/s10646-018-1940-6

Frew, J. A., Brown, J. T., Fitzsimmons, P. N., Hoffman, A. D., Sadilek, M., Grue, C. E., & Nichols, J. W. (2018). Toxicokinetics of the neonicotinoid insecticide imidacloprid in rainbow trout (Oncorhynchus mykiss). *Comp Biochem Physiol C Toxicol Pharmacol*, *205*, 34-42. https://doi.org/10.1016/j.cbpc.2018.01.002

Grefsrud, E. S., Agnalt, A.-L., (HI), L. B. A., NINA, O. D., Dunlop, K. M., (HI), R. E., Peder Fiske NINA, O. F., Glover, K., Grøsvik, B. E., Halvorsen, K., Hannisdal, R., (HI), P. K. H., NINA, K. H., Husa, V., Jansson, E., Johnsen, I. A., (HI), Ø. K., NINA, S. K., Madhun, A. S., . . . (HI), V. W. (2024). *Risikorapport norsk fiskeoppdrett 2024* (Risikorapport norsk fiskeoppdrett

ISSN: 1893-4536). Havforskningsinstituttet. https://www.hi.no/hi/nettrapporter/rapport-fra-havforskningen-2024-4

Grefsrud, E. S., Bjørn, P. A., Grøsvik, B. E., Hansen, P. K., Husa, V., Karlsen, Ø., Kvamme, B. O., Samuelsen, O., Sandlund, N., Solberg, M. F., & Stien, L. H. (2022). *Risikorapport norsk fiskeoppdrett 2022 - kunnskapsstatus* (Rapport fra havforskningen

ISSN:1893-4536). Havforskningsinstituttet. https://www.hi.no/hi/nettrapporter/rapport-fra-havforskningen-2022-13

Hamre, L. A., Eichner, C., Caipang, C. M. A., Dalvin, S. T., Bron, J. E., Nilsen, F., Boxshall, G., & Skern-Mauritzen, R. (2013). The Salmon Louse Lepeophtheirus salmonis (Copepoda: Caligidae) Life Cycle Has Only Two Chalimus Stages. *PLOS ONE*, *8*(9), e73539. https://doi.org/10.1371/journal.pone.0073539

Harris, D. C. L., C. A. . (2020). *Quantitative Chemical Analysis* (Vol. 10). Macmillan International Higher Education.

Havforskningsinstituttet. (2018a, 17.02.2020). *"Generell biologi"*. Havforskningsinstituttet. Retrieved 01.11 from https://www.hi.no/hi/temasider/arter/lakselus/generell-biologi

Havforskningsinstituttet. (2018b, 07.07.2021). *Sea lice: Caligus curtus and Caligus elongatus*. Havforskningsintiuttet. Retrieved 11.05 from https://www.hi.no/en/hi/temasider/species/sea-lice/sea-lice-caligus-curtus-and-caligus-elongatus

Havforskningsinstituttet. (2018c, 27.09.2021). *"Skottelus og torskelus"*. Havforskningsintiuttet. Retrieved 01.11 from https://www.hi.no/hi/temasider/arter/lakselus/skottelus

Havforskningsinstituttet. (2020, 05.07.2021). *"Tema: Lakselus"*. Havforskningsintituttet. Retrieved 01.11 from https://www.hi.no/hi/temasider/arter/lakselus

Hung, A. P. (2014). *Microbial characterization and influence on copepod (Acartia tonsa) nauplii and egg production in water treatment systems with and without membrane filtration* Norwegian University of Science and Technology].

Irish Marine Institute. (2022). *Life cycle of the Salmon Louse*. Foras na mara marine Institute. Retrieved 17.03 from https://www.marine.ie/site-area/areas-activity/aquaculture/sea-lice/life-cycle-salmon-louse

IUPAC. (2019). tandem mass spectrometer. In (3.0.1 ed.). International Union of Pure and Applied Chemistry (IUPAC). https://doi.org/doi:10.1351/goldbook.T06250

Iversen, A., Asche, F., Hermansen, Ø., & Nystøyl, R. (2020). Production cost and competitiveness in major salmon farming countries 2003–2018. *Aquaculture*, *522*. https://doi.org/10.1016/j.aquaculture.2020.735089

Jager, T., Albert, C., Preuss, T. G., & Ashauer, R. (2011). General unified threshold model of survival--a toxicokinetic-toxicodynamic framework for ecotoxicology. *Environ Sci Technol*, *45*(7), 2529-2540. https://doi.org/10.1021/es103092a

Lahnsteiner, F., Kletzl, M., & Weismann, T. (2009). The risk of parasite transfer to juvenile fishes by live copepod food with the example Triaenophorus crassus and Triaenophorus nodulosus. *Aquaculture*, *295*(1-2), 120-125. https://doi.org/10.1016/j.aquaculture.2009.06.038

Forskrift om bekjempelse av lakselus i akvakulturanlegg, (2012). https://lovdata.no/dokument/SF/forskrift/2012-12-05-1140

Liu, W., Zheng, W., Ma, Y., & Liu, K. K. (2006). Sorption and degradation of imidacloprid in soil and water. *J Environ Sci Health B*, *41*(5), 623-634. https://doi.org/10.1080/03601230600701775

Mahapatra, B., Adak, T., Patil, N. K. B., Pandi, G. G. P., Gowda, G. B., Yadav, M. K., Mohapatra, S. D., Rath, P. C., Munda, S., & Jena, M. (2017). Effect of Abiotic Factors on Degradation of Imidacloprid. *Bull Environ Contam Toxicol*, *99*(4), 475-480. https://doi.org/10.1007/s00128-017-2159-6

McHenery, J. G. (2022). *Imidacloprid for salmonids. Environmental assessment in support of an import tolerance request*. F. a. D. Administration. https://www.fda.gov/media/162718/download

McLuckie, C., Moltschaniwskyj, N., Gaston, T., Dunstan, R. H., Crompton, M., Butcherine, P., Benkendorff, K., & Taylor, M. D. (2020). Lethal and sub-lethal effects of environmentally relevant levels of imidacloprid pesticide to Eastern School Prawn, Metapenaeus macleayi. *Sci Total Environ*, *742*, 140449. https://doi.org/10.1016/j.scitotenv.2020.140449

Merck. (2024). *68694 Imidacloprid*. Merck. Retrieved 13.04 from https://www.sigmaaldrich.com/NO/en/product/sial/68694#product-documentation

Motaung, T. E. (2020). Chloronicotinyl insecticide imidacloprid: Agricultural relevance, pitfalls and emerging opportunities. *Crop Protection*, *131*. https://doi.org/10.1016/j.cropro.2020.105097

National Center for Biotechnology Information. (2024a). *PubChem Compound Summary for CID 16212231, Imidacloprid-d4*. Retrieved 13.04 from https://pubchem.ncbi.nlm.nih.gov/compound/Imidacloprid-d4

National Center for Biotechnology Information. (2024b). *PubChem Compound Summary for CID 86287518, Imidacloprid*. National Library of Medicine. Retrieved 05.04 from https://pubchem.ncbi.nlm.nih.gov/compound/Imidacloprid

Ockleford, C., Adriaanse, P., Berny, P., Brock, T., Duquesne, S., Grilli, S., Hernandez-Jerez, A. F., Bennekou, S. H., Klein, M., Kuhl, T., Laskowski, R., Machera, K., Pelkonen, O., Pieper, S., Smith, R. H., Stemmer, M., Sundh, I., Tiktak, A., Topping, C. J., . . . Teodorovic, I. (2018). Scientific Opinion on the state of the art of Toxicokinetic/Toxicodynamic (TKTD) effect models for regulatory risk assessment of pesticides for aquatic organisms. *EFSA J*, *16*(8), e05377. https://doi.org/10.2903/j.efsa.2018.5377

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T. S., Gismervik, K., & Stien, L. H. (2018). Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review. *Reviews in Aquaculture*, *11*(4), 1398-1417. https://doi.org/10.1111/raq.12299

Pang, B. A.-O., Zhu, Y. A.-O., Lu, L. A.-O., Gu, F. A.-O., & Chen, H. A.-O. (2016). The Applications and Features of Liquid Chromatography-Mass Spectrometry in the Analysis of Traditional Chinese Medicine. (1741-427X (Print)). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5121459/

Parsons, A. E., Escobar-Lux, R. H., Saevik, P. N., Samuelsen, O. B., & Agnalt, A. L. (2020). The impact of anti-sea lice pesticides, azamethiphos and deltamethrin, on European lobster (Homarus gammarus) larvae in the Norwegian marine environment. *Environ Pollut*, *264*, 114725. https://doi.org/10.1016/j.envpol.2020.114725

Picone, M., Distefano, G. G., Marchetto, D., Russo, M., Baccichet, M., Zangrando, R., Gambaro, A., & Volpi Ghirardini, A. (2022). Inhibition of Larval Development of Marine Copepods Acartia tonsa by Neonicotinoids. *Toxics*, *10*(4). https://doi.org/10.3390/toxics10040158

Sarkar, M. A., Biswas, P. K., Roy, S., Kole, R. K., & Chowdhury, A. (1999). Effect of pH and Type of Formulation on the Persistence of

lmidacloprid in Water. *Bulletin of Enviromental Contamination and Toxicology*, *63*, 604-609. https://www.researchgate.net/profile/Sankhajit-Roy/publication/338224817_Effect_of_pH_and_Type_of_Formulation_on_the_Persistence_of_lmidacloprid_in_Water/links/5e0a1ca492851c8364a6cd9a/Effect-of-pH-and-Type-of-Formulation-on-the-Persistence-of-lmidacloprid-in-Water.pdf

Sheets, L. P. (2010). Chapter 95 - Imidacloprid: A Neonicotinoid Insecticide. In R. Krieger (Ed.), *Hayes' Handbook of Pesticide Toxicology* (Vol. 3, pp. 2055-2064). Academic Press.

Song, M. Y., Stark, J. D., & Brown, J. J. (2009). Comparative toxicity of four insecticides, including imidacloprid and tebufenozide, to four aquatic arthropods. *Environmental Toxicology and Chemistry*, *16*(12), 2494-2500. https://doi.org/10.1002/etc.5620161209

Statistisk sentralbyrå. (2020a). *07326: Akvakultur. Salg av slaktet matfisk, etter region, fiskeslag, statistikkvariabel og år* [Statistikk]. Statistisk sentralbyrå. https://www.ssb.no/statbank/table/07326/chartViewColumn/

Statistisk sentralbyrå. (2020b, 29.10.2020). *Akvakultur (avsluttet i Statistisk sentralbyrå)*. Statistisk sentralbyrå,. Retrieved 17.03 from https://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/fiskeoppdrett/aar

Stoughton, S. J., Liber, K., Culp, J., & Cessna, A. (2008). Acute and chronic toxicity of imidacloprid to the aquatic invertebrates Chironomus tentans and Hyalella azteca under constant- and pulse-exposure conditions. *Arch Environ Contam Toxicol*, *54*(4), 662-673. https://doi.org/10.1007/s00244-007-9073-6

Suzuki, H., Makino, W., Takahashi, S., & Urabe, J. (2024). Assessment of toxic effects of imidacloprid on freshwater zooplankton: An experimental test for 27 species. *Sci Total Environ*, *927*, 172378. https://doi.org/10.1016/j.scitotenv.2024.172378

Taranger, G. L., Karlsen, Ø., Bannister, R. J., Glover, K. A., Husa, V., Karlsbakk, E., Kvamme, B. O., Boxaspen, K. K., Bjørn, P. A., Finstad, B., Madhun, A. S., Morton, H. C., & Svåsand, T. (2015). Risk assessment of the environmental impact of Norwegian Atlantic salmon farming. *ICES Journal of Marine Science*, *72*(3), 997-1021. https://doi.org/10.1093/icesjms/fsu132

Tišler, T., Jemec, A., Mozetič, B., & Trebše, P. (2009). Hazard identification of imidacloprid to aquatic environment. *Chemosphere*, *76*(7), 907-914. https://doi.org/https://doi.org/10.1016/j.chemosphere.2009.05.002

Veterinærkatalogen. (04.08.2021). *Ectosan Vet*. Felleskatalogen. Retrieved 30.01 from https://www.felleskatalogen.no/medisin-vet/ectosan-vet-benchmark-animal-health-699283

Wang, X., Anadon, A., Wu, Q., Qiao, F., Ares, I., Martinez-Larranaga, M. R., Yuan, Z., & Martinez, M. A. (2018). Mechanism of Neonicotinoid Toxicity: Impact on Oxidative Stress and Metabolism. *Annu Rev Pharmacol Toxicol*, *58*, 471-507. https://doi.org/10.1146/annurev-pharmtox-010617-052429

Wilm, M. (2011). Principles of electrospray ionization. *Mol Cell Proteomics*, *10*(7), M111 009407. https://doi.org/10.1074/mcp.M111.009407

Zuvela, P., Skoczylas, M., Jay Liu, J., Ba Czek, T., Kaliszan, R., Wong, M. W., Buszewski, B., & Heberger, K. (2019). Column Characterization and Selection Systems in Reversed-Phase High-Performance Liquid Chromatography. *Chem Rev*, *119*(6), 3674-3729. https://doi.org/10.1021/acs.chemrev.8b00246

Appendix

Appendix A: Husbandry batches of *Acartia tonsa*, with date of hatching and transferring to holding tank

BATCH	**1**	**2**	**3**
HATCHED (DATE)	02.02.2024	07.02.2024	13.02.2024
HARVESTED (DATE)	04.02.2024	13.02.2024	19.02.2024

Appendix B: Husbandry of copepods protocol given by CFEED

User manual

How to hatch and harvest your copepods

HATCHING CONDITIONS

	26 °C / 79 °F	21 °C / 70 °F
Harvest copepods after:	24 hours	48 hours
Salinity	15 – 40 ppt	15 – 40 ppt
pH	7,8 – 8,5	7,8 – 8,5
Oxygen	> 80% saturation	> 80% saturation
Light	Not necessary	Not necessary
Density	< 1000 ml^{-1}	< 1000 ml^{-1}
Water exchange	0 %	0 %

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PREPARING YOUR HATCHING TANK

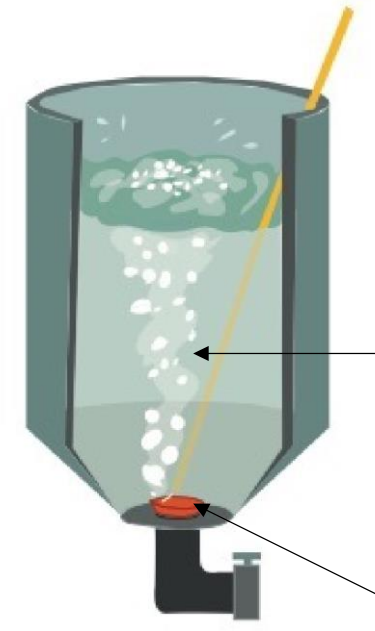
COPEPOD EGGS sediment easy, and sedimentation of eggs may lead to reduced hatching. To prevent the eggs from sedimenting, pay attention to:

- **Tank design**
- **Aeration**
- **Blocking all entrances**

Hatching time depends on th water temperature:

26 °C: 24h hatching
21 °C: 48h hatching

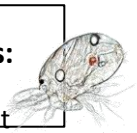
Choose the temperature closest to what is in your production tank

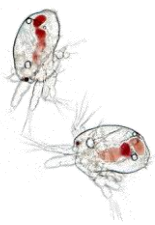


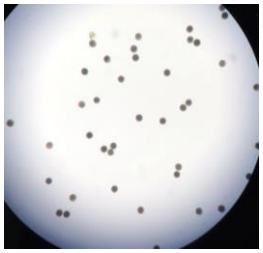
Tank design:
Use tanks with a **conical** or **rounded** bottom to ensure good circulation.
- Avoid tanks with a flat bottom

Aeration:
Heavy aeration, similar to when hatching Artemia, ensures a **good circulation** in your tank and prevent sedimentation of eggs:
- Use an open-ended tube to create **big bubbles**
- Place the tube at the lowest part of the tank

Blocking all entrances:
Eliminate **ALL** openings where eggs can sediment







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1: RECEIVING AND STORING EGGS

The eggs are transported on ice in a Styrofoam box. Upon arrival:

- Check that ice is still present in the box to ensure that the cool chain has been intact.
- **Store the eggs at 1-4 °C / 34-39 °F.**
- Use the eggs within a month after arrival.
- For longer storage, contact CFEED.

2: REMOVAL OF STORAGE MEDIUM

The eggs come in a storage solution containing clay that needs to be removed:

- Remove the bottle you would like to hatch from the cool storage.
- If hatching only part of a bottle: Shake the bottle until the clay and eggs are evenly mixed. While it is mixed, transfer the amount of eggs you want to hatch to a separate container. Place the rest of the bottle back into cool storage.
- Pour the clay mixture into a **50 µm sieve** that will allow the clay to pass through while the eggs remain in the sieve.
- Rinse with seawater until the eggs are clean. Transfer the eggs to a bucket.

3: DISINFECTION

EQUIPMENT:

- A bucket filled with 10L temperate sea water.
- Clean eggs where the storage medium has been removed.
- Aeration for mixing the eggs and chemicals.

DISINFECTION PROCESS:

- Disinfect for 10 minutes using **4 ml NaOCl (14%)**. Aerate and stir to ensure that all eggs are in contact with the chlorine.
- Add dissolved **Na_2SO_3 (8g)** to neutralize the chlorine. Aerate and stir to ensure that all eggs are in contact with the Na_2SO_3. Leave for 10 minutes.
- **Transfer your disinfected eggs into your prepared hatching tank (see page 1 for more information).**

4: LEAVE THE EGGS TO HATCH

Hatching time is dependent on water temperature. See page 1 for more information.

5: CONCENTRATING AND HARVESTING YOUR COPEPODS

Concentrate the hatched copepods before transferring them to your fish larval tank:

- Close off aeration for 20 minutes to let unhatched eggs sediment. **Flush quickly**. • Transfer the copepods to you concentrator.
- **Mesh size: < 65 µm**.
- **Aerate well** while concentrating:
- Prevent the copepods from clogging the mesh.
- No addition of O_2 is necessary.
- If the aeration is good the nauplii can be kept at a density of **15000 ml^{-1}** for up to **24 hours** at **5-6 °C**.
- For longer storage time, do not exceed the limit of 500 per ml. Use the copepods within 24 hours.

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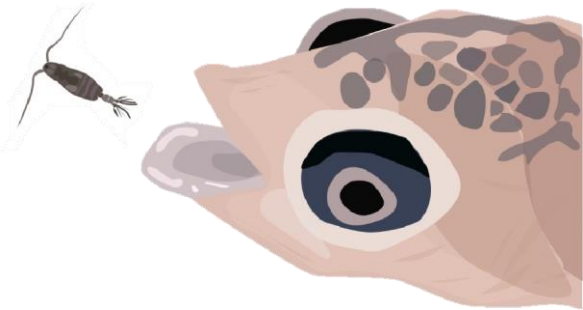
Tip! Giving the copepods microalgae is a great way to **increase the visibility** for the hunting fish larvae:

- Add a small amount of **live microalgae** when the copepods are in the concentrator and wait for 10 - 15 minutes. The guts will be filled and the nauplii are ready to be fed to the fish.
- Contact CFFED if you would like to know more about which types of microalgae to use.



Appendix C: Protocol water samples for 96-hour exposure with nominal concentration 0.2 mg/L - 20 mg/L

Protokoll imidakloprid analyser

Tre laveste nivåer på 96 timers forsøk (forsøk 2) opparbeid 04.04.24

Tillaging standard og intern standard.

Imidakloprid (IMI):

Løsning 1. 10 µg/ml: Ta ut 100 µL av stockløsning IMI 1 mg/ml, fortynn til 10 ml med acetonitril (målekolbe).

Løsning 2. 1 µg/ml: Ta ut 1 mL av løsning 1, fortynn til 10 ml med acetonitril (målekolbe).

Løsning 3. 100 ng/ml: Ta ut 100 µL av løsning 1, fortynn til 1 ml med acetonitril.

Intern standard (IS) IMI-d4:

Løsning 4. 1 µg/ml: Ta ut 50 µL av stockløsning IS 0.2 mg/ml, fortynn til 10 ml med acetonitril (målekolbe).

Analyseoppsett

Prøvene er veldig høy konsentrasjon (0.2, 2 og 20 µg/mL) så fortynner de to høyeste med 1/10: Ta ut 100 µL og fyll opp til 1 ml med sjøvann. Forventer da 0.02, 0.2 og 2 µg/mL (20, 200 og 2000 ng/mL). I tillegg fortynnes de 1/10 under prøveopparbeiding (se tabell) så sluttresultat må ganges opp med 100. Forventet resultat fra instrument er 2, 20 og 200 ng/ml.

For å få filtrert sjøvann, filtreres sjøvann forhånd med en pipette og filter (0,45µm). Filtrerte ca. 30 mL sjøvann. Dette for å gjøre opp for fortynningen til opparbeidingen av prøvene.

Prøve	Sjøvann/ prøve **µL**	Tilsatt IMI (µL) **Bruk løsning 100ng/ml* eller 1 µg/ml**	Kons. IMI **ng/mL**	Tilsatt IS **1 µg/ml**	Kons. IS **ng/mL**	Tilsatt acetonitril **µL**
Blank u/ matriks	0	0	0	50	50	950
Blankt sjøvann	100	0	0	50	50	850
N1 (sjøvann)	100	10*	1	50	50	840
N2 (sjøvann)	100	50*	5	50	50	800
N3 (sjøvann)	100	10	10	50	50	840
N4 (sjøvann)	100	20	20	50	50	830
N5 (sjøvann)	100	50	50	50	50	800
N6 (sjøvann)	100	100	100	50	50	750
N7 (sjøvann)	100	150	150	50	50	700
N8 (sjøvann)	100	200	200	50	50	650
N9 (sjøvann)	100	300	300	50	50	550
Kontroll 1	100	50*	5	50	50	800
Kontroll 2	100	100	100	50	50	750
Kontroll 3	100	200	200	50	50	650
Prøver	100	0	-	50	50	850

Appendix D: Protocol water samples for 96-hour exposure with nominal concentration 0.0002 mg/L - 0.02 mg/L and controls

Protokoll imidakloprid analyser

Tre laveste nivåer på 96 timers forsøk (forsøk 1) opparbeid 19.04.24

Tillaging standard og intern standard.

Imidakloprid (IMI):

Løsning 1. 10 µg/ml: Ta ut 100 µL av stockløsning IMI 1 mg/ml, fortynn til 10 ml med acetonitril (målekolbe).

Løsning 2. 100 ng/ml: Ta ut 100 µL av løsning 2, fortynn til 10 ml med acetonitril.

Løsning 3. 10 ng/ml: Ta ut 100 µL av løsning 3, fortynn til 1 ml med acetonitril.

Intern standard (IS) IMI-d4:

Løsning 4. 1 µg/ml: Ta ut 50 µL av stockløsning IS 0.2 mg/ml, fortynn til 10 ml med acetonitril (målekolbe).

Analyseoppsett

Her er konsentrasjonene på prøvene: 0.0002, 0.002, 0.02 og kontroll. Disser er ikke for høye, dermed fortynnes de ikke i forkant. Derimot fortynnes de 1/10 under prøveopparbeiding (se tabell) så sluttresultat må ganges opp med 10. Forventet resultat fra instrument er ingen, 0.002, 0.02 og 0.2 ng/ml.

For å få filtrert sjøvann, filtreres sjøvann forhånd med en pipette og filter (0.45µm).

Prøve	Sjøvann/ prøve **µL**	Tilsatt IMI **100 ng/ml eller 10 ng/ml***	Kons. IMI **ng/mL**	Tilsatt IS **1 µg/ml**	Kons. IS **ng/mL**	Tilsatt acetonitril **µL**
Blank u/ matriks	0	0	0	20	20	980
Blankt sjøvann	100	0	0	20	20	880
N1 (sjøvann)	100	10*	0.1	20	20	870
N2 (sjøvann)	100	50*	0.5	20	20	830
N3 (sjøvann)	100	10	1	20	20	870
N4 (sjøvann)	100	50	5	20	20	830
N5 (sjøvann)	100	100	10	20	20	780
N6 (sjøvann)	100	150	15	20	20	730
N7 (sjøvann)	100	200	20	20	20	680
N8 (sjøvann)	100	300	30	20	20	580
Kontroll 1	100	50*	0.5	20	20	830
Kontroll 2	100	100	10	20	20	780
Kontroll 3	100	200	20	20	20	680
Prøver	100	0	-	20	20	880

Appendix E: Protocol water samples from both 24-hours and 72-hours pulse exposures

Protokoll imidakloprid analyser

Nivåer fra 24-timers og 72-timers pulse forsøk, opparbeid 04.04.24

Tillaging standard og intern standard.

Imidakloprid (IMI):

Løsning 1. 10 µg/ml: Ta ut 100 µL av stockløsning IMI 1 mg/ml, fortynn til 10 ml med acetonitril (målekolbe).

Løsning 2. 1 µg/ml: Ta ut 1 mL av løsning 1, fortynn til 10 ml med acetonitril (målekolbe).

Intern standard (IS) IMI-d4:

Løsning 3. 1 µg/ml: Ta ut 50 µL av stockløsning IS 0.2 mg/ml, fortynn til 10 ml med acetonitril (målekolbe).

Analyseoppsett

Prøvene er veldig høy konsentrasjon (2 og 20 µg/mL) så fortynner de 1/10: Ta ut 100 µL og fyll opp til 1 ml med sjøvann. Forventer da 0.2 og 2 µg/mL (200 og 2000 ng/mL). I tillegg fortynnes de 1/10 under prøveopparbeiding (se tabell) så sluttresultat må ganges opp med 100. Forventet resultat fra instrument er 20 og 200 ng/ml

For å få filtrert sjøvann, filtreres sjøvann forhånd med en pipette og filter (0,45µm). Filtrerte ca. 30 mL sjøvann. Dette for å gjøre opp for fortynningen til opparbeidingen av prøvene.

Prøve	Sjøvann/ prøve **µL**	Tilsatt IMI **µL (1 µg/ml)**	Kons. IMI **ng/mL**	Tilsatt IS µL **(1 µg/ml)**	Kons. IS **ng/mL**	Tilsatt acetonitril **µL**
Blank u/ matriks	0	0	0	50	50	950
Blankt sjøvann	100	0	0	50	50	850
N1 (sjøvann)	100	10	10	50	50	840
N2 (sjøvann)	100	20	20	50	50	830
N3 (sjøvann)	100	50	50	50	50	800
N4 (sjøvann)	100	100	100	50	50	750
N5 (sjøvann)	100	150	150	50	50	700
N6 (sjøvann)	100	200	200	50	50	650
N7 (sjøvann)	100	300	300	50	50	550
Kontroll 1	100	20	20	50	50	830
Kontroll 2	100	100	100	50	50	750
Kontroll 3	100	200	200	50	50	650
Prøver	100	0	-	50	50	850

Appendix F: LC-QqQ-MS/MS analysis of series 1

	Imidacloprid Quantifier (256.1→ 175)						**Imidacloprid Qualifier (256.1→ 208.9)**	**Imidacloprid-d4 IS (256.1→ 208.9)**		
Name	RT	Resp.	Calc. Conc.	Accuracy	Height	S/N	Ratio	RT	Resp.	S/N
BUM	2.146	1	0.0116		1	0.76	347.7	2.093	1808	1380.26
BMM	2.507	15	0.1745		6	2.29	8.4	2.101	2452	1406.42
C1-T1-24h	1.922	1	0.0102		1	0.39	269.0	2.101	2792	1079.18
C2-T1-24h	1.958	3	0.0267		2	1.29	38.0	2.101	2698	994.74
C1-T2-24h	1.969	1	0.0093		1	0.68	296.6	2.104	2868	606.32
C2-T2-24h	2.069	0	0.0000	0	0	11.76		2.101	2631	2045.48
C1-T1-72h	2.058	2	0.0228		1	0.76		2.101	2845	1146.97
C2-T1-72h	1.969	5	0.0540		3	1.73	20.7	2.104	2903	1121.68
C1-T2-72h	2.168	1	0.0141		2	0.76	236.9	2.101	2984	935.20
C2-T2-72h	1.995	2	0.0228		2	1.24	63.6	2.101	2933	1163.83
N1-24-72h	2.109	1114	11.1119	111	356	198.04	99.0	2.101	2861	1193.53
N2-24-72h	2.106	1990	20.3431	102	652	400.92	95.5	2.101	2791	1599.33
N3-24-72h	2.106	5042	49.3802	99	1658	4439.91	98.3	2.101	2914	833.89
N4-24-72h	2.106	9637	97.8345	98	3145	179.26	97.9	2.101	2811	406.61
N5-24-72h	2.106	14557	152.1670	101	4683	6670.21	96.7	2.101	2730	654.77
N6-24-72h	2.106	19017	201.4275	101	6129	3140.24	96.0	2.097	2694	811.07
N7-24-72h	2.106	27055	297.7358	99	8494	8	97.0	2.101	2593	8
K1-24-72h	2.109	1820	20.7135	104	587	401.47	96.9	2.101	2507	566.61
K2-24-72h	2.106	10265	104.5525	105	3294	1988.89	97.0	2.101	2801	655.89
K3-24-72h	2.106	17869	198.8943	99	5603	285.94	95.3	2.097	2564	931.53
2mg/L-1-T1-24h	2.109	1514	16.2073		509	392.46	95.5	2.101	2665	638.07
2mg/L-2-T1-24h	2.109	1522	16.6282		492	275.92	97.1	2.101	2612	430.14
2mg/L -1-T2-24h	2.106	1593	17.4030		529	249.34	93.7	2.101	2611	901.68
2mg/L -2-T2-24h	2.109	1574	17.4124		507	420.75	96.7	2.101	2580	766.07
2mg/L -1-T1-72h	2.106	1583	16.0322		512	575.76	94.1	2.097	2817	835.59
2mg/L -2-T1-72h	2.106	1550	16.5934		500	302.15	94.8	2.097	2666	4215.41
2mg/L -1-T2-72h	2.106	1819	19.9450		601	965.36	95.1	2.097	2603	738.84
2mg/L -2-T2-72h	2.106	1862	18.9985		610	485.63	96.4	2.101	2797	1301.29
20mg/L l-1-T1-24h	2.106	41916	173.5293		13695	423.07	94.9	2.101	6892	5057.73
20mg/L -2-T1-24h	2.106	50185	177.8424		16167	719.31	94.7	2.101	8052	3467.16
20mg/L -1-T2-24h	2.106	48667	172.7738		15737	791.3	94.8	2.101	8038	3459.87
20mg/L -2-T2-24h	2.106	48808	168.9708		15676	1398.3	94.9	2.101	8242	1222.57
20mg/L -1-T1-72h	2.106	47469	168.9756		15314	921.23	94.8	2.101	8016	4037.53

20mg/L -2-T1-72h	2.106	45730	157.9710		14773	579.38	95.1	2.101	8260	7874.86
20mg/L -1-T2-72h	2.106	56503	196.6028		18141	1402.67	94.5	2.101	8201	4185.62
20mg/L -2-T2-72h	2.106	56299	199.1731		18193	730.53	95.2	2.101	8066	5178.60
K1-24-72h	2.113	5649	20.3735	102	1837	1586.59	96.8	2.104	7911	1777.14
K2-24-72h	2.113	32677	101.5900	102	10575	44753.68	97.2	2.104	9178	5974.13
K3-24-72h	2.113	57497	196.7621	98	18211	555.9	95.6	2.104	8338	3647.33

Appendix G: LC-QqQ-MS/MS analysis of series 2

	Imidacloprid Quantifier (256.1→ 175)						**Imidacloprid Qualifier (256.1→ 208.9)**	**Imidacloprid-d4 IS (256.1→ 208.9)**		
Name	RT	Resp.	Calc. Conc.	Accuracy	Height	S/N	Ratio	RT	Resp.	S/N
BUM-96h	2.036	2	0.007		2	0.77	945.5	2.104	8774	4669.39
BMM-96h	2.05	2	0.0068		2	0.81	295.5	2.104	8929	5836.25
N1-96h	2.109	284	1.0262	103	104	37.48	99.4	2.104	8569	3466.58
N2-96h	2.109	1460	5.4382	109	471	245.92	94.6	2.104	8304	3587.08
N3-96h	2.109	3123	11.7951	118	1009	635.07	97.1	2.104	8187	3186.56
N4-96h	2.109	4600	17.2789	86	1504	770.63	96.1	2.101	8234	3564.24
N5-96h	2.109	13434	51.7371	103	4380	1391.7	94.8	2.101	8030	3040.18
N6-96h	2.109	26805	101.4972	101	8747	7703.9	95.6	2.101	8167	2134.08
N7-96h	2.106	39099	153.1189	102	12671	11036.85	94.8	2.101	7897	923.34
N8-96h	2.106	51943	196.1669	98	16758	7788.14	95.3	2.101	8189	5544.51
N9-96h	2.106	77788	297.9416	99	24881	8261.56	95.8	2.101	8074	6956.11
K1-96h	2.106	1442	5.5755	112	462	143.01	92.4	2.101	7998	2701.94
K2-96h	2.102	26760	101.7503	102	8870	107.12	98	2.093	8133	2221.03
K3-96h	2.102	54742	206.5192	103	17794	6581.09	96	2.093	8197	3051.91
02mg/L -R1-T1-96h	2.102	460	1.5983		143	172.52	101.8	2.093	8906	3075.42
02mg/L -R2-T1-96h	2.098	350	1.2011		119	106.35	90.1	2.093	9008	4825.23
02mg/L -R3-T1-96h	2.102	354	1.2363		114	58.4	84.5	2.093	8848	1461.79
02mg/L -R4-T1-96h	2.102	316	1.0897		98	70.65	102.8	2.093	8956	15069.87
02mg/L -R1-T2-96h	2.098	309	1.0865		104	21.75	106.3	2.093	8802	11647.8
02mg/L -R2-T2-96h	2.102	464	1.6013		145	211.52	99.9	2.093	8959	1356.71
02mg/L -R3-T2-96h	2.098	407	1.401		132	48.35	98.6	2.093	8994	1675.67
02mg/L -R4-T2-96h	2.098	329	1.1213		103	208.3	88.8	2.093	9076	1643.76
2mg/L -R1-T1-96h	2.098	4489	15.8349		1448	762.77	94.1	2.093	8768	37785.7
2mg/L -R2-T1-96h	2.098	2859	10.2034		944	593.29	95.4	2.093	8666	5472.3
2mg/L -R3-T1-96h	2.102	1965	7.0636		652	305.22	93.7	2.093	8602	1242.8
2mg/L -R4-T1-96h	2.102	4393	15.2204		1419	780.15	97.2	2.093	8926	3656.09
2mg/L -R1-T2-96h	2.098	3619	13.3767		1188	738.39	97.5	2.093	8367	1334.88
2mg/L -R2-T2-96h	2.102	2742	9.8394		884	1009.33	96	2.093	8620	3667.34
2mg/L -R3-T2-96h	2.098	4209	14.8269		1362	764.41	96.6	2.09	8779	2964.1
2mg/L -R4-T2-96h	2.098	4404	15.7068		1434	721.43	95.2	2.093	8671	5438.53
20mg/L -R1-T1-96h	2.098	43600	156.0151		13992	6382.17	96	2.09	8642	4534.91
20mg/L -R2-T1-96h	2.102	43902	160.017		14103	8473.48	96.3	2.097	8485	8
20mg/L -R3-T1-96h	2.102	25522	90.6903		8215	3785.28	95.1	2.097	8703	1686.88
20mg/L -R4-T1-96h	2.102	45173	160.8838		14530	6186.91	95.5	2.093	8683	5235.19
20mg/L -R1-T2-96h	2.102	45517	161.7053		14684	12757.87	95.5	2.097	8705	2818.26
20mg/L -R2-T2-96h	2.102	44564	156.2723		14424	14861.86	96.3	2.097	8819	4159.25
20mg/L -R3-T2-96h	2.102	44797	160.5062		14395	6782.31	95.4	2.093	8631	9202.58
20mg/L -R4-T2-96h	2.102	37424	131.5204		12012	5506.94	96.4	2.093	8800	4147.9
K1-96h	2.102	1415	5.3518	107	456	189.74	98	2.093	8178	2584.88
K2-96h	2.102	27096	103.0342	103	8725	2098.2	94.4	2.093	8133	4684.45
K3-96h	2.102	53197	205.4362	103	16957	8590.28	95.2	2.093	8008	2455.65

Appendix H: LC-QqQ-MS/MS analysis of series 3

	Imidacloprid Quantifier (256.1→ 175)						**Imidacloprid Qualifier (256.1→ 208.9)**	**Imidacloprid-d4 IS (256.1→ 208.9)**		
Name	RT	Resp.	Calc. Conc.	Accuracy	Height	S/N	Ratio	RT	Resp.	S/N
BUM	2.039	1	0.0143		1	0,54	23.4	1.699	1357	561.35
BMM	1.951	2	0.0184		1	0,86	138.2	1.699	1322	1139.22
N1	1.708	10	0.1286	129	4	1,49	83.4	1.707	1240	392.72
N2	1.844	18	0.4627	93	7	3,29	106.5	1.847	588	245.26
N3	2.032	45	0.8745	87	14	17,38	115.5	2.031	783	388.11
N4	2.087	405	5.2863	106	127	196,12	93.8	2.082	1179	841.34
N5	2.102	929	9.7763	98	301	214,44	99.9	2.093	1462	1780.26
N6	2.106	1630	15.8299	106	505	429,02	98.0	2.097	1584	451.74
N7	2.109	2784	20.046	100	869	789,30	96.3	2.104	2137	2065.56
N8	2.113	4707	29.6049	99	1411	2674,96	97.6	2.108	2446	659.72
K1 a	2.113	77	0.4114	8	23	6,54	118.0	2.104	2864	649.71
K2 a	2.113	1678	9.5271	95	504	429,42	102.2	2.108	2710	962.30
K3 a	2.113	3613	20.897	104	1076	2186,33	95.3	2.108	2660	622.00
C1-96h-T1	2.058	1	0.0028		1	0,70		2.104	2939	1282.84
C2-96h-T1	2.069	3	0.0178		1	0,84	96.7	2.108	2943	1104.62
C3-96h-T1	2.131	2	0.0095		1	0,67	100.9	2.104	2810	548.22
C4-96h-T1	2.006	0	0.0019		0	0,34		2.108	3014	927.17
C3-96h-T2	2.017	1	0.0064		1	0,93	143.8	2.104	3010	674.35
C4-96h-T2	2.025	0	0.002		0	0,21		2.104	3029	1583.48
0.0002 mg/L-96h-R1-T1	2.095	4	0.02		2	1,07	156.9	2.104	3060	1073.31
0.0002 mg/L-96h-R2-T1	2.087	3	0.0162		2	0,96	157.2	2.104	2953	638.64
0.0002 mg/L-96h-R3-T1	2.12	3	0.0157		2	1,61	118.5	2.101	3088	655.56
0.0002 mg/L-96h-R4-T1	2.128	6	0.0295		2	1,39	100.8	2.101	2910	1762.95
0.0002 mg/L-96h-R1-T2	2.117	4	0.0199		1	0,71	116.4	2.104	2972	2278.22
0.0002 mg/L-96h-R2-T2	2.102	2	0.0082		1	0,66	375.4	2.104	3066	1575.84
0.0002 mg/L-96h-R3-T2	2.12	4	0.0215		2	0,78		2.101	3201	974.61
0.0002 mg/L-96h-R4-T2	2.124	5	0.0275		3	1,20	75.2	2.101	2928	2054.70
0.002 mg/L-96h-R1-T1	2.106	31	0.1627		11	10,86	80.2	2.101	2945	572.98
0.002 mg/L-96h-R2-T1	2.102	34	0.186		11	3,89	67.0	2.101	2825	1633.37
0.002 mg/L-96h-R3-T1	2.095	20	0.1109		6	2,91	128.9	2.101	2739	59.16
0.002 mg/L-96h-R4-T1	2.109	31	0.1632		8	3,27	65.8	2.101	2936	790.96
0.002 mg/L-96h-R1-T2	2.113	27	0.1464		9	11,07	105.2	2.101	2886	7676.74
0.002 mg/L-96h-R2-T2	2.109	27	0.1491		10	5,19	95.7	2.101	2820	1731.14

0.002 mg/L-96h-R3-T2	2.098	40	0.1951		10	12,74	107.5	2.101	3119	583.01
0.002 mg/L-96h-R4-T2	2.098	35	0.178		10	5,12	84.2	2.101	3024	1513.94
0.02 mg/L-96h-R1-T1	2.102	292	1.8201		87	19,84	102.2	2.101	2471	955.52
0.02 mg/L -96h-R2-T1	2.109	276	1.5215		80	120,88	102.2	2.101	2792	720.47
0.02 mg/L -96h-R3-T1	2.109	237	1.3653		74	62,25	100.9	2.101	2671	2541.89
0.02 mg/L -96h-R4-T1	2.109	193	1.101		54	59,38	96.4	2.101	2700	1072.33
0.02 mg/L -96h-R1-T2	2.109	158	0.891		49	44,51	94.4	2.104	2724	1242.91
0.02 mg/L -96h-R2-T2	2.102	207	1.1027		62	23,26	98.5	2.101	2887	1785.06
0.02 mg/L -96h-R3-T2	2.113	255	1.3498		79	21,65	93.9	2.104	2910	703.03
0.02 mg/L -96h-R4-T2	2.109	193	1.0742		55	60,85	90.5	2.108	2759	689.45
2 mg/L -R3-T1-96h	2.106	202	0.9981		62	54,78	86.2	2.104	3114	1942.62
20 mg/L -R3-T1-96h	2.113	187	1.0207		58	101,74	103.1	2.104	2825	1281.05
K1 a	2.102	91	0.4814	96	26	81,78	89.5	2.104	2921	1874.08
K2 a	2.113	1689	9.5641	191	533	54,02	103.0	2.104	2716	1749.33
K3 a	2.109	3540	19.6913	131	1078	33,56	96.0	2.101	2765	1637.92

Appendix I: RCode script for calculating LC

```
install.packages("ggplot2")
install.packages("MASS")
install.packages("drc")
library("drc")
library("MASS")
library("ggplot2")

#Mortality

hours_exp<-read.csv(file.choose())
summary(hours_exp)
names(hours_exp)

imi.96<-subset(hours_exp, Time=="4")

imi.96$Mortality <- imi.96$Dead/imi.96$Total

imi.96$Dose0 <- imi.96$Concentration # need to shift Dose == 0 a bit up, otherwise there are problems with plotting (log10 of zero is infinity)
imi.96$Dose0[imi.96$Dose0 == 0] <- 0.00002 #anywhere there is a zero change it to 10x lower than your lowest dose.

# correcting for mortality in control
d_control <- mean(imi.96$Mortality[imi.96$Dose0 == 0.00002])
imi.96$Corrected_Mortality <- (imi.96$Mortality - d_control) / (1 - d_control)
imi.96$Corrected_Mortality[imi.96$Corrected_Mortality < 0 | imi.96$Dose0 == 0.00002] <- 0

model1 <- drm(Corrected_Mortality ~ Dose0, weights = Total, data = imi.96, fct = LN.2(), type = 'binomial') #probit
summary(model1)
model2 <- drm(Corrected_Mortality ~ Dose0, weights = Total, data = imi.96, fct = LL.2(), type = 'binomial') #logit
summary(model2)

par(mfrow = c(1, 2))
plot(model1)
plot(model2)

mselect(model1, list(LL.2()))

LCvalues<-ED(model2, c(10,50,90), interval = "delta") #calculate LCx values, can choose whatever x values you like, but 10, 50, and 90 most commonly reported
write.table(LCvalues, "clipboard", sep="\t", row.names=FALSE)
```

```
plot(model2)

newdata <- expand.grid(conc=exp(seq(log(0.00002), log(20), length=10000)))
pm <- predict(model2, newdata=newdata, interval="confidence") ## predictions and confidence intervals

# new data with predictions
newdata$p <- pm[,1]
newdata$pmin <- pm[,2]
newdata$pmax <- pm[,3]

theme_set(theme_bw())
p1<-ggplot(imi.96, aes(x=Dose0, y = Corrected_Mortality))+
  theme(axis.text =element_text(size=16),
        axis.title =element_text(size=16),
        panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        axis.ticks = element_line(size = 0.2),
        panel.spacing = unit(1, "lines"),
        plot.margin = unit(c(0.1,0.1,0.1,0.1),"cm"),
        legend.position="none",
        legend.spacing = unit(50,"cm"))+
  geom_point(size=2)+
  geom_ribbon(data=newdata, aes(x=conc, y=p, ymin=pmin, ymax=pmax), alpha=0.2) +
  geom_line(data=newdata, aes(x=conc, y=p), linewidth=1)+
  scale_x_log10(breaks=c(0.00002,0.0002,0.002,0.02,0.2,2,20),      label=c("Control","2e-04"
,"2e-03","2e-02","2e-01","2e+00","2e+01"))+
  xlab(expression(paste("Imidacloprid [mg/L]")))+
  ylab(" Mortality")+
  geom_segment(aes(x=1.853150 , y=0, xend=1.853150 , yend=0.5), linetype = 'dashed',
colour="red", linewidth=1)+ #optional if you want to display LC50 on your graph
  geom_segment(aes(x=0,      y=0.5,      xend=1.853150      ,      yend=0.5),      linetype      =
'dashed',colour="red", linewidth=1)

p1

ggsave(p1, filename = "Dose Mortality.pdf", dpi=600,width = 7, height = 5, units = "in")
#check your working directory is correct before saving

ggsave(p1, filename = "Dose Mortality 72 LC50.tiff", dpi=600,width = 7, height = 5, units =
"in")
```

Appendix J: RCode script for calculating EC

```
install.packages("ggplot2")
install.packages("MASS")
install.packages("drc")
library("drc")
library("MASS")
library("ggplot2")

#Immobilization

hours_exp<-read.csv(file.choose())
summary(hours_exp)
names(hours_exp)

imi.96.I<-subset(hours_exp, Time=="1")

imi.96.I$Immobilized <- imi.96.I$Immobilized/imi.96.I$Total

imi.96.I$Dose0 <- imi.96.I$Concentration # need to shift Dose == 0 a bit up, otherwise there are problems with plotting (log10 of zero is infinity)
imi.96.I$Dose0[imi.96.I$Dose0 == 0] <- 0.00002 #anywhere there is a zero change it to 10x lower than your lowest dose.

# correcting for immobilization in control
d_control <- mean(imi.96.I$Immobilized[imi.96.I$Dose0 == 0.00002])
imi.96.I$Corrected_Immobilized <- (imi.96.I$Immobilized - d_control) / (1 - d_control)
imi.96.I$Corrected_Immobilized[imi.96.I$Corrected_Immobilized < 0 | imi.96.I$Dose0 == 0.00002] <- 0

model1 <- drm(Corrected_Immobilized ~ Dose0, weights = Total, data = imi.96.I, fct = LN.2(), type = 'binomial') #probit
summary(model1)
model2 <- drm(Corrected_Immobilized ~ Dose0, weights = Total, data = imi.96.I, fct = LL.2(), type = 'binomial') #logit
summary(model2)

par(mfrow = c(1, 2))
plot(model1)
plot(model2)

mselect(model1, list(LL.2()))

ECvalues<-ED(model2, c(10,50,90), interval = "delta") #calculate LCx values, can choose whatever x values you like, but 10, 50, and 90 most commonly reported
write.table(ECvalues, "clipboard", sep="\t", row.names=FALSE)

plot(model2)


newdata <- expand.grid(conc=exp(seq(log(0.00002), log(20), length=10000)))
```

```
pm <- predict(model2, newdata=newdata, interval="confidence") ## predictions and
confidence intervals

# new data with predictions
newdata$p <- pm[,1]
newdata$pmin <- pm[,2]
newdata$pmax <- pm[,3]


theme_set(theme_bw())
p1<-ggplot(imi.96.I, aes(x=Dose0, y = Corrected_Immobilized))+
  theme(axis.text =element_text(size=16),
        axis.title =element_text(size=16),
        panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        axis.ticks = element_line(size = 0.2),
        panel.spacing = unit(1, "lines"),
        plot.margin = unit(c(0.1,0.1,0.1,0.1),"cm"),
        legend.position="none",
        legend.spacing = unit(50,"cm"))+
  geom_point(size=2)+
  geom_ribbon(data=newdata, aes(x=conc, y=p, ymin=pmin, ymax=pmax), alpha=0.2) +
  geom_line(data=newdata, aes(x=conc, y=p), linewidth=1)+
  scale_x_log10(breaks=c(0.00002,0.0002,0.002,0.02,0.2,2,20), label=c("Control","2e-04"
,"2e-03","2e-02","2e-01","2e+00","2e+01"))+
  xlab(expression(paste("Imidacloprid [mg/L]")))+
  ylab(" Immobilized")+
  geom_segment(aes(x=4.6939e-04, y=0, xend=4.6939e-04, yend=0.1), linetype = 'dashed',
colour="red", linewidth=1)+ #optional if you want to display LC50 on your graph
  geom_segment(aes(x=0, y=0.1, xend=4.6939e-04, yend=0.1), linetype =
'dashed',colour="red", linewidth=1)

p1

ggsave(p1, filename = "Immobilised.pdf", dpi=600,width = 7, height = 5, units = "in") #check
your working directory is correct before saving
ggsave(p1, filename = "Immobilized E10 72hours.tiff", dpi=600,width = 7, height = 5, units =
"in")
```

Appendix K: RCode script, Step 1 Calibration

```
library(morse)
library(tidyverse)
library(ggmcmc)
library(GGally)

#reminder of EFSA workflow
# Step 1: calibration phase with both GUTS-RED models
# Step 2: validation of both GUTS-RED models
# Step 3: prediction under exposure scenarios of interest

        #### Step 1. Calibration:  GUTS modelling under constant exposure  ####
exp_hour<-read.csv(file.choose())

exp_hour$Mortality <- (exp_hour$Total-exp_hour$Nsurv)/exp_hour$Total

exp_hour$Dose0 <- exp_hour$conc # need to shift Dose == 0 a bit up, otherwise there are problems with plotting (log10 of zero is infinity)
exp_hour$Dose0[exp_hour$Dose0 == 0] <- 0.00002 #anywhere there is a zero change it to 10x lower than your lowest dose.

# correcting for mortality in control
d_control <- mean(exp_hour$Mortality[exp_hour$Dose0 == 0.00002])
exp_hour$Corrected_Mortality <- (exp_hour$Mortality - d_control) / (1 - d_control)
exp_hour$Corrected_Mortality[exp_hour$Corrected_Mortality < 0 | exp_hour$Dose0 == 0.00002] <- 0
exp_hour$Nsurv <- exp_hour$Total-(exp_hour$Corrected_Mortality*exp_hour$Total)
exp_hour$Nsurv <- as.integer(exp_hour$Nsurv)
survDataCheck(exp_hour)
dataset <- survData(exp_hour)
plot(dataset, pool.replicate=FALSE) #plot raw data
summary(dataset)

ggsave(plot(dataset, pool.replicate=FALSE), filename = "Corrected mortality Calibration.tiff", dpi=600,width = 7, height = 5, units = "in", bg ="white")

            ## Fitting GUT-RED-SD Model to constant exposure data ##
fit.cstSD <- survFit(dataset, model_type="SD")
summary(fit.cstSD, EFSA_name = TRUE)
plot_prior_post(fit.cstSD, EFSA_name = TRUE)

plot(fit.cstSD) #model predictions versus data
ppc(fit.cstSD) #checking fit with posterior predictive check

ggsave(plot(fit.cstSD), filename = "plot(fit.cstSD).tiff", dpi=600,width = 7, height = 5, units = "in", bg ="white")
```

```
ggsave(ppc(fit.cstSD), filename = "ppc(fit.cstSD).tiff", dpi=600,width = 7, height = 5, units = "in", bg ="white")

tmpmcmc <- ggs(fit.cstSD$mcmc)
tmppar <- subset(tmpmcmc, tmpmcmc$Parameter=="hb_log10" |
                 tmpmcmc$Parameter=="kd_log10" |
                 tmpmcmc$Parameter=="kk_log10" |
                 tmpmcmc$Parameter=="z_log10")

tmppar$Parameter <- fct_recode(tmppar$Parameter, "zw_log10" = "z_log10")
tmppar$Parameter <- fct_recode(tmppar$Parameter, "bw_log10" = "kk_log10")
ggs_pairs(tmppar, lower = list(continuous = "density"))

# can use models to estimate LCx values either at time points that were included in experimental design or those that were not included
LCx.cstSD.50 <- LCx(fit.cstSD, X = 50, time_LCx = 4) #calculate LC50 based on model predictions at day 4
plot(LCx.cstSD.50)
ggsave(plot(LCx.cstSD.50), filename = "LCx.cstSD.50.tiff", dpi=600,width = 7, height = 5, units = "in", bg ="white")

LCx.cstSD.not <- LCx(fit.cstSD, X = 50, time_LCx = 3) #calculate LC50 based on model predictions at day 1.5 (i.e. time point not included in the experimental design)
plot(LCx.cstSD.not)

LCx.cstSD.5 <- LCx(fit.cstSD, X = 5, time_LCx = 4)  #calculate LC5 based on model predictions at day 4
plot(LCx.cstSD.5)

                    ## Fitting GUT-RED-IT Model to constant exposure data ##
fit.cstIT <- survFit(dataset, model_type="IT")
summary(fit.cstIT, EFSA_name = TRUE, quiet=TRUE)$Qpost
plot(fit.cstIT)
ppc(fit.cstIT) #checking fit with posterior predictive check

plot_prior_post(fit.cstIT, EFSA_name = TRUE)

ggsave(plot(fit.cstIT), filename = "plot(fit.cstIT).tiff", dpi=600,width = 7, height = 5, units = "in", bg ="white")
ggsave(ppc(fit.cstIT), filename = "ppc(fit.cstIT).tiff", dpi=600,width = 7, height = 5, units = "in", bg ="white")


tmptot <- ggs(fit.cstIT$mcmc)
tmppar <- tmptot %>%
  filter(Parameter == "hb_log10" |
```

```
        Parameter == "kd_log10" |
        Parameter == "alpha_log10" |
        Parameter == "beta_log10")
# Retrieve EFSA names for alpha
tmppar$Parameter <- fct_recode(tmppar$Parameter, "mw_log10" = "alpha_log10")
ggs_pairs(tmppar, lower = list(continuous = wrap("density",color = "#ff8c00")))


                        ## comparing the SD and IT models ##
summary(fit.cstSD, EFSA_name = TRUE, quiet=TRUE)$Qpost #compare model parameters
summary(fit.cstIT, EFSA_name = TRUE, quiet=TRUE)$Qpost

ppc(fit.cstSD) # compare posterior predictive check plots
ppc(fit.cstIT)

plot(fit.cstSD) #compare model predictions versus observation data for each concentration
plot(fit.cstIT)

#Compare the LCx values and the confidence intervals between the two models
LCx.cstSD <- LCx(fit.cstSD, X = 90, time_LCx = 4) #compare LC50 values at time point 4
days
plot(LCx.cstSD)
LCx.cstIT <- LCx(fit.cstIT, X = 90, time_LCx = 4)
plot(LCx.cstIT)

LCx.cstSD$df_LCx #compare confidence intervals
LCx.cstIT$df_LCx

LCx.cstSD <- LCx(fit.cstSD, X = 10, time_LCx = 4) #compare LC10 values at time point 4
days
LCx.cstSD$df_LCx
LCx.cstIT <- LCx(fit.cstIT, X = 10, time_LCx = 4)
LCx.cstIT$df_LCx

LCx.cstSD <- LCx(fit.cstSD, X = 50, time_LCx = 4) #compare LC50 values at time point 10
days
plot(LCx.cstSD)
LCx.cstSD$df_LCx
LCx.cstIT <- LCx(fit.cstIT, X = 50, time_LCx = 4)
plot(LCx.cstIT)
LCx.cstIT$df_LCx

LCx.cstSD <- LCx(fit.cstSD, X = 10, time_LCx = 10) #compare LC10 values at time point 10
days
plot(LCx.cstSD)
LCx.cstSD$df_LCx
```

```
LCx.cstIT <- LCx(fit.cstIT, X = 10, time_LCx = 10)
plot(LCx.cstIT)
LCx.cstIT$df_LCx #IT model gives lower LC10 at extrapolated time point

   # Can also use models to make predictions based on a random pulse exposure profile ##
plot(seq(1:10), c(0,0,40,0,0,0,40,0,0,0), type="s", las=1,
    ylim=c(0,60), ylab="Exposure concentration", xlab="Time") #visual of random pulse exposure profile

#predictions based on SD model
data_4pred <- data.frame(time = 1:10,
                    conc = c(0,0,40,0,0,0,40,0,0,0),
                    replicate = rep("pulse",10))
predict.cstSD.4pred <- predict(object = fit.cstSD,
                         data_predict = data_4pred)
plot(predict.cstSD.4pred)

 # How far is the random pulse exposure profile from adverse effects?
 # What is the MFx? ie. how many times greater would the exposure profile need to be to get 50% additional death at time point 10 days

data_40MFx <- data.frame(time = 1:10, conc = c(0,0,20,0,0,0,20,0,0,0)) # MFx for Model GUTS-RED-SD
MFx.cstSD.40MFx <- MFx(object = fit.cstSD, X=50, time= 10,
                   data_predict = data_40MFx, quiet=TRUE)
MFx.cstSD.40MFx$df_MFx
plot(MFx.cstSD.40MFx, log_scale = TRUE)
plot(MFx.cstSD.40MFx, x_variable = "Time")

#predictions based on IT model
data_4pred <- data.frame(time = 1:10,
                    conc = c(0,0,20,0,0,0,20,0,0,0),
                    replicate = rep("pulse",10))
predict.cstIT.4pred <- predict(object = fit.cstIT,
                         data_predict = data_4pred)
plot(predict.cstIT.4pred)

MFx.cstIT.40MFx <- MFx(object = fit.cstIT, # MFx for Model GUTS-RED-IT
                   data_predict = data_40MFx, quiet=TRUE)
MFx.cstIT.40MFx$df_MFx

plot(MFx.cstIT.40MFx, log_scale = TRUE)
plot(MFx.cstIT.40MFx, x_variable = "Time")
```

Appendix L: RCode script, Step 2 Validation

```
require(highlight)
library(tidyverse)
library(png)
library(knitr)
library(morse)
library(ggmcmc) # correlation plot
library(GGally) # to allow color changes with wrap()

#reminder of EFSA workflow
# Step 1: calibration phase with both GUTS-RED models
# Step 2: validation of both GUTS-RED models
# Step 3: prediction under exposure scenarios of interest


                #### Step 2. Validation: GUTS modelling under time-variable exposure  ####

exp_hour_pulse<-read.csv(file.choose()) # load raw data
survDataCheck(exp_hour_pulse)
dataset <- survData(exp_hour_pulse)
plot(dataset, pool.replicate=FALSE) #plotting observed data from pulse exposure
ggsave(plot(dataset, pool.replicate=FALSE), filename = "Step 2 Number of survivors.tiff",
dpi=600,width = 7, height = 5, units = "in", bg ="white")

## Fitting GUT-RED-SD Model to pulse exposure data ##

fit.varSD <- survFit(dataset, model_type="SD")
plot(fit.varSD) #plotting observed survival and model predictions based on pulse exposure
ggsave(plot(fit.varSD), filename = "Step 2 plot(fit.varSD).tiff", dpi=600,width = 7, height = 5,
units = "in", bg ="white")
ppc(fit.varSD)
ggsave(ppc(fit.varSD), filename = "Step 2 ppc(fit.varSD).tiff", dpi=600,width = 7, height = 5,
units = "in", bg ="white")
plot_prior_post(fit.varSD, EFSA_name = TRUE)
summary(fit.varSD, EFSA_name = TRUE)$Qpost

tmptot <- ggs(fit.varSD$mcmc)
tmppar <- subset(tmptot, tmptot$Parameter=="hb_log10" |
                 tmptot$Parameter=="kd_log10" |
                 tmptot$Parameter=="kk_log10" |
                 tmptot$Parameter=="z_log10")
# Retrieve EFSA names for kk and z
tmppar$Parameter <- fct_recode(tmppar$Parameter,
                           "zw_log10" = "z_log10")
tmppar$Parameter <- fct_recode(tmppar$Parameter,
                           "bw_log10" = "kk_log10")
```

```
ggs_pairs(tmppar, lower = list(continuous = wrap("density", color = "#ff8c00")))

#Predict Nsurv from Calibration with observed Nsurv in Pulse
predict_Nsurv_cstTOvar <- predict_Nsurv(fit.cstSD,exp_hour_pulse)
plot(predict_Nsurv_cstTOvar)
ppc(predict_Nsurv_cstTOvar)
ggsave(plot(predict_Nsurv_cstTOvar), filename = "predict_Nsurv_cstTOvar.tiff",
dpi=600,width = 7, height = 5, units = "in", bg ="white")
ggsave(ppc(predict_Nsurv_cstTOvar), filename = "ppc(predict_Nsurv_cstTOvar).tiff",
dpi=600,width = 7, height = 5, units = "in", bg ="white")
predict_Nsurv_check(predict_Nsurv_cstTOvar)$Percent_PPC
predict_Nsurv_check(predict_Nsurv_cstTOvar)$Percent_NRMSE
predict_Nsurv_check(predict_Nsurv_cstTOvar)$Percent_SPPE

#calculate LC50 and LC10 at day 10
LCx.varSD <- LCx(fit.varSD, X = 50, time_LCx = 4, conc_range = c(0,20))
LCx.varSD$df_LCx
plot(LCx.varSD)

LCx.varSD <- LCx(fit.varSD, X = 10, time_LCx = 4, conc_range = c(0,20))
LCx.varSD$df_LCx
plot(LCx.varSD)

## Fitting GUT-RED-IT Model to pulse exposure data ##

fit.varIT <- survFit(dataset, model_type="IT")
plot(fit.varIT) #plotting observed survival versus model predictions
ggsave(plot(fit.varIT), filename = "Step 2 plot(fit.varIT).tiff", dpi=600,width = 7, height = 5,
units = "in", bg ="white")
ppc(fit.varIT)
ggsave(ppc(fit.varIT), filename = "Step 2 ppc(fit.varIT).tiff", dpi=600,width = 7, height = 5,
units = "in", bg ="white")
plot_prior_post(fit.varIT, EFSA_name = TRUE)
summary(fit.varIT, EFSA_name = TRUE)$Qpost

tmptot <- ggs(fit.varIT$mcmc)
tmppar <- tmptot %>%
  filter(Parameter == "hb_log10" |
         Parameter == "kd_log10" |
         Parameter == "alpha_log10" |
         Parameter == "beta_log10")

tmppar$Parameter <- fct_recode(tmppar$Parameter, "mw_log10" = "alpha_log10")
ggs_pairs(tmppar, lower = list(continuous = wrap("density",color = "#ff8c00")))

#Predict Nsurv from Calibration with observed Nsurv in Pulse
```

```
predict_Nsurv_cstTOvar <- predict_Nsurv(fit.cstIT,exp_hour_pulse)
plot(predict_Nsurv_cstTOvar)
ppc(predict_Nsurv_cstTOvar)
ggsave(plot(predict_Nsurv_cstTOvar), filename = "predict_Nsurv_cstTOvar_IT.tiff", dpi=600,width = 7, height = 5, units = "in", bg ="white")
ggsave(ppc(predict_Nsurv_cstTOvar), filename = "ppc(predict_Nsurv_cstTOvar_IT).tiff", dpi=600,width = 7, height = 5, units = "in", bg ="white")
predict_Nsurv_check(predict_Nsurv_cstTOvar)$Percent_PPC
predict_Nsurv_check(predict_Nsurv_cstTOvar)$Percent_NRMSE
predict_Nsurv_check(predict_Nsurv_cstTOvar)$Percent_SPPE

# can use models to estimate LCx values either at time points that were included in experimental design or those that were not included

LCx.varIT <- LCx(fit.varIT, X = 50, time_LCx = 3, conc_range = c(0,20))
LCx.varIT$df_LCx
plot(LCx.varIT)

LCx.varIT <- LCx(fit.varIT, X = 10, time_LCx = 4, conc_range = c(0,20))
LCx.varIT$df_LCx
plot(LCx.varIT)

        ## Use models to make predictions for a random constant exposure profile ##

# (1) Create constant exposure profile or upload
data_4pred <- data.frame(time = 0:4, conc = rep(16, 10),
                         replicate = rep("constant", 20)) # argument `replicate` is used to provide several profiles of exposure
par(mar=c(4,4,0.2,0.2))
plot(data_4pred$time, data_4pred$conc, type="s", las=1,
    ylim=c(0,20), ylab="Exposure concentration", xlab="Time")

# (2) Prediction with parameter estimates from the constant profiles
predict.cstSD.4pred <- predict(object = fit.cstSD, data_predict = data_4pred) #with model SD
plot(predict.cstSD.4pred)

predict.cstIT.4pred <- predict(object = fit.cstIT, data_predict = data_4pred) #with model IT
plot(predict.cstIT.4pred)

# (3) Prediction with parameter estimates from the time-variable profile
predict.varSD.4pred <- predict(object = fit.varSD, data_predict = data_4pred) #with model SD
plot(predict.varSD.4pred)

predict.varIT.4pred <- predict(object = fit.varIT, data_predict = data_4pred) #with model IT
plot(predict.varIT.4pred)
```

Use models to make predictions for a random time-variable exposure profile

```
# (1) Create time-variable exposure profile or upload
data_4pred <- data.frame(time = 1:10,
                         conc = c(0,0,20,0,0,0,20,0,0,0),
                         replicate = rep("pulse", 10))
par(mar=c(4,4,0.2,0.2))
plot(data_4pred$time, data_4pred$conc, type="s", las=1,
    ylim=c(0,35), ylab="Exposure concentration", xlab="Time")

# (2) Prediction with parameter estimates from the constant profile
predict.cstSD.4pred <- predict(object = fit.cstSD, data_predict = data_4pred) #with model SD
plot(predict.cstSD.4pred)
predict.cstIT.4pred <- predict(object = fit.cstIT, data_predict = data_4pred) #with model IT
plot(predict.cstIT.4pred)

#  (3) Prediction with parameter estimates from the time-variable profile
predict.varSD.4pred <- predict(object = fit.varSD, data_predict = data_4pred) #with model
SD
plot(predict.varSD.4pred)
predict.varIT.4pred <- predict(object = fit.varIT, data_predict = data_4pred) #with model IT
plot(predict.varIT.4pred)

data_4MFx <- data.frame(time = 1:10, conc = c(0,0,20,0,0,0,20,0,0,0))
MFx.varSD.4MFx <- MFx(object = fit.varSD, data_predict = data_4MFx,
                      X=50, quiet=TRUE)
MFx.varSD.4MFx$df_MFx
plot(MFx.varSD.4MFx, log_scale = TRUE)
```

Appendix M: RCode script, Step 3 Prediction

EFSA validation of GUTS modelling under time-variable exposure

```
#reminder of EFSA workflow
# Step 1: calibration phase with both GUTS-RED models
# Step 2: validation of both GUTS-RED models
# Step 3: prediction under exposure scenarios of interest

                                #### Step 3. Predictions ####

## Compute qualitative and quantitative model performance criteria suitable for GUTS

# Prediction with parameter estimates from the constant profile  using SD model #
predictSD_Nsurv_cstTOvar <- predict_Nsurv(fit.cstSD, exp_hour_pulse)
load("predictSD_Nsurv_cstTOvar.RData") #saves doing prediction, as can take quite long
plot(predictSD_Nsurv_cstTOvar)
ppc(predict_Nsurv_cstTOvar) #want to have approx 50% green

#checking the validity of predictions
predict_Nsurv_check(predictSD_Nsurv_cstTOvar)
predict_Nsurv_check(predict_Nsurv_cstTOvar)$Percent_PPC
predict_Nsurv_check(predict_Nsurv_cstTOvar)$Percent_NRMSE #needs to be less than 50%
predict_Nsurv_check(predict_Nsurv_cstTOvar)$Percent_SPPE

# Prediction with parameter estimates from the constant profile using IT model #
predictIT_Nsurv_cstTOvar <- predict_Nsurv(fit.cstIT, propiconazole_pulse_exposure)
load("predictIT_Nsurv_cstTOvar.RData") #saves doing prediction
plot(predictIT_Nsurv_cstTOvar)
ppc(predictIT_Nsurv_cstTOvar)

#checking the validity of predictions
predict_Nsurv_check(predictIT_Nsurv_cstTOvar)
predict_Nsurv_check(predictIT_Nsurv_cstTOvar)$Percent_PPC
predict_Nsurv_check(predictIT_Nsurv_cstTOvar)$Percent_NRMSE #needs to be less than
50%
predict_Nsurv_check(predictIT_Nsurv_cstTOvar)$Percent_SPPE

# now need to decide which is better model , SD looks better in this case

##  make predictions for new exposure profiles ##

# Imagine we want to predict the survival rate at non tested concentrations (eg, 21) or under a
time-variable profile.
data_4pred <- data.frame(time = c(1:10, 1:10),
                         conc = c(c(21,21,21,21,21,21,21,21,21,21),
                                  c(0,0,40,0,0,0,40,0,0,0)),
```

```
                    replicate = c(rep("constant",10), rep("pulse",10)))

# Use the fit on constant exposure propiconazole with model SD
predict.cstSD.4pred <- predict(object = fit.cstSD,
                               data_predict = data_4pred)

#plot the two exposure profiles
plot(1:10,
    c(0,0,40,0,0,0,40,0,0,0), type="s", las=1,
    xlab="Time", ylab="Concentration", lwd=2)
lines(1:10, rep(21,10), lwd=2, col="purple")
legend("topright",  legend=c("Constant","Pulse"),  pch=NA,  lty=1,  col=c("purple","black"),
bty="n", lwd=2)

#Plot the predicted survival rate under the new exposure profiles.
plot(predict.cstSD.4pred)

#Removing background mortality from predictions.
predict.cstSD.4pred.hbOUT <- predict(object = fit.cstSD,
                                     data_predict = data_4pred,
                                     hb_value = FALSE, hb_valueFORCED = 0)
plot(predict.cstSD.4pred.hbOUT)

## get a b
```