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Short communication

# Time-to-response toxicity analysis as a method for drug susceptibility assessment in salmon lice

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ABSTRACT

The salmon louse *Lepeophtheirus salmonis* (Krøyer, 1837) is an ectoparasite causing infections of wild and farmed Atlantic salmon (*Salmo salar* L.) in the Northern hemisphere. While *L. salmonis* control at commercial mariculture sites increasingly employs non-medicinal approaches, such as cage designs reducing infection rates and biological control through cleaner fish, anti-parasitic drugs are still a requirement for effective fish health care. With only a limited range of salmon delousing agents available, all of which have been in use for more than a decade, drug resistance formation has been reported for different products. Successful resistance management requires reliable susceptibility assessment, which is usually achieved through *L. salmonis* bioassays. These tests involve the exposure of parasites to different drug concentrations and require significant numbers of suitable *L. salmonis* stages. The present study reports an alternative bioassay that is based on time-to-response toxicity analyses and can be carried out with limited parasite numbers. The assay determines the median effective time ($ET_{50}$), i.e., the time required until impaired swimming and/or attachment behaviour becomes apparent in 50% of parasites, by conducting repeated examinations of test animals starting at the time point where exposure to a set drug concentration commences. This experimental approach further allows the estimation of the apparent drug susceptibility of individual *L. salmonis* by determining their time to response, which may prove useful in experiments designed to elucidate associations between genetic factors and the drug susceptibility phenotype of parasites. Three laboratory strains of *L. salmonis* differing in susceptibility to emamectin benzoate were characterised using standard 24 h bioassays and time-to-response toxicity assays. While both the median effective concentration ($EC_{50}$) and the $ET_{50}$ showed variability between experimental repeats, both types of bioassay consistently discriminated susceptible and drug-resistant *L. salmonis* laboratory strains.

*Statement of relevance:* Infections by sea lice cause significant costs to the global salmon farming industry, which have been estimated to exceed €300 million per year worldwide. Control of sea lice still relies to a significant extent on chemical delousing; however, chemical control is threatened by resistance formation. Resistance can be combated by rotation between different drugs and strategic implementation of non-medicinal strategies. However, resistance management requires reliable and feasible methods of susceptibility assessment.

The present study is a technical note introducing a novel approach to susceptibility assessments in sea lice. The method can be applied in susceptibility assessments on farms, where it offers the advantage of a reduced requirement of parasites for testing. In addition, the novel method allows deriving the times of parasite require to show a response after drug treatment has started, thus providing a variable characterizing the drug susceptibility phenotype of individual parasites. Accordingly, the bioassay approach presented here will be useful for studies aiming at unravelling the genetic determinants of drug resistance.



## 1. Introduction

Sea lice (Caligidae: Copepoda) are marine fish ectoparasites feeding on the host's mucus and skin tissues (Boxaspen, 2006). Sea louse infections of farmed Atlantic salmon (*Salmo salar*) mostly involve *Lepeophtheirus salmonis* (salmon louse) and *Caligus elongatus* in the Northern hemisphere, and *Caligus rogercresseyi* in Chile (Costello,

*Abbreviations:* EMB, emamectin benzoate; $EC_{50}$, median effective concentration; $LC_{50}$, median lethal concentration; $ET_{50}$, median effective time; $LT_{50}$, median lethal time; PEG 300, polyethylene glycol 300.

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2006). Sea louse control on salmon farms relies mainly on the use of veterinary drugs, supplemented by farm management measures (Costello, 2009) and non-medicinal approaches such as the deployment of cleaner fish (Sayer et al., 1996) and modified cage designs reducing the likelihood of infection (Stien et al., 2016).

Only a restricted range of licensed anti-sea louse drugs are currently available (Burridge et al., 2010). The continued use of the same or a few types of control agents, however, can favour the development of drug resistance in parasites (Denholm et al., 2002). Drug resistance is well documented in *L. salmonis*, and compounds for which loss of efficacy has been reported include the organophosphates dichlorvos and azamethiphos (Jones et al., 1992; Kaur et al., 2015; Roth et al., 1996), the pyrethroid deltamethrin (Sevatdal and Horsberg, 2003), the non-specific oxidising agent hydrogen peroxide (Treasurer et al., 2000) and the macrocyclic lactone emamectin benzoate (EMB) (Lees et al., 2008). Resistance to different drugs has further been found in *C. rogercresseyi* (Agusti et al., 2016; Bravo et al., 2010). Accordingly, there is an urgent need for efficient resistance prevention and management strategies in sea lice (Aaen et al., 2015).

A key requirement for such effective sea louse control strategies is an accurate assessment of the drug susceptibility status of sea louse populations. Such assessments are usually achieved by conducting bioassays, which typically involve subjecting batches of preadult or adult parasites to a dilution series of the drug (Sevatdal and Horsberg, 2003; Westcott et al., 2008). The internal exposure of an aquatic organism taking up a toxicant from water will increase both with increasing toxicant concentration and increasing length of exposure. Traditional aquatic bioassays typically employ different toxicant concentrations to achieve gradually varied exposures, while keeping the length of the exposure period constant. Results are expressed as the median lethal or median effective concentration ($LC_{50}$, $EC_{50}$), i.e., the concentration theoretically causing a toxic effect in 50% of the tested population. In an alternative approach called time-to-response toxicity analysis, gradual exposure levels are achieved by combining a fixed toxicant concentration with variable exposure periods. In this approach, the susceptibility of the population to the toxicant is expressed as the median lethal or effective time ($LT_{50}$, $ET_{50}$) (Robertson and Preisler, 1991). Time-to-response bioassays have been used in susceptibility assessments of terrestrial arthropod pests (Robertson and Preisler, 1991) and ecotoxicity studies in aquatic invertebrates and fish (Pascoe and Edwards, 1989; Rand, 2008).

The availability of sea louse stages suitable for bioassays can be restricted at production sites. Alternative bioassays involving only one drug concentration and a fixed exposure period have been proposed to allow for drug susceptibility assessment under such circumstances (Helgesen and Horsberg, 2013a; Whyte et al., 2013). While single-dose bioassays can be highly useful as a tool in fish health management, their ability to resolve susceptibility differences between parasite populations is by design limited. Time-to-response toxicity analyses could provide a complementary approach allowing characterisation of the susceptibility status at greater depth when sea louse availability is restricted. In addition to supporting veterinary decisions on fish farms, drug susceptibility assessments in sea lice are central to experimental plans aiming at identifying genetic determinants of drug resistance, which often require the determination of the susceptibility phenotypes of individuals (Besnier et al., 2014). Differentiation between susceptible and resistant parasites has been previously achieved by rating toxic responses following exposure to a diagnostic drug concentration for a set time period (Ljungfeldt et al., 2014). Using a similar approach, but additionally implementing repeated observations to determine the time to response for individual parasites, would permit a more graduated characterisation of the drug susceptibility phenotype than achievable with a test design employing a one concentration/one exposure time cut-off criterion to define resistance/susceptibility.

The aim of the present study was to investigate the potential of time-to-response toxicity analyses as an alternative approach to conducting sea louse drug sensitivity assessments. Time-to-response toxicity analyses were compared to standard bioassays with respect to their ability to differentiate between well-characterised laboratory strains of *L. salmonis* showing different degrees of resistance to the salmon delousing agent EMB.

## 2. Materials and methods

### 2.1. Salmon louse (*L. salmonis*) strains and husbandry

Three *L. salmonis* laboratory-maintained strains established from field isolates of egg strings without further selection in the laboratory were used in this study. The drug-susceptible strain IoA-00 (previously called "S″) (Heumann et al., 2012) was established in 2003 from a Scottish farm site where no chemical control agents other than hydrogen peroxide had been used. The EMB-resistant strain IoA-01 (previously called "PT" or "R") (Heumann et al., 2012, 2014) and the multi-resistant strain IoA-02, which is hyposensitive to both EMB and deltamethrin, were created in December 2008 and September 2011, respectively, from other Scottish sites where there had been reports of variable treatment efficacies. These strains have since been cultured under identical laboratory conditions using Atlantic salmon as host fish, as described in detail elsewhere (Heumann et al., 2012). To propagate cultures, *L. salmonis* egg strings were collected from gravid females and allowed to hatch and develop to infective copepodids, which were used to inoculate tanks containing fresh host fish. To collect *L. salmonis*, host fish were either euthanised under a UK Home Office approved Schedule 1 method, or anaesthetised with 100 mg $L^{-1}$ 2-phenoxyethanol (99%; Sigma-Aldrich, Dorset, UK) in seawater for 3 min. Previous experiments assessing the effect of anaesthesia on bioassay results did not find significant differences between the two sea lice collection methods (data not shown). Parasites were removed from fish into clean aerated seawater using fine forceps, and fish were transferred into clean seawater with aeration for recovery. Infection rates were maintained at levels compatible with good fish welfare according to MERL Good Laboratory Practise (GLP) Standard Operating Procedure (SOP) protocols. All laboratory infections were carried out under UK Home Office licence and appropriate veterinary supervision.

### 2.2. Standard bioassays

Experiments used adult male or preadult II female *L. salmonis*. After collection from host fish, parasites were allowed to recover for 2–4 h in filtered aerated seawater at 12 °C before use in bioassays. Exposures were performed in a temperature-controlled incubator set to 12 °C, using 150 mL plastic Petri dishes holding 70 mL of exposure solutions and containing ten sea lice. EMB (technical grade, a gift from MSD Animal Health) was solubilised using polyethylene glycol of a number average molecular weight ($M_n$) of 300 (PEG 300, pH. Eur., Merck Chemicals, UK) before being diluted in seawater. Exposure solutions contained a final concentration of 0.05% (*v*/v) PEG 300. Each test comprised a geometrical dilution series of EMB of at least five concentrations in addition to seawater and solvent controls, the latter containing 0.05% (v/v) PEG 300. Sea lice were assigned to treatments randomly. At least two replicate Petri dishes were used per combination of strain and drug or control treatment. After 24 h of exposure, sea lice were visually examined and rated according to their attachment and mobility behaviour. Prior to rating, beakers were re-labelled with codes by laboratory staff not involved in the recording of experimental outcomes to allow for observer-blinded rating. In experiments conducted in 2011 and before, salmon lice were rated as normally motile (unaffected) or immotile (affected) upon visual examination and stimulation with a fine brush (Heumann et al., 2012). Later experiments used rating criteria initially proposed by Sevatdal and Horsberg (2003) and Westcott et al. (2008) and modified by Igboeli et al. (2012), where parasites are rated "live" when firmly attached to the surface of the Petri dish or swimming normally, "weak" when swimming irregularly and failing to attach to surfaces firmly