Received: 21 June 2017 | Revised: 27 September 2017 | Accepted: 15 November 2017

DOI: 10.1111/jfd.12768

SPECIAL ISSUE

WILEY Journal of Fish Diseases

Sensitivity assessment of sea lice to chemotherapeutants: Current bioassays and best practices

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Abstract

Traditional bioassays are still necessary to test sensitivity of sea lice species to chemotherapeutants, but the methodology applied by the different scientists has varied over time in respect to that proposed in "Sea lice resistance to chemotherapeutants: A handbook in resistance management" (2006). These divergences motivated the organization of a workshop during the Sea Lice 2016 conference "Standardization of traditional bioassay process by sharing best practices." There was an agreement by the attendants to update the handbook. The objective of this article is to provide a baseline analysis of the methodology for traditional bioassays and to identify procedures that need to be addressed to standardize the protocol. The methodology was divided into the following steps: bioassay design; material and equipment; sea lice collection, transportation and laboratory reception; preparation of dilution; parasite exposure; response evaluation; data analysis; and reporting. Information from the presentations of the workshop, and also from other studies, allowed for the identification of procedures inside a given step that need to be standardized as they were reported to be performed differently by the different working groups. Bioassay design and response evaluation were the targeted steps where more procedures need to be analysed and agreed upon.

KEYWORDS

azamethiphos, emamectin benzoate, hydrogen peroxide, pyrethroids, standardization of bioassay methodology

1 | INTRODUCTION

The sea lice species *Lepeophtheirus salmonis* (Krøyer) and *Caligus rogercresseyi* (Boxshall & Bravo 2000) are the main ectoparasites of Atlantic salmon (*Salmo salar* L.) in fish farms in the Northern and Southern Hemispheres, respectively. Most fish farms use husbandry practices and chemical treatments to keep parasites below the levels set by the authorities in each country (Helgesen, Romstad, Aaen, & Horsberg, 2015). To do this, chemical treatments have been and are currently indispensable, even though the use of non-chemical methods is increasing (Jansen, Grøntvedt, Tarpai, Helgesen, & Horsberg, 2016). Chemical treatments have been the most predictable and efficacious to reduce parasite load leading to the extensive use of the available chemicals, which in turn has resulted in resistance to chemical treatments (Aaen, Helgesen, Bakke, Kaur, & Horsberg, 2015).

Bioassays for sensitivity testing of sea lice provide an indication of the degree of susceptibility of sea lice to chemotherapeutants, while avoiding the possibility of the efficacy results being influenced by field factors, such as adverse treatment conditions and inappropriate dosage (Denholm et al., 2002). Alterations in the efficacy results by such factors could lead to incorrect interpretations (Marín, Ibarra, Medina, & Jansen, 2015). Since chemical treatment may in itself constitute a means of generating resistance among parasites (Aaen et al., 2015), and given the limited range of active ingredients used in antiparasitics against caligid copepods, appropriate management is needed to maintain their efficacy (Lees, Baillie, Gettinby, & Revie, 2008), to delay resistance from emerging (Denholm et al.,

2002; Marín et al., 2015), and for early detection of parasite resistance to the chemotherapeutants (Brooks, 2009).

The methodology to perform bioassays consists of exposing individuals to a gradient of at least five different concentrations plus one control. To calculate the concentration that immobilizes 50% of the parasites, namely the half maximal effective concentration (EC_{50}) value, the highest concentration should cause 100% of the expected response (Chapman, Crane, Wiles, Noppert, & McIndoe, 1996). This methodology was used to evaluate *L. salmonis* sensitivity to pyrethroids (Sevatdal & Horsberg, 2003) and later used to develop a handbook to perform sensitivity bioassays of *L. salmonis* to different chemotherapeutants (Search 2004), last updated in 2006 (Search 2006). From 2006, bioassays have been used to systematically monitor parasite response to chemotherapeutants and to relate parasite response to treatment efficacy and local treatment intensity (Helgesen et al., 2015; Jansen et al., 2016). These studies have provided valuable information on factors that contribute to differences in sensitivity among parasites, such as gender (i.e. Helgesen, Bravo, Sevatdal, Mendoza, & Horsberg, 2014; Igboeli, Burka, & Fast, 2014; Igboeli, Fast, Heumann, & Burka, 2012; Whyte, Westcott, Jimenez, Revie, & Hammell, 2014) and season (Lees et al., 2008; Westcott, Stryhn, Burka, & Hammell, 2008).

However, there have also been reports on difficulties performing the bioassay that together may impose severe limitations on carrying out sensitivity bioassays: (i) a large number of sea lice is required (Helgesen & Horsberg, 2013a; Marín et al., 2015; Whyte, Westcott, Elmoslemany, Hammell, & Revie, 2013), (ii) labour-intensive and requires well-trained observers to determine parasite condition (Helgesen & Horsberg, 2013a; Marín et al., 2015), (iii) it is expensive (Whyte et al., 2013), and (iv) results are highly sensitive to parasite condition on arrival at the laboratory, and the management carried out during the bioassay may affect the possibility of obtaining significant results (Marín et al., 2015; Whyte et al., 2013). These difficulties have been addressed by scientists differently, all of which may make intercomparisons difficult in some cases.

Traditional six-concentration bioassays may be required in the initial stages of a given product's use, to detect sensitivity changes when small changes need to be detected, because as tolerance develops within a sea lice population, it appears to reach a "tipping point," after which the tolerance spreads rapidly through the remaining population and less sensitive instruments may be used (Whyte et al., 2013). Although both *L. salmonis* and *C. rogercresseyi* show reduced sensitivity to several chemotherapeutants, there are still some chemotherapeutants showing high efficacy in particular geographical areas. For example, *C. rogecresseyi* is still sensitive to azamethiphos in most of the salmon farm areas in Chile (Marín et al., 2015) as well as hydrogen peroxide (Marín et al., 2017). Grøntvedt, Jansen, Horsberg, Helgesen, and Tarpai (2015) indicated a reduced sensitivity to azamethiphos and pyrethroids among *L. salmonis* in every county in Norway except Finnmark. Resistance of *L. salmonis* to emamectin benzoate has not been reported in British Columbia (Saksida et al., 2013). However, a sea lice outbreak in 2015 raised concern about the prospect of chemical resistance (Bateman et al., 2016). In addition, while development of biochemical and molecular tools for determination of resistance mechanisms continues to advance, not all resistance mechanisms in sea lice have been elucidated for all chemotherapeutic agents (Aaen et al., 2015). Presently, molecular techniques remain labour-intensive and are not yet fully developed for sea lice-monitoring programmes (Aaen et al., 2015).

The use of traditional bioassays is still necessary to test sensitivity of sea lice species, but published studies report different methodologies in respect to that proposed in the Handbook in 2006. For example, the use of single-dose bioassay (Helgesen & Horsberg, 2013a; Marín et al., 2015; Whyte et al., 2013) and the use of four categories to categorize sea lice health condition (Igboeli et al., 2012). These divergences motivated the organization of a workshop during the Sea Lice 2016 conference titled "Standardization of traditional bioassay process by sharing best practices," from which there was an agreement to update the 2006's handbook to be presented during the Sea Lice Conference 2018. Thus, the objective of this article is to provide a baseline analysis of the methodology being currently applied to perform traditional bioassays based on the material presented during the workshop to identify those aspects that need to be addressed to standardize the protocol for azamethiphos, pyrethroids, emamectin benzoate and hydrogen peroxide.

2 | METHODS

The workshop "Standardization of traditional bioassay process by sharing best practices" took place on 29 September of 2016 in Westport, Ireland. A total of 81 people and nine speakers participated in the workshop. Six of the nine presentations were related to traditional bioassays and were included in this baseline summary (Sea Lice Conference 2016).

To summarize those aspects of the methodology that were identified in the workshop as needed to be standardized to update the handbook "A handbook in resistance management," the following activities were performed:

- A synthesis of the information available regarding sea lice in vitro response to the different chemotherapeutants, difficulties associated with bioassay application, and any other methodology consideration.
- A step-by-step analysis of the methodology to perform the traditional bioassay based on the available information and presentations given during the workshop to identify those aspects that are being carried out differently among scientists and that should be analysed to agree on a common procedure.
- A step-by-step analysis of the methodology to identify those aspects where best practices can be implemented.
- Identification of those methodological aspects of the bioassay that are common to all chemotherapeutants and those that are specific for a given chemotherapeutant.

The four sources of information listed previously were used to summarize the key aspects of the traditional methodology that should be addressed to update the handbook. In particular, for the four chemotherapeutants a unique set of steps was defined for the protocol (bioassay design; material and equipment; sea lice collection, transportation and laboratory reception; preparation of dilutions; parasite exposure; response evaluation, data analysis and report). Each step was analysed based on the four sources of information listed previously, and differences among chemotherapeutants were identified.

3 | RESULTS AND DISCUSSION

From among the eight steps included as part of the protocol (Search, 2006 modified), the bioassay design and evaluation of sea lice response are the aspects that need agreement for consistency (Table 1). Sources of information on each step of the methodology are also listed in Table 1. Many studies have given support for the need to define whether sensitivity testing should be performed separately for males and females (Table 2).

TABLE 1 Synthesis of those aspects of the bioassay methodology that need to be addressed to reach an agreement in order to update the handbook: "Sea lice resistance to chemotherapeutants: A handbook in resistance management"

Bioassay step	Topic to be addressed for the standardization of sensitivity protocols	Reference
1. Bioassay design	Concentration selection: 6 or 8 (including control). Include among the concentrations that which the manufacturer recommends for treatments.	Search (2006); Roy (2016); Hausdorf (2016)
	Deltamethrin: recommendations regarding treatment concentration and exposure time are different among manufactures	
	Developmental stage to perform bioassay for sea lice species	Search (2006)
	Adjustment of the set of concentrations as the maximum does not causes 100% of expected response	Hausdorf (2016); Marín (2016)
	Estimate EC_{50} separately for males and females	Various authors (See Table 2)
	Cancel bioassay if there is not enough parasites to perform a six-concentration bioassay with three replicates per concentration	Marín (2016)
	Acclimatization time of sea lice prior to exposure (6, 8, 12, 18, 24)	Sevatdal and Horsberg (2003); Search (2006); Igboeli et al. (2012); Whyte et al. (2013); Saksida et al. (2013); Marín et al. (2015); Whyte et al. (2014); Helgesen et al. (2015); Downey (2016)
2. Material and equipment	Glass containers or other material to expose parasites and prepare solutions	Search (2006); Helgesen & Horsberg (2012)
	Type of container: strainers, Petri dish	Westcott et al. (2008); Helgesen & Horsberg (2013b); Igboeli et al. (2012); Tudor and Bouchard (2016); Downey (2016)
3. Sea lice collection, transportation, and laboratory reception	Type of information to be collected from the farm	Hausdorf (2016); Marín (2016)
4. Preparation of dilutions	Check nominal versus achieved concentration	Hausdorf (2016); Marín (2016)
	Criteria to easily identify live parasites	Hausdorf (2016)
5. Parasite exposure	Treatment order and randomization of the test subjects	Marín (2016); Tudor and Bouchard (2016)
	Time after exposure observation (24 hr, 24 and 48 hr)	Search (2006); Bravo et al. (2008); Sevatdal and Horsberg (2003); Sevatdal et al. (2005); Igboeli et al. (2012, 2013); Saksida et al. (2013); Marín et al. (2015); Helgesen et al. (2015); Marín et al. (2017); Tudor and Bouchard (2016)
	Hydrogen peroxide: immediately, 30 min and 24 hr post-exposure	
6. Response evaluation	Classification of sea lice condition: Live, moribund, and dead or alive, week, moribund and dead	Search (2006); Igboeli et al. (2012); Roy (2016)
	Hydrogen peroxide: Use red neutral technique, or other, to identify easily the alive parasites (red colour) and dead parasites (natural colour).	Hausdorf (2016)
	Discharge the bioassay if 20% of mortality is observed in the control after 24 hr post-exposure	Helgesen and Horsberg (2013a), Marín et al. (2015)
7. Data analysis	Decision about the software used to estimate EC_{50}: SPSS, PoloPlus; Minitab, R, regarding type of correction applied by the software	Search (2006); Tudor and Bouchard (2016); Roy (2016)
8. Report	Basic statistics to be shown on the report	Horsberg (2016); Marín (2016); Roy (2016)

3.1 | Bioassay design

Available information shows that the response of sea lice species to the chemotherapeutants varies between genders and that the less sensitive gender varies depending on the chemotherapeutant (Table 2).

The set of concentrations cannot be fixed or standardized since response to a given concentration gradient may differ among both sea lice species and populations because of differences in treatment intensity. For example, reduced sensitivity to azamethiphos was reported to be widespread along the Norwegian coast with some exceptions where parasites were sensitive (Grøntvedt et al., 2015). In a latter study based on data from Grøntvedt et al. (2015), Jansen et al. (2016) associated high local intensity of treatments with lower mortality rates from bioassays. However, in Chile, response of *C. rogercressyi* to azamethiphos bioassays caused mortalities larger than 80% in most of the sampled areas (Marín et al., 2015). For hydrogen peroxide, EC_{50} reported for *L. salmonis* in Norway was also associated with treatment efficacy, with values ranging from 538 to 2,127 ppm (Helgesen et al., 2015). In Chile, the reported EC_{50} value is 709.8 ppm (Marín et al., 2017), similar to areas in Norway with a history of treatment, but without having experienced reduced treatment efficacy (Helgesen et al., 2015). Thus, the actual set of concentrations may need to be adjusted as sea lice response changes.

It may be important that the set of concentrations used includes the recommended concentration for treatments given by the manufacturer, especially when there is no antecedent of reduced sensitivity. This will provide information that may be related to the response observed after treatments on farms, having considered that treatment results may be influenced by several factors (Denholm et al., 2002) and that parasite exposure to the chemotherapeutant in the bioassay and on the farm is different (Sevatdal & Horsberg, 2003).

It should also be discussed whether the bioassay should be performed if there are too few parasites for the defined set of concentrations and replicates. It may be possible to agree upon a minimum number of parasites needed to perform the bioassay for the result to remain useful using statistical procedures. It has been indicated that to have a reliable full scale dose–response experiment 120 test subjects are required, but a study with fewer than that, for example with 60 test subjects, can be successful as long as the doses are selected carefully (Robertson & Preisler, 1992). However, Sevatdal and Horsberg (2003) indicated that bioassays performed with less than 100 individuals are less reliable since the probit analysis may be unable to estimate confidence interval.

Developmental stage to be evaluated was standardized for *L. salmonis* in Search (2006) indicating that pre-adult II is a defined stage in the development and all tests performed with this stage can be compared, since differences associated with age and history of exposure to chemotherapeutants will be reduced. However, there have been studies in which the adult stages, or pre-adult and adult stages have been used (Igboeli et al., 2012; Whyte et al., 2013). For *C. rogercresseyi*, only adults have been used as there is no pre-adult stage, which makes result comparison difficult. Thus, further studies should propose a standardization for developmental stage of this sea lice species. An additional reason to standardize developmental stage for bioassays relates to variation in cuticle processes and its composition across sea lice development. Boxaspen (2006) indicated that knowledge about these aspects may contribute to understand variation in sensitivity of sea lice to chemotherapeutants. In particular, Sevatdal, Copley, Wallace, Jackson, and Horsberg (2005) indicated that the main route of entry for deltamethrin is the cuticle, specifically extremities of the ventral surface, probably because a combination of thinner cuticle and greater surface area of ventral side. Based on this, Whyte et al. (2014) suggested female *L. salmonis* larger size or their thicker cuticle could explain the lower sensitivity compared to males.

F1 bioassays, in which females are collected from farms and their eggs are cultivated to produce a new generation, may facilitate obtaining the required number of parasites, and also allows uniformity of the developmental stage and age of the sea lice to which sensitivity will be evaluated, especially for *C. rogercresseyi*, species that only have one mobile stage. This practice has been used previously (Carmona-Antoñanzas et al., 2017; Helgesen et al., 2015; Igboeli et al., 2013, 2014; Poley, Sutherland, Jones, Koop, & Fast, 2016; Roy, 2016; Sevatdal & Horsberg, 2003), and inclusion in the handbook should be discussed. As indicated by Sevatdal and Horsberg (2003), this type of bioassay raises questions about the level of representativeness of response of the F1 with respect to the farm strain, where sea lice have many different parents and will exhibit varying levels of resistance to chemotherapeutants. However, because the egg strings are cultivated from farming lice with different parents, the heterogeneity of response of the parents will remain as the F1 generation will retain their inherited resistance or sensitivity to chemotherapeutants. These have been demonstrated for *L. salmonis* subjected to hydrogen peroxide bioassay (Helgesen et al., 2015), emamectin benzoate (Igboeli et al., 2014) and deltamethrin (Carmona-Antoñanzas et al., 2017). Thus, sea lice sample from a given location should represent the sensitive and resistance fractions of the population at that location.

Time for sea lice to acclimate prior to exposure should be defined, and criteria for deciding the current acclimatization times should be discussed since reported times vary from 6 to 24 hr (references are presented in Table 1).

3.2 | Material and equipment - Water conditions for all steps of the bioassay

Various characteristics of water, including the amount of suspended solids and dissolved organic matter, salinity and temperature, may vary among sea lice collection locations and throughout the seasons (i.e. amount of organic particles and temperature are expected to be high in the summer and lower in the winter).

The presence of particulate and dissolved organic matter can alter bioassay results in varying degrees, as suspended solids and dissolved organic matter will affect bioavailability of the chemotherapeutants, as being indicated for deltamethrin (Yang et al. 2007). For hydrogen peroxide, it has been reported that aeration and organic

TABLE 2 Reports of gender-related differences in sensitivity to chemotherapeutants for the sea lice species *Lepeophtheirus salmonis* and *Caligus rogercresseyi*

Differences in sensitivity between genders	Source
Lepeophtheirus salmonis	Downey (2016)
Lepeophtheirus salmonis. Gender-related differences in EMB susceptibility indicated that pre-adult stage female sea lice exhibited a significantly larger sensitivity towards EMB than males	Westcott et al. (2008); Whyte et al. (2013)
Lepeophtheirus salmonis. Female sea lice are more sensitive to emamectin benzoate than males	Igboeli et al. (2013)
Lepeophtheirus salmonis Males are more sensitive than females to deltamethrin	Whyte et al. (2014)
Caligus rogercresseyi. Females were more resistant than males when were exposed to deltamethrin	Helgesen et al. (2014)
Caligus rogercresseyi. A reduction in sensitivity observed to pyrethroid in females may be due to a greater expression of the ATP-binding cassette transporter P-glycoprotein (Pgp) than males	Valenzuela-Muñoz, Nuñez-Acuña, and Gallardo-Escárate (2014)
Caligus rogercresseyi. Survival of females subjected to a bioassay was higher than that of males for pyrethroids and azamethiphos	Marín et al. (2015)
Caligus rogercresseyi. Females showed a larger survival when exposed to Hydrogen peroxide at concentrations larger than 1,443 ppm than males	Marín et al. (2017)
Caligus rogercresseyi. Females were less sensitive to a concentration of 2,000 mg/L Hydrogen peroxide	Chávez-Mardones, Asencio, Latuz, and Gallardo-Escárate (2015)

matter in the water decrease its concentration to undetectable levels 1 hr post-exposure, as organic matter acts as an active catalyst (Tort, Fletcher, Wooster, & Bowser, 2003). To reduce variability in bioassay results caused by variation in source water quality, a standard seawater filtration protocol should be developed. The protocol would have to be sufficient in removing any organics and other substances that may sequester the chemotherapeutant, thus decreasing the exposure concentration of the chemotherapeutants during the bioassay. Then, the filtered water should be aerated to ensure proper levels of dissolved oxygen during the bioassay. For hydrogen peroxide, no oxygen should be supplied since nominal concentration during the bioassay may be reduced (Tort et al., 2004).

Salinity and temperature should be standardized at the optimal value of the sea lice species to avoid variability on bioassay results caused by prior exposure to suboptimal conditions. Since sea lice collected from farms may have been exposed to suboptimal conditions, the acclimatization period should consider the time required for physiological processes of sea lice to return to normal conditions. The use of incubators where temperature can be controlled allows maintaining sea lice under optimal temperature. For hydrogen peroxide bioassay, the set of concentrations to be cused must consider the effect of the interaction between temperature and exposure time. (Thomassen, 1993; Treasurer, Wadsworth, & Grant, 2000;). Salinity may need to be adjusted, and to do that, an artificial salt can be used to increase salinity and dilution with filtered sea water to decrease salinity. These aspects were not formally discussed; however, they are important aspects of the bioassay protocol that would be addressed in further discussion.

3.3 | Material and equipment - Preparation of dilutions

Variations among protocols are concentrated on the type of material in which bioassay solutions are prepared and parasite exposed to these solutions. However, most of the studies reported on both the literature and presentations on the workshop use glass (Table 1). This arises from the information that chemotherapeutants have different adherence to materials affecting the actual concentration to which parasites are exposed (Helgesen & Horsberg, 2013b). It is also important to note that the material and equipment used to carry out the bioassay should reduce sea lice handling to ensure that healthy individuals are tested for sensitivity.

3.4 | Sea lice collection, transportation and laboratory reception

These three steps should be executed by experienced professionals in (i) identifying both sea lice species and developmental stage, (ii) collecting the sea lice from fish without inflicting damage to the individuals and (iii) securing the appropriate conditions for sea lice transportation, sea lice reception and maintenance in the laboratory before subjecting them to the bioassay. Sea lice sampling should be designed to collect a sample that represents the heterogeneity of sea lice response to chemotherapeutants by randomly selecting the cages from which sea lice will be collected. Sampling and transportation should be carried out in the shortest possible time to avoid affecting parasite condition. Water quantity, temperature, oxygen and salinity should be monitored during transportation. If deviations from the optimum range for sea lice occurs for temperature, salinity and oxygen during transportation, measures to re-establish them should be available, as well as water level (i.e. carrying additional water during transportation, gel packs, battery-operated air pumps). Once sea lice arrive to the laboratory, water temperature, salinity and oxygen should be registered, as well as parasite condition of the overall sample. The holding period, or acclimatization period, prior to bioassay was suggested by Search (2006) to be 6 hr. However, there are differences among studies, from 6 hr to cases in which the bioassay has been performed within the 24 hr after sea lice

collection, as a result of distances between farm and laboratory (Marín et al., 2015) (Table 1).

This information raises the question about what should be the appropriate acclimatization time, and what would be the effects on bioassay response of the total time elapsed from sampling to bioassay. To answer these questions, additional studies are required to identify physiological indicators informing about sea lice condition that can be related to stress induced by transportation conditions and/or extended acclimatization time.

Some of the presented protocols in the workshop emphasized collecting information from the farm when parasites are obtained (Horsberg, 2016; Marín, 2016; Roy, 2016). Table 3 shows an a priori list of variables that could be obtained during sea lice collection. This should be something to incorporate for all the working groups so that a better interpretation of bioassay results can be obtained.

3.5 | Parasite exposure

Parasite health prior to chemotherapeutant exposure should be made based on the criteria that define the category "alive parasite" for the evaluation of parasite response to the chemotherapeutant (Search 2006, Westcott et al., 2008). Using the same criteria to evaluate prior and after exposure condition of the parasite is consistent with the expectation that a parasite not affected by the chemotherapeutant should be observed as being as healthy as prior to exposure. A practical condition factor should be developed for healthy adult parasites, or even the live condition (Hausdorf, 2016). Assignment of parasites to a given treatment and replicate is complicated as the number of subject units is large (concentration*replicate), and it may not be possible to perform this all at the same time. This topic needs to be addressed in more detail.

3.6 | Response evaluation

Evaluation time depends on the chemotherapeutant. Thus, the effects on the parasite of exposure to emamectin benzoate, pyrethroids and azamethiphos have being reported for 24 hr post-exposure, but also for 48 hr post-exposure (Sevatdal & Horsberg, 2003; Marín et al., 2015; Table 1). This 48-hr evaluation is focused on evaluating mortality, since 24 hr post-exposure is a short period of time to evaluate mortality, which for treated group increases 48 hr after exposure suggesting that moribund lice die during after 24 post-exposure (Sevatdal & Horsberg, 2003). If response 48 hr post-exposure is being included as part of the bioassay, statistical comparisons of mortality between 24- and 48-hr results should indicate no significant differences. Thus, mortality 48 hr post-exposure can be attributed to the chemotherapeutant. It should be further evaluated if it is necessary to keep this step for monitoring sea lice sensitivity, since it adds significant time to the procedure. Evaluation for hydrogen peroxide bioassay includes evaluation immediately after exposure and 1 hr post-exposure (Helgesen et al., 2015; Marín et al., 2017), but the objective of the evaluation at different times should be defined. For example, if the objective is to estimate EC_{50}, then the evaluation should be done immediately after exposure since parasites will begin to recover shortly (30–60 min; Treasurer & Grant, 1997; Marín et al., 2017). Thus, evaluating the response later may underestimate the effect of hydrogen peroxide, as recovery increases with time. However, a later evaluation made continuously allows recovery characteristics to be observed. For example, using an observation frequency of half hour during the first 3 hr after exposure, and a last observation 25 hr post-exposure, Marín et al. (2017) reported recovering curve of the exposed individuals, the total number of individuals that recovered, differences in the curve between males and females, and how active are recovered individuals. These characteristics may contribute to evaluate the potential for reinfestation of fish on farms after treatment. Bravo, Treasurer, Sepúlveda, and Lagos (2010) suggested that the large capability of *C. rogercresseyi* to swim when detached from its host and the proximity of farming sites in Chile may increase the potential for host reinfestation after treatment with hydrogen peroxide.

The classical categorization of sea lice condition presented in the handbook (2006), alive, moribund and dead, has gained precision by the contributions of different authors. Westcott et al. (2008) define specific criteria based on the handbook (2006) such as inability to swim and floating in the petri dish for the dead category. A new category labelled "weak" has been introduced (Igboeli et al., 2012) and it should be analysed if this new categorization improves the accuracy to categorize parasite condition, and as a consequence allows a better data fit to estimate of EC_{50}. Comparing results of a given bioassay obtained using Westcott et al. (2008) and Igboeli et al. (2012) categorization and criteria could be an approach to this analysis.

Discarding the bioassay if mortality in the control group 24 hr post-exposure is larger than 20% has been a practice adopted by several scientists studying sea lice sensitivity, but it has not been made explicit by all. Therefore, it needs to be agreed upon as a practice to be included in the update of the handbook.

3.7 | Data analysis

To estimate the EC_{50}, the sea lice scored as moribund and dead are combined as the responders, where the LC_{50} is estimated using only

TABLE 3 Information to be obtained when collecting sea lice to test chemotherapeutant sensitivity

Farm information	Water	Host	Sea lice
Date and time of sampling	Temperature	Species name	Mean abundance of mobile stages and ovigerous females
Company name			
Farm identification	Salinity	Weight	Previous treatments
Month of the production cycle	Oxygen concentration and saturation	Cage from which fish was sampled	
Locality		Current disease challenges	

dead lice as the responders. The addition of other sea lice condition category, for example weak, will require standardization of how this additional category is grouped and identified as responders. The estimations of the EC_{50} and LC_{50} are calculated using probit analysis that can be conducted in various statistical packages (e.g. Polo Plus, SPSS, JMP, SAS and R). The probit analysis is a type of regression analysis that takes the sigmoid shape (s-shape) of binomial response variables and transforms it to a linear response. Then, a regression is conducted on the transformed data to estimate the concentrations of the antiparasitic needed for various response percentages (e.g. 50% responding), and the lower/upper confidence intervals. The use of standard effect percentages, like 50%, allows for easy comparison of bioassays across time within a chemotherapeutant and across chemotherapeutants. A Pearson goodness-of-fit test is also conducted with the probit analysis to quantify how well the response data fit the sigmoid shape. The closer the percentage response data fit the sigmoid shape, the better the estimates of the EC_{50} and LC_{50}. Some statistical programs will use correction factors to account for poor fit of the response data to the sigmoid shape (i.e. SPSS v. 24 uses a heterogeneity factor in the calculations of the confidence limits), while other programs do not (e.g. Polo Plus). Utilizing these correction factors may be of importance as many factors can contribute to poor fit of bioassay data, especially when collecting sea lice from the field, such time since last chemotherapeutant exposure, general sea lice condition and insufficient sample size. In addition, when comparing data across different bioassays, it is important to know when these correction factors were used, and if the same statistical program was used for these analyses. Further discussion is needed to determine the most appropriate software to use for analysing bioassay data so that results can be easily compared.

3.8 | Report

Besides reporting EC_{50} with its 95% confidence interval, it will be useful to report significance of the test to check data fit to the model, and the concentration–response curve predicted by the model and actual data. This will allow observation of the response observed under the recommended concentration for treatment.

4 | CONCLUSION

Much information has been generated from 2006 that may be used to update the current handbook: "Sea lice resistance to chemotherapeutants: A handbook in resistance management." For methodological procedures where there is no clear agreement, it may be necessary to design new studies that provide the information needed to continue improving the handbook.

It remains essential to establish standardized sea lice-monitoring programmes on a continuum, specific to geographical location, in order to survey emerging resistance patterns. Standardization of the traditional bioassay using instituted best practices would provide a solid foundation for creating effective sensitivity sea lice-monitoring programmes. Currently, only Norway has a country-wide surveillance programme utilizing lice bioassays. Other salmon-producing countries have organized Integrated Pest Management (IPM) plans, but monitoring for lice sensitivity to therapeutic agents has been discontinuous, and reporting of this work sparse as indicated previously by Aaen et al. (2015).

ACKNOWLEDGEMENTS

We thank MSD Animal Health for sponsoring this workshop and Marine Institute, Ireland, for organizing the workshop. We are grateful for the contributions of Tor Einar Horsberg, William Roy, Elan Downey. We appreciate the contributions made by the anonymous referees that greatly improved this manuscript. Thanks to other contributors of the workshop and to the audience for their interest and useful comments and suggestions. First author thank the Graduate School of Universidad Austral de Chile for funding the collaboration of JM.

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How to cite this article: Marín SL, Mancilla J, Hausdorf MA, Bouchard D, Tudor MS, Kane F. Sensitivity assessment of sea lice to chemotherapeutants: Current bioassays and best practices. *J Fish Dis*. 2017;00:1–9. https://doi.org/10.1111/jfd.12768