The salmon louse larval black box: evaluating fecundity and enumerating planktonic stages with an aquaculture management perspective

Cameron Thompson

Thesis for the degree of Philosophiae Doctor (PhD)
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SCIENTIFIC ENVIRONMENT

The candidate, Cameron Thompson, is a doctoral student in the Faculty of Mathematics and Natural Sciences at the University of Bergen. The doctoral work was conducted while positioned as a PhD fellow at the Institute of Marine Research (IMR) in the Disease and Pathogen Transmission research group.

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UNIVERSITY OF BERGEN

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All the way back in 2013, I was invited by my then supervisor, Jeffrey Runge, to visit Norway as part of a collaboration with Howard Browman, Anne Berit Skiftesvik, and David Fields. This PhD would not have happened without them and all those talented individuals at the Austevoll Research Station.

Ellie, you selflessly joined me on this journey. It has been challenging in more ways than we could have ever anticipated and you have been there every step of the way. I am lucky to have you and our children, Linnea and Felix, who keep me grounded and joyful.

FOREWORD

Why this copepod?

The salmon louse, *Lepeophtheirus salmonis salmonis* (Krøyer 1837; Skern-Mauritzen et al., 2014) is an obligate ectoparasite of salmonids. The parasitic stages feed on the mucus, tissue and blood of their host causing sores, immunosuppression and reduced feed conversion efficiency (Thorstad et al., 2015; Fjelldal et al., 2020). It is a major pest of salmon aquaculture and poses an environmental threat to wild salmonids (Costello, 2009b, 2009a; Torrissen et al., 2013; Vollset et al., 2018). The three previous statements are ubiquitous in the salmon louse literature, justifying the immense research effort that has gone into studying this otherwise ordinary copepod parasite. Between 1969 and 2021, the topic of salmon lice has garnered 1520 articles, 36% of which were published in those last 5 years[1]. That wealth of knowledge has been utilized by the Norwegian authorities to form the most rigorous research driven management institution of its kind (Evaluation Committee, 2021). Nevertheless, gaps in knowledge on the planktonic stages of the salmon louse remain (Brooker et al., 2018), and the current regime managing salmon aquaculture growth (The Traffic Light System) faces expert and stakeholder criticisms for its inherent uncertainties and lack of legitimacy (Osmundsen et al., 2020; Evaluation Committee, 2021; Sønvisen & Vik, 2021).

Following the common justification, the impetus for my PhD work was to improve knowledge on salmon louse planktonic stages which would then improve salmon aquaculture management. However, I have found that justification insufficient; this realization came slowly but was punctuated by a few experiences. My introduction to salmon lice was in their role as a model organism for an

[1] **Web of Science Search Result**. Number of articles and review articles with search terms in all fields: "salmon lice" OR "salmon louse" OR "*Lepeophtheirus salmonis*" OR "*Caligus rogercresseyi*" OR "*Caligus elongatus*"

investigation into the effects of ocean acidification on copepods (see Thompson et al., 2019). They were a very interesting and useful model organism, and through that study I learned about their outsized role in salmon aquaculture. I would ultimately pursue that topic in my PhD, and during the editing of my first article I had a minor epiphany about why we were studying the salmon louse. A collaborator had edited a line to refer to salmon lice as a '*disease* challenge' instead of what I wrote describing them as an '*environmental* challenge'. I changed it back realizing that at one point in the past salmon lice were primarily a disease challenge but we were now interested in them because they had become an environmental challenge more akin to pollution. And, there was no way to effectively measure their planktonic abundance, but we figured it out! Creating the novel fluorescence enumeration methodology (**Paper III**) was an immensely satisfying success, but I was surprised by the ambivalent response from potential industry partners: it is interesting and impressive, but there is no market for it. I was confused because much of the work behind the traffic light system is in determining how many infectious salmon lice copepodids are in the water column, and here was a tool to do that.

Thus, one of the goals here is to explore the problem of salmon lice as it relates salmon aquaculture in Norway. Doing so puts the thesis work into the proper context of post-normal-science (see Ravetz, 1999), and highlights where the knowledge gained through this thesis can be most useful. To accomplish this task, we trace the history of the industry and its governance; explore the various environmental challenges of aquaculture and responses to them; and delve into the workings of the traffic light system and the placement of salmon lice within it. Although salmon lice are also a disease challenge and environmental challenge for other salmon producing nations the focus here is on the Norwegian context.

ABSTRACT

Modern salmon aquaculture began in 1971 with the innovation of at-sea fish pens which precipitated a rapid growth in production. The expansion of the industry and increased number of farmed fish concentrated within the open net-pens has produced conditions that foster environmental and disease problems. Among the various pathogens impacting the industry, the salmon louse (*Lepeophtheirus salmonis*) presents a unique challenge due to its proliferation on farms, welfare impacts on host fish, the threat it poses to wild populations of salmonids, and for the cost of and its resistance to control efforts. Norway, the world leader in salmon production, has responded to the persistent challenge of salmon lice with the implementation of a management regime (Traffic Light System) that links permitted aquaculture production to louse induced mortality of wild Atlantic salmon populations. Those management decisions are reliant on an understanding of salmon louse distribution throughout the Norwegian coast, but aspects of the copepod's life history and biology which determine their planktonic abundance remain understudied. Nevertheless, to meet the needs of the management regime modelers must forecast salmon louse reproduction and planktonic dispersal from salmon farms. Although these models are validated with observations of salmon louse infections on fish, there is a lack of empirical evidence on the distribution and abundance of planktonic stages. Due to the difficulty of enumerating planktonic lice in a mixed zooplankton sample they are almost unobservable and thus exist in a 'black-box'.

This thesis seeks to shed light on the salmon louse larval black-box within the context of the aquaculture management in Norway through two approaches. A greater knowledge of the planktonic stages can be gained through a better understanding of the salmon louse's life history, and through empirical data on their planktonic abundance and distribution. This thesis addresses the first approach by refining the current understanding of salmon louse fecundity and the second through the development of a novel method for enumeration of planktonic stages.

In paper I, we investigated fecundity by examining egg clutch size of salmon lice collected from farmed salmon, wild salmon, and sea trout from multiple farms and fields sites throughout Norway. The investigation revealed the predominant determinate of clutch size is the body size of females, which is dependent on rearing temperature. We further found that a third of adult female lice on farmed salmon were not sexually mature and 10% of the mature females were not egg-bearing. The female lice parasitizing sea trout were less fecund then those on Atlantic salmon with lower rates of egg-bearing and smaller clutch sizes.

In papers II and III, we develop a novel method of planktonic salmon lice enumeration which used fluorescence to differentiate the lice within a mixed zooplankton sample so they could be rapidly identified. First the fluorescence profiles of lice and non-target copepods were examined to identify a unique and reliable fluorescence signal, then a methodology using that signal was developed and tested. The fluorescence signal was found to be strongest using an excitation wavelength of 470 nm and an emission filter of 525 nm. After storage in formalin preservation the salmon lice copepodids had a fluorescence intensity that was 2.4 times greater than non-target copepods. When a mixed zooplankton sample was illuminated with the excitation light the salmon lice would fluoresce brighter than most other animals in the sample and could be quickly discovered. Participants in a blind trial processed standard zooplankton samples in a mean of 31 minutes and identified the lice with an accuracy of 82%. Compared to traditional taxonomic identification, the novel method was 20 times faster, thus providing a practical tool for the study of lice and monitoring of their planktonic stages.

The management of salmon aquaculture is dependent on accurate understanding and modeling of the distribution of planktonic salmon lice. The work of this thesis can reduce the inherent uncertainties of those models through better parameterization and through a new tool which enables validation with direct observation of planktonic abundance. However, for the aquaculture industry to continue to grow in Norway this thesis concludes that a prevention priority must replace the current paradigm of salmon louse control through treatment.

LIST OF PUBLICATIONS

Paper I

Thompson, C.R., Bui, S., Dalvin, S., and Skern-Mauritzen, R., (*in preparation*). Disentangling the key drivers of salmon louse fecundity through an examination of field samples, with a focus on the role of temperature and body size

Paper II

Thompson, C.R., Bron, J.E., Bui, S., Dalvin, S., Fordyce, M.J., Furmanek, T., á Norði, G. and Skern-Mauritzen, R., (2021). Illuminating the planktonic stages of salmon lice: A unique fluorescence signal for rapid identification of a rare copepod in zooplankton assemblages. *Journal of Fish Diseases*, *44*(7): 863-879.

Paper III

Thompson, C.R., Bron, J., Bui, S., Dalvin, S., Fordyce, M.J., á Norði, G. and Skern-Mauritzen, R., 2022. A novel method for the rapid enumeration of planktonic salmon lice in a mixed zooplankton assemblage using fluorescence. *Aquaculture Research*.

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LIST OF ABREVIATIONS

IMR – Institute of Marine Research

MAB – Maximum Allowable Biomass

NALO – National monitoring of salmon lice on wild salmonids

NFSA – Norwegian Food Safety Authority

TLS – Traffic Light System

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1. INTRODUCTION

1.1 The Blue Revolution and Growth of Salmon Aquaculture in Norway

The cultivation of aquatic animals is an ancient practice, the beginnings of which can be traced back to China 4000 years ago. Aquaculture expanded into many cultures and regions throughout the world but was primarily restricted to the pond culture of a few species that could be reared from wild caught fry. In the late 19th century an increased demand for recreational fisheries precipitated a surge of new hatcheries, particularly for salmon and trout which had good survival rates due to their large yolk sacs. Through the development of new innovations for hatching and rearing fish, aquaculture continued to progress. However, it was primarily an artisanal practice, and it was not until the mid-20th century that it began to change into modern technological and scientific discipline we know today (Tidwell, 2012). Although it started slowly, the boom in aquaculture began in the 1950s and 1960s. It was fueled by a growing population and demand for fish that fisheries could not meet because wild stocks had reached or exceeded their sustainable limit (Boyd & McNevin, 2015). In the 1970s and 1980s a number of innovations increased productivity and decreased costs which made aquaculture economically competitive with animal protein. These advances spurred a further growth in aquaculture described as the 'blue revolution' following the similarly named 'green revolution' in agriculture (Garlock et al., 2020).

Since 1970, global aquaculture production has been growing at a rate of 7.5% per year with the total global fish production reaching 82 million tonnes in 2018 (FAO, 2020). In 2015, aquaculture surpassed wild fisheries as the primary source of fish for human consumption. It is now viewed as a vital source of production necessary to meet the demand of a growing world population that is also increasing its consumption of protein rich food. Furthermore, aquaculture is an important sector of economic activity and a stable source of income and employment (Garlock et al., 2020). The sector generated 250 billion dollars in first sale value and employed 20 million people in 2018 (FAO, 2020). Despite these widely-recognized benefits,

aquaculture has been criticized for its consumption of resources in terms of feed and area use, and for the emission of waste and pollutants into the environment (Ottinger et al., 2016). As the sector increased in scale and adopted more industrial practices, it also began to encounter greater levels of disease. Viruses, bacteria, and parasites proliferate in the high-density conditions, leading to reduced welfare, higher mortality, and reduced growth for the cultivated animals (Ashley, 2007). Nevertheless, to serve the needs of a growing population with a growing demand for seafood, aquaculture must continue to innovate to overcome challenges and increase production (FAO, 2020).

The development of aquaculture in Norway has largely followed the global pattern, except that the industry in Norway is almost entirely focused on high value salmonids. In 2020, 99.6% of aquaculture production volume and 99.6% of the sale value was from salmonid farms of which 95.1% were Atlantic salmon (*Salmo salar*) with the remaining percentage coming from rainbow trout (*Oncorhynchus mykiss*) (Anon, 2021). Thus, in referencing the industry the terms 'salmon farming' and 'salmon aquaculture' are used to mean both cultivated salmonid species. Norway is the production leader of farmed Atlantic salmon, accounting for nearly 1.4 million of the total 2.6 million tonnes produced worldwide in 2019 (FAO-FIGIS, 2021). The next greatest producer of farmed salmon is Chile, followed by the United Kingdom, Canada, and the Faroe Islands (FAO, 2020). Although sea lice are a common problem for all the salmon farming nations the focus of this thesis is on Norway alone. In 2020, the salmon aquaculture industry in Norway generated approximately 8 billion USD in first hand value and was responsible for the employment of 54 400 people, 14 500 of whom worked directly in aquaculture (Johnsen et al., 2021). The success of the industry in Norway can partly be explained by its favorable location for salmon aquaculture with beneficial water conditions for growth and an especially long coastline of 103 000 km. Also important to its success are the numerous technological innovations, a good governance structure, functioning regulatory frameworks, and Norway's position as a first mover in the industry with a good knowledge base (Osmundsen et al., 2017; Osmundsen et al., 2020; Hersoug, Mikkelsen, et al., 2021).

1.2 Development of Salmon Aquaculture in Norway

1.2.1 Establishing an Industry and Limited Entry Licenses

The first forays into modern commercial aquaculture in Norway began in the 1960s. Early pioneers established hatcheries and attempted a number of grow out methods which included the damming of small inlets and the use of fish ponds on land using freshwater (Hersoug, 2021). Those efforts were largely a commercial failure, but success came in 1971 with the innovation of at-sea, flexible, fish pens which were open to the flow of water. Following that initial development there was great excitement for the growth of Atlantic salmon aquaculture (Hersoug, 2005). The potential was quickly recognized by authorities who then proceeded to set up a committee, the Lysø commission, to establish how the new industry would be developed and regulated. The committee established a temporary aquaculture act that emphasized societal considerations with a focus on maintaining a strong market for the farmed fish and supporting rural coastal communities. The market concern was especially pressing because public and private financiers had previously lost money on loans that had defaulted when those earlier efforts failed. To accomplish their goals, the commission adopted the well-established limited entry regime of modern fisheries with the issuing of licenses or concessions (Hersoug, 2005). Thus, the licensing tool was adopted early in the governance of salmon aquaculture, and throughout the development of the sector up to and including the traffic light system it would remain central to management of production in the industry.

Along with limiting entry, the first licenses issued were used to designate the area where farming was allowed and the maximum volume of the pens (Gullestad et al., 2011). Initially the volume was set to 8000 m^3, by 1975 new licenses were restricted to 5000 m^3 which was then reduced to 3000 m^3 in 1981 and adjusted again up to 5000 m^3 in 1985 before being increased to 12 000 m^3 in 1988. Still limited by the number of licenses and the volume that could be farmed, farmers stocked fish in high concentrations and used liberal amounts of feed. Authorities recognized that this was a welfare and environmental issue and instituted a density regulation to control the maximum number of fish per cubic meter (Hersoug, 2021).

Throughout the early period of salmon aquaculture growth there was a continual issuing of new licenses and an increase in production due to the upward adjustment of the volume restriction. Aided by easier access to financing and technological development salmon aquaculture became a highly profitable enterprise and expanded rapidly (Hersoug, 2005). Unfortunately and despite management's stated goal to prevent it, the market demand did not grow along with the supply and that overproduction eventually led to a crash in price in the early 1990s. Compounding the overproduction problem, Norway began to face legal challenges from the United States and the European Union. Norway was accused of unfairly subsidizing the salmon aquaculture industry and dumping product on those export markets, the aggrieved parties issued punitive tariffs and mandated minimum prices and maximum export volumes. Ultimately, the salmon agreement of 1996 with the European Union limited the growth of the industry to 10% per year which was achieved through a feed quota regime and further density limits; this system would last through 2004 (Gullestad et al., 2011; Hersoug, 2021).

1.2.2 Industry Restructuring and Consolidation

In the beginning, much of the concern was over equity, who was granted licenses (concessions) to farm salmon and where was it taking place. The government's policy goal was to support rural coastal communities that were hard hit by the restructuring and rationalization of the fishing industry which resulted in an economic loss in those areas (Hersoug, 2005). Thus, management restricted licenses to an owner operator arrangement and a mandatory sales union was created to buy their fish and bring it to market. When the market price plummeted in the 1990s, the union went bankrupt and with it many farmers who's finances were tied to the union (Gullestad et al., 2011; Hersoug, 2021). The market challenges faced by the industry coincided with a critical management decision in 1992 to remove the rule restricting ownership to a single license. Further changes in regulations liberalized the exchange and sale of licenses. Following these rule changes, a thorough restructuring of the industry began that would see small family owned operations give way to large corporations (Asche et al., 2013). There were 772 licenses held by 668 companies in 1992, and as a result of the consolidation there were just 220 companies holding 869

permits in 2005 (Gullestad et al., 2011). Along with the general reduction in the number of companies, there was a consolidation into progressively larger firms. In 1996 the top 10 largest companies were responsible for 18.9% of the sale volume, and by 2009 they were responsible for 65.7% (Anon, 2021) (Fig. 1.). Thus, the original concern over equity and the desire to avoid a market collapse due to overproduction has largely been abandoned by management but the licensing regime remained in place.

The structural changes in the industry which led to the formation of fewer but much larger companies enabled those firms to take advantage of economies of scale and better financing. At the same time and in part due to the greater capacity of these companies to innovate, technical efficiencies in the industry improved greatly. Productivity increased both because of better practices put in place on the farms and because of improvements in the inputs such as feed and smolts (Asche, 2008; Asche & Roll, 2013; Asche et al., 2013). Altogether these changes transformed the nascent salmon aquaculture industry, releasing a seemingly unquenchable desire for growth that would result in it becoming a vital economic sector. Between 1992 and 2012 salmonid aquaculture production increased by a factor of 10, from 130 thousand tonnes to 1.3 million tonnes (Fig. 1; Anon, 2021). Meanwhile the number of licenses also increased but at a much slower rate, going from 772 in 1992 to 963 in 2012, a 25% increase (Gullestad et al., 2011; Anon, 2021). Thus, much of the production increase was not due to an increase in licenses but a greater and more efficient utilization of the licenses.

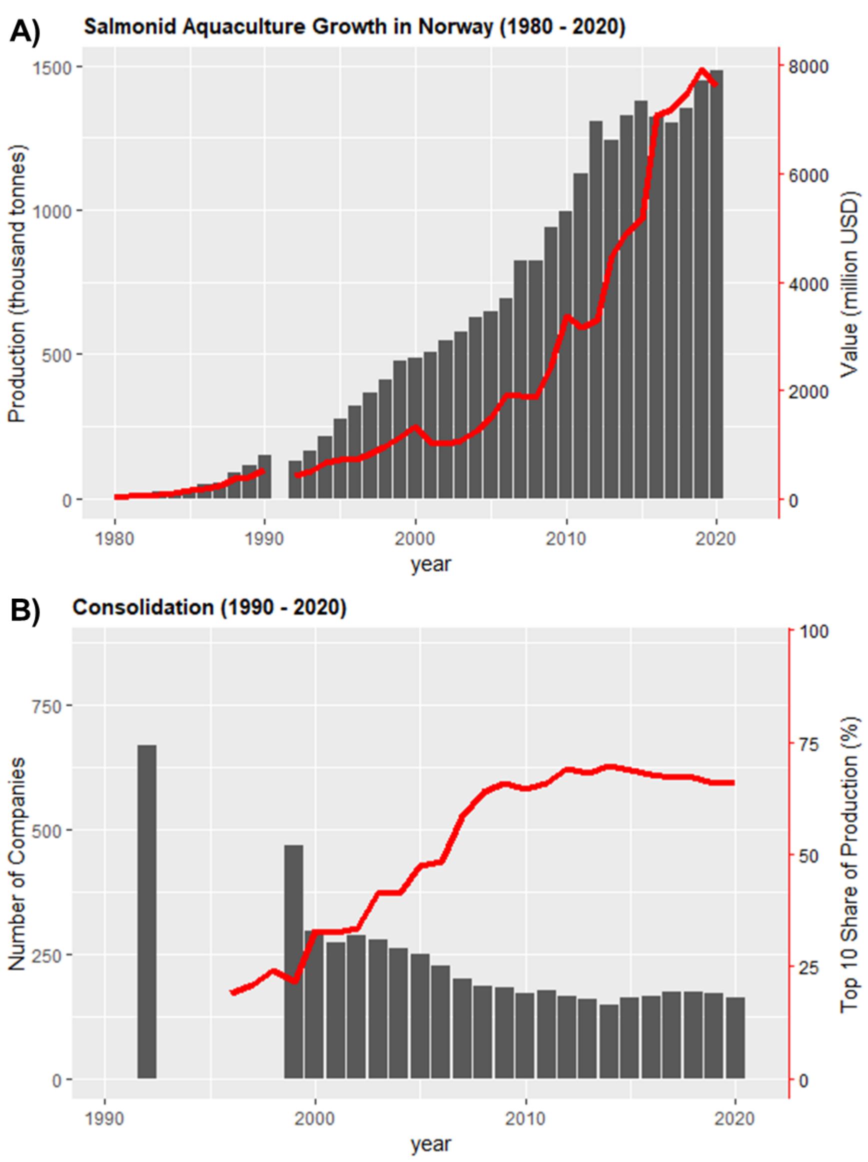

Figure 1. A). Growth of Salmon Aquaculture in Norway. Left y axis is the production weight sold that year in thousands of tonnes, right y axis is the sale value for that year in millions of USD, converted from NOK with a 0.111 NOK to USD exchange rate. B) Consolidation of the industry. Left y axis is number of companies with grow out operations and the right y axis is the percentage of production produced by the top 10 largest companies. Data sources: (Hersoug, 2005; Gullestad et al., 2011; Anon, 2021)

1.2.3 Regulating Maximum Allowable Biomass

The greater production efficiency in the sector intersected with the feed quota regulation and limited licenses to create an ethical dilemma that management was forced to address in 2001. In an effort to maximize production, farms were stocked with more fish than could be sustained by the amount of feed provided which led to an increase in mortality and poor welfare outcomes (Hersoug, 2021). Ultimately an output control system of maximum allowable biomass (MAB) was adopted in 2005, replacing the input control system of feed quotas and volume limits. Previously, a license allowed a farmer to grow out fish in 12 000 m^3 of space with a maximum fish density and feed allotment. That license was converted to a maximum allowable biomass of 900 tonnes in Troms and Finnmark, and 780 tonnes elsewhere (Gullestad et al., 2011). The stocking density limits remained but nevertheless the rule change led to an immediate 30% increase in production. The MAB has been criticized for its difficulty to monitor by authorities and for the burden it places on farmers who must plan years long production cycles, but the MAB system has the advantage of providing a single regulatory currency. It is more flexible to changes in the industry and technology, can be used for regulation of both the production licenses and locality licenses (see section 1.3.4), and is resilient to market and political pressures (Hersoug, 2021).

The evolution of the salmon aquaculture governance system can be viewed as an example of institutional path dependency. Limited entry licenses were already a familiar tool in Norway, and once established the production licenses were never abandoned but co-opted to serve the needs and crises of the moment. The MAB system was adopted in part because it could preserve the value of the licenses by converting the previous regulatory regime (see Hersoug, 2005; Hersoug, 2022). Despite the need to adapt to subsequent environmental challenges, this regulatory framework of production licenses with MAB is still in effect today within the traffic light system. Arguably, the path dependency is also partly responsible for salmon lice becoming the central governable object within the new system. The risk of salmon lice induced mortality on wild salmon is linked to their planktonic abundance which depends on the abundance of their hosts, and that abundance is readily

represented by MAB (Osmundsen et al., 2020). Thus, a salmon louse indicator could be adapted to the system as it existed but before it could be integrated it would first need to be recognized as a problem and turned into a management object.

1.3 The Salmon Louse Threat

Leading up to 2012, the salmon aquaculture industry had experienced a nearly 10% increase in production year over year, and many experts believed that while the rate of growth would decrease, production would still grow tremendously. The conservative projection was that by 2050 production would quadruple to reach 5 million tonnes (Olafsen et al., 2012). However, that optimism would face the reality of a near immediate stop in the allocation of new licenses due to environmental concerns over farm escapes and lice (Hersoug, 2015). Disease and environmental challenges had been ever present in the development and growth of the salmon aquaculture industry, and were highlighted in the same document that forecasted the 5 million tonne goal (Olafsen et al., 2012). In the development of the aquaculture industry these challenges had been largely overcome through technical innovation and effective regulations. However, the use of control rules over operations at individual farms was no longer sufficiently addressing the challenge of salmon lice, and authorities were confronted with the need for a new governance system to ensure a predictable growth of the industry (Anon, 2015; Osmundsen et al., 2020). The traffic light system that was eventually established placed the salmon louse at the center of management decisions, recognizing it as a critical environmental threat to wild salmon (Vollset et al., 2018). Thus, the salmon louse, an otherwise ordinary copepod parasite, has curiously become an object of incredible import.

1.3.1 The Copepod Parasite

The salmon louse, *Lepeophtheirus salmonis*, is one of an estimated 60,000 species of marine copepods, only a quarter of which have been described. Copepods are the most numerous animals on the planet, more numerous than insects, and weighing 150 times that of the entire human population (Schminke, 2007). On average a copepod can be found in every liter of ocean water though local

concentrations can be far greater, especially during blooms at higher latitudes (Boxshall, 1998). World over, they serve an essential ecological function as consumers of plankton and as a food source for many other animals, transferring primary production energy to higher trophic levels. Copepods are ubiquitous, they are found in every marine and freshwater aquatic habitat, and the many species have adapted to nearly as many ecosystem niches. A third of copepod species are believed to be parasitic and most of them, including the salmon louse, feature a life cycle that includes a free-living larval stage (Ho, 2001). Each species of copepod parasite must find a suitable host and attach or enter that host to parasitize it so that the parasite can mature and produce its own offspring, repeating the cycle (Marcogliese, 2005)

Copepod parasites are ubiquitous pests of finfish aquaculture with 61% of the reported species belonging to the *Caligidae* family which includes 509 species in 30 genres (Johnson et al., 2004; Hemmingsen et al., 2020). Among the *Caligidae*, the *Lepeophtheirus spp.* and *Caligus spp.* are particularly known for their impact on farmed fish and the economic damage they cause (Costello, 2006). The salmon louse, *Lepeophtheirus salmonis,* has a high degree of host specificity parasitizing the farmed species *Salmo salar* (Atlantic salmon) and *Oncorhynchus mykiss* (Rainbow trout)*,* as well as *Salmo trutta* (Sea trout) and *Salvelinus alpinus* (Arctic char) (Bjørn, 2002). Another species of *Caligidae*, *Caligus elongatus* can also be found on those host fish, but it has lesser impact and host specificity, having been reported on over 80 different species of fish (Dojiri & Ho, 2013; Hemmingsen et al., 2020).

The sea lice *Lepeotheirus salmonis* and *Caligus elongatus* both have a life cycle that includes 8 developmental stages but with notable differences in their ontogeny. After hatching from an egg the lice begin as a planktonic free-living larva, this first naupliar stage quickly develops into a second naupliar stage, and then a copepodid stage which finds and attaches to the host fish. While *Caligus elongatus* develops through four chalimus stages and then onto the adult stage, the *Lepeotheirus salmonis* life cycle features two chalimus stages followed by two pre-adult stages and then the final adult stage (Piasecki, 1996; Hamre et al., 2013; Venmathi Maran et al., 2013). The adult stages of *Lepeotheirus salmonis* are much larger than those of *Caligus elongatus,*

but their planktonic stages are approximately the same size and have similar morphology. Differentiating the species of the planktonic stages through taxonomic identification is possible but can be challenging (see Schram, 2004). Thus, when attempting to enumerating the planktonic stages of salmon lice the presence of *Caligus elongatus* should also be accounted for.

At 10°C it takes less than 4 days (38 degree days) for the salmon louse (*Lepeotheirus salmonis*) to develop into the infective copepodid stage. Prior to attaching to the host, the planktonic stages are all non-feeding and metabolize maternally derived lipids which enables the salmon louse to survive 150 degree days or more (Samsing et al., 2016; Thompson et al., 2019). The prolonged non-feeding planktonic stage enables the louse to disperse widely from its maternal origin (Asplin et al., 2014), and due to the effects of various currents the vertical distribution of the lice greatly influences their dispersion (Johnsen et al., 2014).

Typically, the infectious copepodid stages will occupy shallow depths where they are more likely to encounter their target hosts (Costello, 2006; Coates et al., 2020; Coates et al., 2021; and references therein). While the planktonic stages move passively in the horizontal plane they exhibit vertical swimming behaviors in response to numerous environmental stimuli to maintain an optimal position in the water column. However, these behaviors differ by stage with the nauplii exhibiting a greater depth preference according to temperature and salinity. Meanwhile, the infectious copepodids prefer the upper water column (Crosbie et al., 2019; Crosbie et al., 2020), and swim towards chemical and mechanical cues which simulate the presence of a potential host (Fields et al., 2018). The behaviors of the planktonic stages can be observed under laboratory conditions, but measuring the resulting vertical distributions in the field is laborious and few studies have attempted to do so (see Nelson et al., 2018; Skarðhamar et al., 2019).

Enumerating the planktonic stages of salmon lice in the water column is a laborious task that is rarely attempted because finding them is a needle in the haystack problem (see Bui et al., 2021). In a mixed zooplankton sample collected using standard

methodologies, salmon lice are rare in comparison to the numerous other copepod and other zooplankton species. Several studies have attempted to describe the distribution and abundance of the planktonic salmon lice. They have shown that nauplii occur in greater numbers with their abundance decreasing with distance from farms (e.g. Nelson et al., 2018), and copepodids can be found aggregating in shallow estuarine waters near the mouths of waterways (Penston et al., 2008). Altogether these works have measured planktonic lice abundances ranging from 0.075 to 0.70 m^{-3} (Penston et al., 2011; Salama & Rabe, 2013; á Norði et al., 2015; Nilsen, 2016; Byrne et al., 2018; Nelson et al., 2018; Skarðhamar et al., 2019). In comparison, a standard zooplankton sample from the west coast of Norway collected with a vertical net can be expected to contain 5000 m^{-3} or more animals (T. Falkenhaug, personal communication, 6 June 2020), global mean density of free-living copepods is estimated to be 1000 m^{-3} (Boxshall, 1998), and along the Norwegian coast *Calanus fnmarchicus* and *Metridia spp.* concentrations have been found between 10 000 and 100 000 m^{-3} (Halvorsen et al., 1999 cited in Skarðhamar et al., 2019). Thus, to find a single salmon louse using the traditional technique of light microscopy taxonomic identification it may be necessary to sort through 1.4 thousand to 1.4 million animals. Overcoming this needle and the haystack problem is one of the objectives of this thesis.

1.3.2 A Pest and Disease

Contrary to the accepted view today, the salmon louse was considered an indicator of health. When it was found on a fish caught far upstream from the mouth of a river it was believed that the host salmon must be a healthy, strong swimmer to have come so far so quickly. Otherwise, prior to the establishment and growth of salmon aquaculture, the salmon louse was given little thought beyond it being a naturally occurring parasite of salmonids. (Misund, 2019; Osmundsen et al., 2020). Aquaculture has greatly disrupted the typical host parasite dynamic, especially salmonid aquaculture. The growth of open net-pen aquaculture in Norway increased the opportunities for the salmon louse to find and infect a host, and by the mid-1970s they were already recognized as a disease problem on farms (Heuch & Mo, 2001; Heuch et al., 2005).

In the 1970s and 1980s, research and industry focus on salmon lice was primarily concerned with their elimination from farms, which was accomplished through chemical therapeutics (Misund, 2019). Multiple drugs were developed and these kept the salmon louse in check since farmers could treat their farms with reasonable efficacy before the parasite caused excessive problems (Igboeli et al., 2014; Aaen et al., 2015). Although the infection levels at farms were a disease problem they weren't hindering the growth of the industry. However, by 1992 it was observed that salmon lice could spread to and negatively impact sea trout (*Salmo trutta*) and possibly wild salmon (*Salmo salar*) (Heuch & Mo, 2001). As the industry expanded throughout the 1990s, concern grew over salmon lice epidemics on farms and their spread to wild fish. In a consensus response by farmers, fish health personnel, and the Norwegian Food Safety Authority (FSA), the 'National Action Plan Against Salmon Lice' was put into effect in 1997 (Heuch & Mo, 2001). It was meant to reduce harmful effects of lice with part of the plan focused on coordinating control efforts and another part focused on data gathering. Prior to 2000, a seasonal maximum threshold allowed up to 5 adult female lice per farm fish in the summer and autumn, and 2 in the spring. Under the new action plan that threshold was reduced to 0.5 for the winter and spring, if a farm exceeded the limit then a treatment would be required, and any treatment would then be reported to authorities (Heuch et al., 2005). By the early 2000s salmon lice were treated as a costly disease of concern by the industry and authorities, but the uncontrollable threat they posed would not be widely appreciated until the end of the decade. Data was being gathered and the problem was being monitored, but other environmental threats such as fish escapes and pollution were the management focus (Anon, 2007).

1.3.3 One Challenge Among Many

Disease and environmental problems have broadly followed the development and growth of aquaculture due to the concentration of animals and inputs such as feed to a single location (Ashley, 2007; Ottinger et al., 2016). Along with salmon lice, farmers have faced numerous disease challenges which have largely been mitigated through technical innovations and better management. In the 1980s outbreaks of bacterial infections, causing vibriosis and furnunculosis, plagued the industry and

resulted in the liberal use of antibiotics. Aided by government intervention, effective vaccines were developed in the early 1990s and mass vaccination campaigns were promoted. The vaccination together with the implementation of sanitization practices such as fallowing and the spatial separation of sites alleviated the problem and antibiotic treatment has dropped (Midtlyng et al., 2011). Sanitation practices, selective breeding for resistance, and vaccines have also helped reduce the outbreak and spread of viral diseases including the particularly serious infectious salmon anemia (ISA) and pancreas disease (PD). These technical developments helped reduce the costs of production and fuel much of the industry growth through the 1990s and 2000s (Kumar & Engle, 2016). In the same period, multiple innovations to feed and feed systems were made which improved fish nutrition, reduced the reliance on fishmeal, reduced wastage, and reduced pollution (Torrissen et al., 2011; Kumar & Engle, 2016). Many of these innovations which increased the productivity of the industry also improved its sustainability and limited its impact on the environment but further management action would be needed.

Sustainability as a goal for the aquaculture industry was codified into law in 1992 with an amendment to the farming act using the Brundtland (1987) definition: "meeting the needs of the present without compromising the ability of future generations to meet their needs" (Gullestad et al., 2011). In practice, management has prioritized environmental sustainability and a good economic model over social aspects of sustainability such as legitimacy and equity (according to Hersoug, Mikkelsen, et al., 2021; Sønvisen & Vik, 2021). The sustainability goal has since intersected with a particular focus on wild salmon resulting in that species dominating much of the environmental sustainability discourse. Approximately one third of Atlantic salmon spawning grounds are in Norway. Recognizing their responsibility, the population decline of the wild stocks, and their disappearance from many waterways, the Norwegian government has a number of international agreements and policy directed to protecting wild salmon (Anon, 2009).

Among the threats to the sustainability of wild Atlantic salmon populations is the risk that they may interact with escaped farmed fish. Escapees present an

environmental threat to the wild salmon because the farmed fish could compete for resources, spread diseases, and reproduce with wild stocks resulting in progeny poorly adapted to the natural environment. The challenge of farmed salmon escaping sea pens had long been recognized and a monitoring program was initiated in 1989, but by the 1990s the number of escapes was reaching hundreds of thousands per year (Thorstad et al., 2008; Glover et al., 2017). In response, Norway has initiated a number of efforts to mitigate the problem, including: technical requirements for the net-pens to prevent escapes, tracing escapes to the farm origin using molecular techniques, removal of escaped fish from waterways, and National Salmon Fjords (Mahlum, 2020).

The National Salmon Fjords are a form of marine spatial planning that designated certain waterways and their adjacent fjords as protected areas in which new farms are not allowed and the few grandfathered farms are strictly regulated. The program established in 2003 replaced an earlier one established in 1989 that fulfilled a similar role but was deemed too small to be effective (Thorstad et al., 2008). Despite an increase in salmon aquaculture production, those initiatives have led to a reduction in the reported number of escapes from a high of approximatley 9 hundred thousand in 2008 down to less than two hundred thousand in 2018 (Glover et al., 2019). The proportion of escapees detected in rivers has also shrunk, nevertheless half of the wild salmon populations show evidence of introgression from the farmed salmon (Glover et al., 2017; Glover et al., 2019). In the case of escapees, technical improvements together with management actions have alleviated the threat, which follows the pattern seen in the development of the industry in which innovation has been used to overcome environmental and disease challenges. Nevertheless, escapees remain a threat to wild populations.

1.3.4 Governing the Environment at Every Locality

Spatial planning is a well-known tool of aquaculture management in Norway, every farm must have a production license to grow the salmon and another locality license to operate. The locality license is part of a larger system of governance which manages the coastal space and thus involves the coordination of multiple authorities,

each with their own set of regulations and priorities. In Norway, it is primarily the responsibility of the local municipalities to navigate the differing needs of multiple stakeholders and conduct coastal zone planning through which sites are allocated for farms. Before a locality license can be issued it needs to be certified as meeting statute requirements by those other authorities, including the Norwegian Food Safety Authority (NFSA), Environment Agency, and Coastal Administration. Through this process, environmental, sanitation, and welfare regulations are enforced (Sandersen & Kvalvik, 2014; Kvalvik & Robertsen, 2017; Hersoug, Mikkelsen, et al., 2021).

When fish pens were first deployed in Norway, they were often placed at sites with poor water circulation resulting in excessive buildup of discharge and poor water conditions for the animals. Now, before a locality licenses can be approved a site survey is conducted to determine its carrying capacity based on numerous factors including: water exchange, sediment condition, benthic composition, currents, and organic load among others. Although these factors can be measured, every site is unique and authorities have relied on an imperfect calculation of carrying capacity which is measured in MAB. Thus, MAB serves as the currency to connect the locality license to the production licenses, which uses the entirely separate traffic light system to determine MAB (Hersoug, 2021).

The coastal zone planning and the system of thoroughly regulated locality licenses has reduced environmental and sanitary problems in the aquaculture industry, but finding good sites is becoming more challenging. In response to the stricter regulatory regime and to the greater MAB allowed on a single site, there has been a pattern of intensification to the best possible sites over the past few decades. Between 1999 and 2019 the number of farming sites reduced from 1866 to 966, while the overall production nearly tripled. Farmers are still able to find good sites and willing municipalities for new locality licenses, but there is growing concern that space is limited and could easily become a bottleneck to future growth (Sandersen & Kvalvik, 2014; Kvalvik & Robertsen, 2017; Huserbråten et al., 2020; Hersoug, Mikkelsen, et al., 2021).

1.3.5 The Perpetual Production License

Previously production licenses were allocated for the purposes of limited entry (where and who could farm) and the growth of the industry, while the locality licenses were used to regulate other matters. Once rules such as volume limits and feed quotas went into place the production license was no longer the only means of controlling the growth of the industry. Instead they could be used to address environmental and equity concerns such as employment opportunities. Prospective farmers would have to apply for a license and allocations were issued to the best applicants. Before the traffic light system was even proposed this system was already criticized as a 'beauty contest'; it was resource demanding for authorities, inevitably subjective, and based on unverified promises by applicants. The license system was also criticized by stakeholders for the irregularity of the allocation rounds and for how unpredictable the allocation rules and volumes would be (Hersoug, 2015). Thus, when proposed the traffic light system presented a more predictable means of regulating the growth of production in each zone, and auctioning those concessions would be a more objective means of allocation (Anon, 2015).

The purpose of the production license system has changed greatly since its inception. When considering the structural changes in the industry, the coexisting locality licensing system, and additional operational regulations, the production licenses utility from an outside perspective is questionable. However, governance systems are at the mercy of path dependency and the license system is fully entrenched with many stakeholders dependent upon it (Hersoug, 2005, 2021; Hersoug, Mikkelsen, et al., 2021). In 2019 there were 1187 production licenses which would each cost 150-200 million NOK at an allocation auction and represents a total invested value of around 200 billion NOK (Anon, 2021). Now, with the traffic light system that immense wealth and the entire salmon aquaculture industry has been tied to the ordinary parasitic copepod, *Lepeophtheirus salmonis*.

1.3.6 The Environmental Crisis of Salmon Lice

Salmon lice became the center of the traffic light system instead of any other environmental threat because of the inability to control them at the local level and

because of the sustainability discourse that developed around them. All the previously mentioned environmental and disease challenges that the industry encountered were successfully handled through technical innovations and spatial management. Up until salmon lice were recognized as an environmental problem needing special attention, they were handled similarly to other pathogens. As explained previously (section 1.3.2), pesticides had been in use since the 1970s, farms followed sanitation practice, the lice were monitored, and their intensity at farms limited through treatment. However, the salmon lice developed resistance to the drugs which the industry relied upon to control them (Igboeli et al., 2014; Aaen et al., 2015). The number of lice on farms and number of epidemics grew, and recognizing the growing problem the Salmon Lice Directive came into force in 2009 to specify how lice should be monitored and controlled (Osmundsen et al., 2020). As stewards of animal welfare, the responsibility to implement the directive fell to the NFSA. They introduced a rigorous new protocol including stricter lice limits, requirements for treatment plans, and the possibility of sanctions for the worst performers (Hersoug, 2015; Thorvaldsen et al., 2019). Rather than solving the problem this action marks the beginning of a period between 2009 and 2014 in which the discourse on salmon lice shifted and they went from being a disease problem to an environmental and societal problem.

The decline of wild salmon, concurrent growth of aquaculture, and a growing environmental awareness had previously sensitized society to the impacts salmon farming has on wild populations. Due to the growing challenge salmon lice became the center of public discourse and by 2014 were defined as the most critical problem of the aquaculture industry (Misund, 2019). Beyond being an environmental problem, the salmon louse is a symbol of the potentially dangerous aspects of aquaculture and makes for an easily communicated story of how aquaculture is connected to sustainability. Unlike those other environmental and disease challenges, salmon lice are resistant to control and their population growth has a direct relationship with the total number of fish on farms. They are also easily monitored and counted on farms (Osmundsen et al., 2020). Furthermore, aquaculture experts and authorities were aware of how salmon lice differed from other environmental challenges and could be used as an indicator of sustainability (Gullestad et al., 2011; Anon, 2015). The threat

of salmon lice has a direct relationship with MAB, which was already the currency of the licensing system prior to the traffic light proposal and thus could be readily adapted (Hersoug, 2021).

The traffic light system was officially proposed to government in 2015 and faced push back from industry groups but was ultimately accepted and implemented in 2017 due to a lack of alternatives and pressure from environmental interests (Vollset et al., 2018; Hersoug, 2021, 2022). Thus, the salmon louse became the center of salmonid aquaculture management and a newly created governmental technology. As a technological object the salmon louse now serves a purpose for governance beyond its intrinsic copepod and parasitic nature. While they are a measurable parameter, and those numbers can be communicated and compared across space and time, that has the effect of simplifying them and reducing the apparent uncertainty (Osmundsen et al., 2020). The next section explores how the traffic light system works, where the salmon louse biology is being simplified, and where the uncertainties exist.

1.4 The Traffic Light System

1.4.1 Determining Permitted Salmon Aquaculture Production

The traffic light system (TLS) is a means of determining the permitted salmon aquaculture production capacity across 13 zones using sustainability indicators. Although more indicators can be added and it was the stated intention to do so, there is only one indicator in use – the salmon louse induced wild salmon mortality. Following the traffic light metaphor, the system has three assessment levels corresponding to the threshold level of lice induced mortality, green for less than 10%, yellow for 10-30% and red for over 30% of the population in the production zone likely dying due to lice infestations. The traffic light indicator then has an associated action rule for each level, when it is green production capacity can increase by 6%, when yellow production stays the same, and red requires that production be decreased by 6% (Vollset et al., 2018). Every year the likelihood of wild fish mortality is determined for each of the production zones, and every other

year a color is assigned and the action rule is enforced. Although the traffic light was turned on in 2017 and a threshold mortality level was determined for each production zone, it was considered a trial year and the action rule for the red level was not enforced. The first enforcement would follow the assessment round in 2019, in which two of the production zones were red. Thus, this would be the first time the rule would be enforced, and it would end up reducing production by 17 000 tonnes and cost farmers 850 million NOK. Many of the effected farmers sued the government over the lost production but ultimately lost in court. Although the legality of the system was upheld its legitimacy was challenged, and the credibility of the science was questioned (Sønvisen & Vik, 2021).

Following the lawsuit, an independent and international committee was asked to evaluate how scientific assessments are made in the TLS. They acknowledged that it is a sophisticated and evidence driven program but within the system there are uncertainties. The greatest source of uncertainty comes from the calculation of wild salmon mortality as it relates to the salmon louse infection intensity (Evaluation Committee, 2021). The research used to derive the mortality thresholds comes from tank experiments investigating the physiological effects, and from field studies measuring the lice loads of wild fish. The physiological experiments showed that an infection of just 0.1 lice/g fish weight will cause problems and salmon smolt will die with around 10 lice per fish. The tank experiments were supported by field observations since post-smolts had not been observed with more than 10 lice and those with 10 lice were in very poor condition (Taranger et al., 2011; Taranger et al., 2012). From that evidence base, Taranger and others (2012) proposed a calculation for the estimated lice induced mortality, which they characterized as a first generation method of quantification. Despite acknowledgement that further work was needed, the originally proposed calculation is still in use in the TLS because no new research has thus far challenged the basis of the calculation (Vollset et al., 2019).

1.4.2 Evaluating Infection Intensity on Wild Salmon

The majority of effort in the traffic light system is directed towards the evaluation of infection intensity on wild salmon from which their likely mortality can

then be estimated. It is ultimately up to the appointed expert group to make the assessment of likely mortality for each production area. As the name suggests, the members of the group possess an expertise in the field and must have the ability to conduct an analysis of the gathered data to make an assessment, but otherwise they should have a broad background (Vollset et al., 2019). The assessment is based on multiple sources of information including monitoring data from farms (Jansen et al., 2012) and wild caught salmonids (Serra-Llinares et al., 2014), sentinel cages with fish (Bjørn et al., 2011), and several models (Sandvik et al., 2016; Vollset et al., 2019). The monitoring data is generated from two distinct programs operating under different authorities. The farm monitoring falls under the authority of the NFSA, and the monitoring of wild fish is done through the salmon lice surveillance program for wild salmonids (NALO) which is administered by the Institute of Marine Research (IMR). In accordance with the NFSA regulation over the control of lice, farms must count the lice on at least 10 fish per pen and report the average. If the water temperature is above 4°C this is done weekly, below 4°C it is done every other week (Anon, 2012). While systemic falsifying of reports is believed to be unlikely due to the potential for significant jailtime and heavy fines, counting lice is a difficult manual task done by farm workers and thus susceptible to bias and noise. Depending on the farm, the procedures used to collect fish may differ as well as the counting procedure. The accuracy of stage identification will depend on the counters' ability and any number of extraneous elements such as the lighting and weather (Thorvaldsen et al., 2019). There is a likewise potential for variation to influence the commensurability of wild fish data obtained from NALO though the sampling is done by biologist trained to count lice. Several operations are used in NALO for collecting samples and monitoring infection pressure on wild fish. In designated fjord systems, a weeks-long trawling effort is conducted to capture wild Atlantic salmon smolts as they migrate out of their rivers towards the open ocean. Another set of observations comes from the deployment of sentinel cages, which are deployed in fjord systems, stocked with salmon smolts, left for 2 weeks, and then sampled. The last operation involves targeted fishing and release of sea trout using traps and nets in areas with high expected salmon lice infestation pressure and in control areas with little

pressure. In all cases the morphological measurements are made of the salmonids and a thorough lice count is performed (Karlsen et al., 2021).

While the expert group examines the monitoring data from the farms and NALO, the data also feeds into a set of sophisticated models which are then used for the assessment. Notably, the monitoring programs only generate data on the attached parasitic stages not the planktonic infectious copepodid stages which are the focus of the models (Myksvoll et al., 2018; Sandvik et al., 2021). There are four model products which estimate the salmon louse infestation pressure and the risk of mortality to wild salmon: the IMR infection pressure map, the IMR virtual smolt model, SINTEF's model system, and the Veterinary Institute's model system (Vollset et al., 2019). All of them rely on the same louse reproduction model following the work of Stien and others (2005) which was acknowledged by the authors as being based on an incomplete understanding of louse fecundity.

1.4.3 Salmon Louse Fecundity and the Production of Lice

Fecundity is a function of the rate of egg clutch production, the proportion of those eggs that are viable, and the number of eggs per clutch (Stien, Bjorn, et al., 2005). Before producing eggs the salmon louse female must also develop into the adult stage, reach sexual maturity, and mate. Mating follows the general pattern in *Caligid* copepods with the adult males, finding a preadult female and waiting until it molts to its adult stage so that a spermataphore can be deposited (Ritchie et al., 1996; Pike & Wadsworth, 1999). The spermatapore obstructs subsequent mating, but multiple spermataphores have been observed and multiple paternity has been confirmed (Todd et al., 2005; Hamre et al., 2009). It has been further observe that, 19% of female lice on salmon farms carry no spermataphores while only 10% of females on wild fish have none (Todd et al., 2005). Although exceptions have been observed under laboratory settings (see Eichner et al., 2008; Skern-Mauritzen et al., 2009), Ritchie (1996) suggests mating is necessary prior to the production of egg strings. Lack of mates may be an issue when densities are low, but on farms the mobile stages of salmon lice are able to switch hosts, possibly alleviating the issue (Bui et al., 2020). Regardless of mating status, eggs cannot be produced by an adult

female until a process of maturation is complete in which egg yolk proteins are transcribed and the genital segment expands and lengthens (Eichner et al., 2008). Thus, not every adult female salmon louse observed on a host fish is necessarily capable of producing fertilized egg strings, yet that is what the louse reproduction model assumes. Egg production and the releases of newly hatched nauplii from farms is calculated from the number of fish, the number of adult females per fish, temperature, and a constant number of eggs per female (Ådlandsvik et al., 2017).

Temperature has a negative relationship with hatching and development time in salmon lice (Samsing et al., 2016; Hamre et al., 2019), which follows the general pattern seen in ectotherms including copepods (Angilletta Jr et al., 2004). Ectotherms also follow the temperature-size rule with animals reared at colder temperatures growing slower but ultimately reaching a greater body size at maturity (Atkinson, 1994). Body size effects all aspects of an organism's biology and ecology, with larger sizes generally granting greater fitness within a population due in part to the increased fecundity (Kingsolver & Huey, 2008; Horne et al., 2016). Generally, a relationship has been observed between temperature, season, salmon louse body size, and clutch size. Larger female lice are found in colder months, and those females have larger clutch sizes (Ritchie et al., 1993; Gravil, 1996). By conducting exhaustive laboratory experiments rearing lice at various temperatures Samsing (2016) fit development rate, body size, and clutch size to a universal temperature function. Samsing (2016) found the smallest clutch sizes and females occurred at the highest temperatures then sizes increased with lower temperatures until the lowest temperatures where sizes decreased again. However, the function chosen may not provide the best fit (see Quinn, 2017) and the lowest temperature treatments may have been below thermal tolerances (see Brooker et al., 2018). Furthermore, Samsing (2016) was able to control temperature throughout the lifecycle of the salmon louse, but in the wild a louse will experience a range of temperatures. Disentangling the various factors influencing fecundity and better parameterizing it is necessary to understanding salmon louse biology (Brooker et al., 2018) and is an objective of this

thesis. A better parameterized louse reproduction model would improve the prediction of salmon lice infestation intensity for assessing louse induced mortality.

1.4.4 Modeling Salmon Louse Induced Mortality

Using the production of lice at farms as a starting point, the two IMR model products predict the distribution and abundance of infectious copepods as a common model input. The input model couples hydrodynamic models with a salmon louse biology model that treats them as particles with growth, mortality, and behavior parameters, such as development rate and vertical positioning (Vollset et al., 2019). Unsurprisingly, the physical parameters predicted by the hydrodynamic model have a higher correlation with measured values than the infestation pressure as measured by the monitoring data. Part of the challenge stems from the lack of abundance data on salmon lice in the water column and a lack of knowledge of their movements at small scales, nevertheless the output of the model has good correlation with monitoring data, especially at higher scales (Myksvoll et al., 2018). The uncertainties around the parameterization of the salmon lice model are acknowledged and the model system is continuously being updated to incorporate the best available science on salmon lice. Some parameters like mortality remain a major source of uncertainty as it is simply set at 17% per day following Stien (2005) (Sandvik et al., 2019). Exemplifying the iterative improvements, recent work by Crosbie and others (2019) described a low salinity avoidance behavior by salmon lice that was then incorporated into an improved version of the IMR distribution and abundance model (Sandvik et al., 2020).

The salmon louse distribution and abundance model is used by two IMR model products, the infection pressure map which is calibrated using the sentinel cage data, and virtual smolt model. The virtual smolt model simulates the migration and infection of smolt from river to the open ocean through the previously calculated infectious copepodid distribution, and calibrates its results to the trawl data (Sandvik et al., 2019; Vollset et al., 2019). The third model is SINTEF’s SINMOD, which is designed similarly to the virtual smolt model but is independently operationalized. A concentration field of infectious copepodids is calculated from hydrodynamic models

and a louse model, then a simulated smolt migrates from river to the sea and mortality is calculated based on the probable infection. The final model is provided by the Norwegian Veterinary Institute and includes a simple calculation of decreasing salmon louse density with distance from farms. Through a series of complex steps it then models infestation, the smolt migration, and ultimately smolt mortality (Vollset et al., 2019). The models are a necessary component of the TLS because it is prohibitively impractical to monitor wild fish throughout the coast of Norway and they give a full spatial coverage. However, they are also being used to predict the distribution of planktonic salmon lice without having any direct field observations on the distribution of those stages.

1.4.5 The Needle in the Haystack Problem

Enumerating the planktonic stages of salmon lice in a mixed zooplankton sample is a needle in the haystack problem because they are relatively rare compared to the other co-occurring species (see section 1.3.1). The traditional method of taxonomic identification through light microscopy is too labor-intensive for most endeavors, which has motivated some research into alternative methods. Bui et al. (2021) investigated several alternative approaches to enumeration, including molecular techniques, but found limited success in overall throughput, cost, and accuracy. One of the methods Bui (2021) investigated was the use of fluorescence illumination as a modification of taxonomic identification, which followed preliminary work by Fordyce (2017). The intention of the method is to fluoresce the target salmon lice without fluorescing non-target animals, which makes it easier to find and identify the lice. The method was not successful, but the wavelengths were also chosen haphazardly and the salmon louse may still have a unique fluorescence signal.

Fluorescence occurs when a molecule is exposed to a light source that raises its energy level (excitation) and as the energy level of the molecule lowers back to its ground state light is emitted (emission). The excitation wavelengths of the light are always shorter and higher energy than those of the emission wavelengths. Fluorescing molecules, called fluorophores, have an intrinsic pattern of excitation and emission

spectra defined by their wavelengths and the amount of energy emitted and absorbed. Those characteristics of fluorescence make it a predictable phenomenon that can be used for identification and quantification of fluorophores through spectroscopy (Lakowicz, 2006). When it is not possible to purify a compound, the relative contribution of its constituent fluorophores can be measured through an excitation and emission matrix, in which the fluorescence intensity of each combination of wavelengths is recorded (Coble, 1996). In a mixed zooplankton sample that technique would not enable identification of the lice because their low abundance would not produce a signal. However, by exploring the excitation and emission matrices of target salmon lice and non-target zooplankters it is possible to find the wavelengths where their fluorescence intensities differ. Identifying that unique fluorescence signal is one of the objectives of this thesis, as is the development of a novel method for rapid enumeration using fluorescence. Overcoming the needle in the haystack problem through novel methods would enable direct monitoring of salmon louse planktonic distribution and abundance. The resulting data could be used in the TLS assessment and for the improvement of the existing models through validation of the infection pressure maps.

1.4.6 An Expertly Judged Assesment

The TLS model results for each production zone are evaluated by the expert group along with the monitoring data in order to make an assessment of louse induced mortality of wild salmon. Considered in the expert group's assessment is the past status of the production zone and the uncertainties contained within the different information sources (Vollset et al., 2019). Ultimately, the Ministry of Trade and Industry, as the regulatory authority, decides which color each production zone will be and whether production capacity should be adjusted (Anon, 2020). The evaluation committee (2021) has noted that the final steps in the assessment are highly reliant on the judgement of the expert committee, but the TLS assessment understates the role of judgement while promoting the role of scientific evidence. When the science is presented, in the form of model products, the TLS also 'black-boxes' the results by making the complexities less visible while promoting its apparent objectivity (Osmundsen et al., 2020). If stakeholders begin to view these aspects of the TLS as

lacking scientific credibility, it may hamper the governance system's ability to effectively manage salmon aquaculture (see Cash et al., 2002).

Under the TLS, the health of wild populations and the future growth of the salmonid aquaculture industry is reliant on accurate parameterization of salmon lice life histories. Such information is vital for creating reliable estimates of louse production from farms, modeling infection intensity on wild fish, and for understanding how best to treat and prevent lice epidemics (Brooker et al., 2018). Furthermore, the legitimacy of the TLS is currently being scrutinized by stakeholders partly because of the scientific uncertainties within it. Specifically, stakeholders critical of the system have pointed towards the wild salmon mortality calculation and the use of models to estimate infection pressure rather than direct observation (Evaluation Committee, 2021; Sønvisen & Vik, 2021). Stakeholder acceptance and perceived legitimacy of an institution is critical for the successful administration of a regulatory regime, and despite the vested interests in the TLS by industry and authorities it remains controversial (Hersoug, 2022). Thus, there is an immediate need to improve our understanding of those aspects of salmon louse life history that remain understudied.

2. AIMS

This thesis seeks to improve the state of knowledge on the planktonic stages of the salmon louse *Lepeophtheirus salmonis.* Better parameterization of the salmon louse life history and enumeration of the planktonic stages will increase the biological understanding of copepod parasites, improve salmon louse reproduction and dispersal models, and inform management decisions. Specifically, the first aim of this work is to support the traffic light system by reducing uncertainties in the salmon louse larval reproduction models. The salmon louse reproduction model is the starting point for the estimation of their distribution and abundance, but fecundity of the adult females remains a source of uncertainty. Further uncertainty in the TLS stems from the inability to validate the salmon louse reproduction and distribution models with *in-situ* measurements of their planktonic abundance. The planktonic stages of salmon lice are not typically enumerated in the water column because of the needle in the haystack problem. Overcoming that challenge and developing a practical means of enumerating planktonic salmon lice stages is the second aim of this thesis which will also support the TLS by providing input data to distribution models.

Specific Objectives of Thesis:

1) Examine salmon louse fecundity using field samples collected from farmed and wild fish throughout Norway over the course of 2 years.
2) Investigate the fluorescence profiles of sea lice and non-target copepod species to find a unique fluorescence signal with the greatest potential for rapidly identifying salmon lice.
3) Evaluate the performance of a novel method for the fluorescence aided identification and enumeration of salmon lice in mixed zooplankton samples.

3. SUMMARY OF WORK

3.1 Investigation of Salmon Louse Fecundity

We investigated salmon louse fecundity through an examination of female lice collected from farmed salmon, wild salmon, and sea trout from multiple farms and fields sites throughout Norway. Fecundity in salmon lice is a product of the rate of egg string production, the proportion of eggs that are viable, and the number of eggs per string. Here we focused on examining the number of eggs per string (the clutch size), and the morphology of the female louse. This work is described in detail in Paper I, the following is a brief overview of the methods and findings.

3.1.1 Comments on Methods

Samples were stored in saline ethanol and two sets of images were taken of all animals for fecundity and size measurements: a macro image of the lice and a micro image of the eggs. Image analysis was conducted using ImageJ (Rasband, 2011) to take the following measurements: total animal length, genital segment length, cephalothorax width, and length of egg strings. Following Eichner and others (2008), the ratio of cepthalothorax to genital segment length was calculated and used as an indicator of sexual maturation. Sexual maturity status was also determined by the current presence of egg strings or evidence of their previous presence.

Using statistical models the response variables clutch size, female louse size, and egg bearing status were examined as they related to multiple covariates: environmental conditions, sampling location and date, the fish host origin, and maternal characteristics. Covariates chosen for inclusion in the statistical models had been shown in previous studies to have a relationship to salmon louse fecundity. Since the sampling design was hierarchical with observations nested within a sample set from a single fish that is nested within a sampling event, a mixed effects model approach was utilized using the ‘glmmTMB’ function (Brooks et al., 2017; R Core Team, 2020). Due to the fragmented nature of the sampling design the dataset was not continuous over all host species, latitudes, and seasons. Thus, to avoid statistical

problems the dataset was partitioned accordingly for inclusion into the various statistical models. For instance, the analysis of clutch size was limited to the observations collected from farms because farm data was collected year-round while the field data was only collected during a few weeks in summer.

The Akaike information criterion (AIC) was used for selecting the best performing model for each of the various data sets. This approach produces the model which best predicts the response variable by including covariates which reduce the unexplained variance. As a result, these multiple regression models can include covariates whose relationship to the response variable is not well understood, that may be collinear with undescribed variables not included in the model, and that are not statistically significant according to their p-values. Complicating matters, there was a large amount of variability in the data which is unsurprising for a set of observations from samples collected in the field. Thus, interpretation of the model results can be challenging, especially when searching for causal relationships.

3.1.2 Results

A review of the literature uncovered that observations of clutch size had been previously related to many of the factors identified in this investigation, but a comprehensive analysis had not previously been attempted. Those earlier studies had also correctly identified temperature as the driving force of fecundity in salmon lice but had neglected to account for life histories. The statistical models of the farm sourced dataset identified body size of the female louse as the predominate determinant of clutch size (**Paper I**). In copepod life histories, body size results from the rearing temperature experienced by the animal with colder temperatures leading to larger adults. Further analysis revealed that the total length of female salmon lice was partially explained by temperature at time of sampling but rearing temperature is unknown since salmon lice may live for many months. In the case of farm observations from northern latitudes, temperatures were lower and had a reduced range of values, and it was not possible to predict total female length from sampling temperature. Similarly, the size of females collected on wild fish was not modeled because sampling temperature was not reflective of the rearing temperature over the

limited temporal scope of observations. As had been reported elsewhere, the female lice collected from wild salmon were far larger than those collected from farmed fish. Fitting the clutch size model to the wild fish dataset resulted in predictions that matched the observations from wild salmon, but not sea trout. The clutch size of females on sea trout was lower than expected, but we note that sea trout were typically captured in lower salinity water which may impact salmon louse fecundity. Furthermore, based on an analysis of sexual maturity as indicated by the development of the genital segment, female lice on sea trout had lower rates of expected egg-bearing than those on farmed salmon. The adult female lice on farmed salmon were found to be sexually mature 66% of the time, and 10% of those mature females were not egg-bearing.

3.1.3 Future Perspectives

The management of salmon aquaculture is reliant on the modeling of salmon louse (*Lepeophtheirus salmonis salmonis*) reproduction and dispersion, but aspects of the copepod's fecundity remain understudied. The investigation here revealed that female adult size is a key determinate of clutch size, but obtaining that measurement requires cumbersome manual examination. However, innovations in hyperspectral imaging now enable the identification of parasitic louse stages on fish (Pettersen et al., 2019), and that technology could be adapted to measure the size of lice on fish and used to calculate clutch size. Otherwise the size of the female louse and resulting clutch size could be modeled for a better parameterization of the TLS reproduction model. Here it was also shown that female lice on sea trout produce less eggs than those on farmed salmon and we hypothesize that low salinity is the underlying cause. Further work through a controlled experiment is needed to investigate whether low salinity does have a negative impact on fecundity and to what degree.

3.2 Developing the Fluorescence Enumeration Method

Overcoming the needle in the haystack problem in order to identify and enumerate planktonic salmon lice larvae requires the development of novel methods. While fluorescence aided taxonomic identification had shown potential, the set of

fluorescence filters used in previous efforts were chosen haphazardly and it did not perform better than traditional methods (Fordyce, 2017; Bui et al., 2021). Here we systematically developed the fluorescence method by identifying a unique signal and then testing its application. This work is described in detail in Papers II and III, the following is a brief overview of the methods and findings.

3.2.1 Comments on Methods

First, we investigated the fluorescence profiles of salmon lice and non-target copepod species with excitation emission matrices. Fluorescence intensity was measured with a Shimadzu RF-6000 Spectrofluorometer which sequentially changes the filter wavelengths along both spectra and measures emitted light intensity, producing a matrix of fluorescence data over the wavelengths 200 - 600 nm. We then identified fluorescence peaks in the salmon lice profiles where fluorescence intensity was particularly high and determined whether these peaks differed in intensity from non-target copepods. The fluorescence profiles of live animals and those fixed in formalin and ethanol were examined but only the formalin preserved samples and their respective fluorescence peaks were selected for further investigation.

Next, the fluorescence peaks with potential to be used as a unique signal were investigated with fluorescence microscopy using various excitation and emission filter sets. The earlier measurements with the spectrofluorometer were useful for identifying where sufficiently large and consistent differences in fluorescence intensity existed between profiles, but those measurements were normalized to the maximum intensity observed. The fluorescence microscopy enabled images to be taken of individual animals under controlled conditions and any difference in fluorescence can be attribute to the animal and its characteristics rather than to variation in the measurement. The images were processed to quantify the total fluorescence, the number of pixels fluorescing, and their mean fluorescence or intensity. Differences in fluorescence intensity were analyzed with respect to variation in sea lice species, age, stage, host fish origin, heat treatment, and length of storage. Thus, we were able to describe the factors which influence the fluorescence

signal and determine which conditions would be optimal for distinguishing between salmon lice and non-target animals.

Finally, a methodology for rapid salmon louse identification and enumeration was developed around the fluorescence signal identified and the optimal conditions described. We tested the performance of the method through a blind trial in which participants attempted to rapidly enumerate all the salmon lice copepodids contained within mixed zooplankton samples. The samples were collected in the spring and autumn from locations in Norway, Scotland and the Faroe Islands, and then spiked with a known number of lice. The performance in terms of accuracy and time was then analyzed as it related to the participant, sample treatment, and characteristics of the sample such as background fluorescence.

3.2.2 Results

In developing the novel methodology much of the initial work was dedicated to identifying the fluorescence peaks and conditions which could substantially and consistently differentiate planktonic salmon lice from non-target copepods **(Paper II**). Not all of those findings are relevant to the application of the methodology, but were nonetheless important for its development. For instance, the fluorescence signal will only be produced after prolonged preservation in formalin. The signal in live animals was deemed too weak for application and it was discovered that the fluorophore was soluble in the ethanol resulting in it leaking out of the animals and into the solution. Ultimately, using fluorescence microscopy and a targeted filter set (excitation/emission wavelengths of 470/525 nm), we found that the salmon louse has a fluorescence intensity 2.4 times greater than non-target animals after 90 days in formalin storage. No difference in fluorescence intensity was found between lice sourced from a laboratory strain, a Norwegian farm, and a Faroese farm. However, salmon lice sourced from wild sea trout did have a lower intensity while still having a greater intensity than non-target copepods. Similarly, the *Caligus elongatus* copepodids fluoresced with greater intensity than the non-target copepods but less than the salmon lice (*L. salmonis*). The intensity of the fluorescence signal also decreased with the age of the animal with nauplii having the greatest intensity,

followed by 6, and then 12 day-post-hatch copepodids. The pattern of decreasing intensity with age suggests that the responsible fluorophore is linked to the lipid stores which are metabolized by the non-feeding planktonic lice. In an effort to decrease the required storage time in formalin a heat treatment was applied to increase to rate of the reaction producing the fluorophore. After 7 days at 42° C the fluorescence intensity of salmon louse copepodids was 3.6 times greater than non-target copepodids. Overall, the formalin induced fluorescence signal in salmon lice was shown to be robust to multiple conditions and a good candidate for method development.

In the next step of the method development the fluorescence signal was used as an aid to traditional zooplankton taxonomic identification (**Paper III**). A Nikon SMZ18 stereomicroscope was equipped with a fluorescence attachment and a standard set of filters that would produce the fluorescence signal. Rather than identify every animal within the sample the participants sorted through and found intensely fluorescing particles similar in size and shape to planktonic salmon lice. Identification of the louse could then be confirmed upon closer inspection. Through this approach participants processed entire zooplankton samples in a mean of 31 minutes and enumerated the lice with a mean accuracy of 81.8%. Compared to previously published findings (see Bui et al., 2021), the novel method is more than 20 times faster than traditional light microscopy approaches.

3.2.3 Future Perspectives

The fluorescence aided method enables workers to overcome the needle in the haystack problem and rapidly enumerate planktonic salmon lice in mixed zooplankton samples. As described in Paper III, the method is a useful tool for processing zooplankton samples that are collected with a standard net tow or plankton pump, and can be used in the investigation of salmon louse biology and distribution. Nevertheless, the method is challenged by the need for large sampling volumes to capture the low-density salmon lice and to overcome any patchiness which might distort the findings (see Wiebe & Wiebe, 1968; Downing et al., 1987; Postel et al., 2000). It is recognized that both physical and biological mechanisms drive the

observed patchiness which differs according to scale (see Pinel-Alloul, 1995), but for salmon lice our understanding of those mechanisms and the observed patchiness is limited (section 1.4.5). While a typical sample may not contain any salmon lice, one vertical net tow collected by Nelson el al. (2018) contained 255 copepodids in a sample volume of approximately 2 m^3, which suggests that salmon louse patchiness can be very high.

A more targeted sampling protocol and additional pre-processing of the tow could be implemented to avoid non-target animals such as *Calanus spp.,* improving accuracy and reducing enumeration time. There are numerous more ways the method could be improved in its current form, but for it to be useful for high throughput surveillance the sampling and enumeration would need to be automated. We suggest that such an instrument can be developed for automated deployment, but it would require addition research and development. The instrument would need to collect a sample, fix it in formalin, heat and store it for some unknown minimum time, take fluorescence images of the particles in the sample, and classify them as salmon lice or not. Among those steps, the imaging and classification are the most readily achievable through the application of machine learning which has advanced considerably over the past few decades (Irisson et al., 2021). Prioritizing those steps would facilitate the processing of manually collected samples and produce greater knowledge on the patchiness of planktonic salmon lice. Then the engineering challenge of collecting and fixing a representative sample could be addressed. Regardless of these proposed advances, the fluorescence method in its current form provides a quantum leap over current practices and will enable workers to broaden the scope of research into the planktonic stages of salmon lice.

4. SYNTHESIS AND GENERAL DISCUSSION

The traffic light system, the salmon louse, and our scientific understanding of its effect on wild salmon sits at the center of the governance regime through which salmon aquaculture industry is managed. This is the boundary between science and policy, and the decisions that result from it are accepted because they are salient, scientifically credible, and viewed as legitimate by stakeholders (see Cash et al., 2002; Cash et al., 2003). Throughout the history of salmon aquaculture development in Norway, this boundary has shifted to focus on other concerns such as escaped salmon and pollution. And at the start of modern salmon aquaculture, environmental issues were secondary to equity and market concerns. Eventually salmon lice became the most salient environmental concern and was adopted as the object through which sustainability would be managed. Nevertheless, as we've seen through this history the governance structure is constantly in flux, and the current traffic light system may not last (see Hersoug, 2022). Here, we will examine some of the various threats the current traffic light system faces and how the results of this thesis may mitigate them.

4.1 Improving the Traffic Light System

Stakeholder critics of the traffic light system frequently cite the outsized role models have in the assessment of infestation pressure. While the lice counts and limits on fish at farms are viewed as a relatively objective measure, the models and resulting mortality assessments are viewed as encompassing too much uncertainty (Olaussen, 2018; Osmundsen et al., 2020; Sønvisen & Vik, 2021). Although, the manual counts have their own uncertainties (Thorvaldsen et al., 2019) and the models are necessary to assess a vast territory that cannot be monitored directly (Vollset et al., 2019), the criticisms can still be addressed. Findings from the fecundity study (Paper I) can be used to better parameterize the models while the novel method described in **Paper III**, makes it possible to overcome the needle in the haystack problem to rapidly enumerate salmon lice in mixed zooplankton samples. As described in the paper, standard zooplankton samples can be taken and analyzed through a simple modification of traditional techniques or with further work the

method could be automated to provide *in-situ* monitoring. The method could be employed in the same manner as NALO with certain fjord systems targeted for regular sampling, and specific areas of high and low infestation targeted following the lice distribution model. Although the dataset may be redundant with some of the NALO data already collected and it would similarly not be deployable throughout the Norwegian coast, it would provide a credible direct measure of lice abundance. Currently, the TLS relies on indirect measures of infestation pressure to calibrate its models, including lice counts on wild caught fish and the use of sentinel cages (section 1.4.2). The wild fish data is primarily collected from sea trout rather than Atlantic salmon and the capture location serves as a proxy for the unknowable location of infection. Sentinel cages manage to control for both the host species and location issue but may not reflect a natural infection intensity and have the consequence of poor welfare outcomes for the fish (Karlsen et al., 2021). A direct measure of planktonic salmon louse abundance avoids the issues of using those indirect ones. That data could be used to further tune the models, and it would be further informative to see where the different datasets diverge, e.g. there may be areas of high planktonic lice abundance and lesser infection intensity on wild fish. Discovering the mechanisms behind such differences would then be valuable from a management perspective and for the possible treatment and prevention of lice.

The new tool (**Paper III**) could be used to investigate many more aspects of salmon lice behavior and biology that could then be incorporated into the lice model to improve the performance of the model products. The swimming behavior and vertical distribution of salmon lice is particularly relevant to know for parameterizing distribution models which primarily rely on experimental observations (e.g. Myksvoll et al., 2018). Depth stratified sampling in the field would provide knowledge on the abundance distribution through the water column that could then be compared to the laboratory results. Estimating mortality (in the planktonic stages) is an even more vexing problem since results from laboratory experiments are not available. It is also a difficult parameter to measure through field samples but there are a few methods available such as the vertical life table method (Aksnes & Ohman, 1996; Thompson,

2012). It involves the repeated sampling of a copepod cohort with defined stages, based on knowledge of the development rates the difference between the expected stage structure and the observed is attributed to mortality. Certainly, there are more questions that can be asked and investigated so long as the salmon louse can be practically found and enumerated in zooplankton samples. All that information would then contribute to an improved data input to the TLS.

The results in **Paper I** exemplifies how the TLS assesments can be improved by addressing some of the uncertainties contained within them. The production model of salmon lice emanating from farms is based on a simplified formulation of fecundity. The results of **Paper I** show that the size of the female louse rather than temperature is the most important determinant of the clutch size. Although, the reproduction model could be improved by incorporating that finding into a model of louse size, it would be better to measure the size of lice directly. As described in the paper it might be possible to do with further development of imaging technology. Nevertheless, it raises the question: with respect to estimating salmon louse induced wild salmon mortality, how much more need or can the TLS be improved? Considering the role of judgement in the TLS assessment, the TLS evaluation committee also observed that the benefit of improving the model products might be limited (Evaluation Committee, 2021).

4.2 The Treatment Spiral

The traffic light system determines the permitted production capacity in each zone based on the action rule and level of salmon lice induced mortality on wild salmon. The system has thus been criticized by stakeholders as a form of collective punishment because actors with high lice loads on their farms are sanctioned in the same manner as all other actors in a zone (Osmundsen et al., 2020). Reducing the production of lice from farms is a collective action problem that the TLS is ill-suited to overcome. It is up to the NFSA to set the lice limit regulations and enforce them. However, the lice limits are not specifically designed to incentivize farmers to prevent lice infestations and the release of planktonic salmon lice. Each farmer has an

incentive to maximize profits and while preventative measures may greatly reduce the risk of salmon louse infection, it will always have a cost (economic and decreased fish welfare). Alternatively, it may be possible to proceed through an entire grow out cycle with few or no treatments, which costs less than the preventative measures. Unsurprisingly, treatment of lice has been the focus of decades of research, development, and application while prevention of infection has been neglected (Barrett et al., 2020).

Through most of its history, the salmon aquaculture industry had relied on chemotherapeutics treatments to control lice, but in recent years non-medical treatments have come into greater use. Starting in the 1980s, organophosphates were the only available pesticide, and then in the 1990s a number of other treatments were developed including hydrogen peroxide, chitin inhibitors, pyrethroids, and emamectin benzoate (Myhre Jensen et al., 2020). While the latter is administered as a feed additive the rest are done through bath treatments, which involves the reduction of the water volume through a tarpaulin or transfer well boat. The pesticide is added to the bath treatment and following a set time the fish are released back into their pen which also releases the therapeutic into the environment (Overton et al., 2019). After being released, these chemicals persist in the water column, disperse to the sediments, and have negative impacts on non-target species (Urbina et al., 2019). Salmon lice began to develop resistance to the limited number of chemotherapeutics, one after another until the efficacy of all those drugs was depleted. This drug resistance started to emerge as a problem by 2008, and instigated a rapid innovation of non-medical delousing technologies that overtook the market by 2015. These preferred de-lousing treatments included the bath treatment of fish with hydrogen-peroxide, the addition of cleaner fish in pens, mechanical removal, thermal de-lousing, and freshwater baths (Overton et al., 2019; Myhre Jensen et al., 2020). Hydrogen peroxide has been a known treatment option since the 1993, but only recently saw an increase in use since its efficacy was still quite good. Nevertheless, there is evidence that resistance has been detected in salmon lice (Myhre Jensen et al., 2020).

Three different mechanical de-lousing technologies have been developed, all of which require fish to be crowded and then pumped into the device. The systems differ in design, using high pressure water, turbulence, and brushes to remove the lice. Although limited information is available, reports indicate that the treatment has high efficacy but also causes scale removal, gill bleeding, wounds, and mortality. Thermal de-lousing is based on the principal that while both species are stressed, the larger sized host fish enables it to withstand high temperatures for longer than the parasite. The salmon undergoing the treatment are first crowded, pumped into a water strainer, and then placed in a 28-34°C sea water bath for 20-30 seconds causing the mobile lice to fall off. The thermal treatment has good efficacy but negatively impacts fish welfare and increases mortality. Freshwater treatments have also been developed and are designed to work similarly to the thermal treatments, but their efficacy and welfare impact has not been well documented. While the legacy treatment options resulted in an increase in post treatment mortality of less than 14% these increasingly used non-medicinal options cause up to 31% post treatment mortality (Overton et al., 2019). Although these novel treatments have little environmental impact, the increased mortality is greatly concerning, as are the understudied welfare impacts. Another treatment option, the use of cleaner fish, has minimal welfare complications for the salmon, but presents a unique ethical dilemma for the numerous impacts it has on the 5 different species of cleaner fish (Overton et al., 2020)

The history of salmon aquaculture governance indicates that large regulatory changes can happen in the face of crises of all sorts, and here the industry may face another such imposition. Past welfare challenges were met with the enactment of density regulations, lice limits, and the elimination of feed quotas and shift to MAB. In the current situation, NFSA is the department with immediate responsibility for deciding if more rules and standards are necessary. Regulations could be enacted to mitigate the welfare and mortality issues by enforcing best practices, but that wouldn’t necessarily disincentives the practice and the problem would persist but to a lesser extent. However, if the welfare issue gains in salience the salmon aquaculture industry may face even greater scrutiny and NFSA could be forced to make drastic

changes. Although the TLS is seemingly distant from the welfare issue, without effective treatment tools available the risk of salmon louse induced mortality could balloon, and the TLS may lack the legitimacy to enforce such a large-scale reduction in MAB. Whether the previous scenario or any similar pressure disrupts the current paradigm, the welfare impact of the current treatments calls for a different strategy in combating the lice challenge.

4.3 Prevention Priority

Infection prevention rather than treatment is the pathway that will enable the salmon aquaculture industry to reach the 2050 goal of 5 million tonnes, and it is here that the work in **Paper III** would prove useful. Several prevention technologies already exist and are in use, primarily in the form of physical barriers that separate the salmon from infectious copepodids. These 'skirts' and 'snorkels' require that a fine mesh material be placed around the fish pen, with the former being placed in a simple circular arrangement and the latter having a smaller opening at the surface and greater volume at depth. Although these devices are water permeable, the overall water exchange is reduced which can cause a reduction in local oxygen concentration among other problems. Thus, they are not deployed much below 10 m, which enables the farmed salmon to go to depth for better water conditions (Stien et al., 2018). Salmon lice are typically distributed in the upper water column and having the barriers primarily in the upper water column prevents lice from entering the pen. However, there is a concern that the lice may adapt to occupy lower depths (Coates et al., 2021). Therefore, it would be beneficial to study the *in-situ* vertical distribution of salmon lice using the method described in **Paper III.** An automated device based on the fluorescence method would also enable active monitoring that informs farmers when best to deploy barriers. That responsive approach, would reduce labor and maintenance costs, and limit the potential harmful effects on salmon. However, much more methodological development would be needed before such an instrument could be deployed.

Having a better understanding of the existing distribution, and the mechanisms which determine that distribution would aid efforts to prevent infection through spatial management (see Samsing et al., 2019; Barrett et al., 2020). As described with the TLS above (section 4.1), the improved parameterization of the louse model combined with validation of model forecasts would improve dispersion models. The suggestion here is that rather than simply use the information for an assessment of induced salmon louse mortality, it be used to lessen the likelihood of infections at farms through better sitting. Spatial management is already practiced for the designation of marine protected areas in the National Salmon Fjords (see section 1.3.3), for the issuing of locality licenses (see section 1.3.4), and through the regulation of minimum distances between sites for sanitation purposes (see section 1.3.3). The model of infection pressure should also be used as a guide for determining whether or not a site is appropriate for a farm. Otherwise a plan should be put in place by the farmer which involves some infection prevention measures. These requirements can easily be added to the current locality licensing regime. The current models may not be accurate at the scale necessary for that level of spatial planning (see Myksvoll et al., 2018; Huserbråten et al., 2020), but they could be improved and their predictions could be verified by sampling of the zooplankton.

More rigorous spatial planning would reduce the likelihood of salmon lice infections at farms, but the space available to do so is limited. Overcoming this bottle neck and achieving the desired production of 5 million tonnes will require further innovation (Hersoug, Mikkelsen, et al., 2021). Beyond the barrier technologies there are fully closed pens, which are situated like a normal pen but pump lice-free water from depth (Nilsen et al., 2017). More capital intensive innovations include the placement of offshore sea pens (Hvas et al., 2021), and land based RAS (Bjørndal & Tusvik, 2019). The amount of time a salmon is in the open ocean exposed to infection risk can also be reduced through the on-growing of post-smolts from the typical 150g to 1000 g (Bjørndal & Tusvik, 2020). These innovations are still in the early stages of development and will need to be scrutinized further. Regardless, of their infection prevention efficacy and welfare benefits, neither the existing barrier technologies or

the capital-intensive systems will be widely adopted unless their use is properly incentivized.

The directorate of fisheries has maintained a pathway around the TLS called special purpose licenses through which they can incentivize different activities, including the research and development of new technologies. However, Hersoug and others (2021; 2022) contend that they are regularly exploited to increase permitted production without having to go through the regular process, which undermines the TLS and does not properly incentivize innovation. For instance, once the project goals of a development license have been achieved it can be converted to a standard license one for less than a 10th of the regular cost, and then the license holder can go back to using typical open net pens. Ultimately development licenses were only available for a short application window (2015-2017) because they proved to be immensely complicated and burdensome to administer (Hersoug, Olsen, et al., 2021). Also outside the TLS and implicitly promoted by authorities are offshore facilities and land based RAS projects. The capital-intensive projects may be important for future growth of salmon aquaculture, but the industry is primarily reliant on the open net pen which is the most cost effective means of production. As seen from this brief overview, incentivizing farmers to adopt more environmentally sustainable practices through production licensees is challenging and fraught with administrative and political discretion (see Hersoug, 2022). It may be necessary to reduce the risk profile for large capital projects by eliminating the license requirement, but such a scheme is ill-suited for the common farm with open net pens.

4.4 Conclusion

The aim of the thesis was to improve knowledge on the planktonic stages of salmon lice, doing so would support salmon aquaculture management and the traffic light system. The aim was accomplished directly with the improved understanding of fecundity and indirectly through the creation of a new tool for the measurement of salmon louse abundance in mixed zooplankton samples. However, the description of the governance structure shows how the scientific benefit to the current traffic light

system is limited, a conclusion shared by the independent evaluation committee (Evaluation Committee, 2021). Nevertheless, the direct measurement of salmon louse abundance in the water column would support the longevity of the traffic light system through its added legitimacy. Following the historical pattern, the traffic light system itself may be upended or changed significantly as stakeholders' central concern shifts and the legitimacy of the current system is challenged.

The growing welfare concern following de-lousing treatments is a likely catalyst for the next crisis. Heading off that challenge calls for a shift to a prevention first strategy in which knowledge of planktonic stages (and the work of this thesis) will play a greater role. It is also vitally important that the prevention strategy be properly incentivized. The conditional issuing of production licenses is an ill-suited motivator because of administrative burden and subjectivity. Rather, the locality licensing system along with the NFSA should require a prevention first strategy that entails spatial management and an understanding of salmon louse planktonic distribution.

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RESEARCH ARTICLE

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Illuminating the planktonic stages of salmon lice: A unique fluorescence signal for rapid identification of a rare copepod in zooplankton assemblages

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Abstract

Monitoring of planktonic salmon louse (*Lepeophtheirus salmonis salmonis*) abundance and parameterization of key life-history traits has been hindered by labour-intensive and error-prone quantification using traditional light microscopy. Fluorescence illumination has been proposed as a means of improving visualization, but prior to this study adequate investigation of the relevant fluorescence profiles and measurement conditions has not been undertaken. We investigated the fluorescence profiles of *L. salmonis* and non-target copepod spp. with excitation and emission matrices (200–600 nm) and identified unique fluorescence signals. Fluorescence microscopy using excitation wavelengths of 470 ± 40 nm, and emission wavelengths of 525 ± 50 nm, showed that after 90 days of formalin storage salmon lice have a mean fluorescence intensity that is 2.4 times greater than non-target copepods (copepodid and adult stages). A 7-day heat treatment of 42°C in formalin increased the difference between salmon louse copepodids and non-target copepods to a factor of 3.6, eliminating the need for prolonged storage. Differences in the fluorescence signal and endogenous fluorophores were investigated with respect to variation in sea lice species, age, stage and host fish origin. Under the conditions outlined in this paper, the fluorescence signal was found to be a reliable means of visualizing and differentiating salmon lice from non-target zooplankters. Adaptation of the fluorescence signal would greatly expedite traditional methods of enumerating salmon louse larvae in plankton samples and could provide a means of automated detection.

KEYWORDS

aquaculture, Atlantic salmon, caligidae, excitation and emission matrix, *Lepeophtheirus salmonis*

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1 | INTRODUCTION

The salmon louse, *Lepeophtheirus salmonis salmonis* (Krøyer 1837; Skern-Mauritzen et al., 2014), is an obligate ectoparasite of salmonids and a major constraint to Atlantic salmon (*Salmo salar*) aquaculture. Salmon aquaculture has expanded rapidly from a few thousand tonnes of fish produced in 1980 to the 2.4 million tonnes produced in 2018 (FAO, 2020). Norway is currently the largest producer of salmon at 1.28 million tonnes produced in 2018 (FAO, 2020), but due to environmental challenges, principally infestation by *L. salmonis*, growth of the industry has stagnated since 2012 while costs continue to rise (Bjørndal & Tusvik, 2019). Estimates of the economic impact of *L. salmonis* range from 6.2% to 8.7% of productive value (Abolofia et al., 2017; Costello, 2009a), suggesting the losses for the global salmon farming industry to be in excess of $1.26 billion USD.

Parasitic stages of *L. salmonis* feed on the mucus, tissue and blood of their host causing sores, immunosuppression and reduced feed conversion efficiency (Thorstad et al., 2015). In Norwegian waters, wild Atlantic salmon smolt migrating from rivers towards the sea are infected by *L. salmonis* copepodids suggested to primarily derive from infested farms (Fjørtoft et al., 2019; Kristoffersen et al., 2018), and the resulting lice loads increase their risk of mortality (Taranger et al., 2014). The growth of salmon aquaculture and resulting rise in *L. salmonis* infestations have been associated with declines of some wild salmonid populations, which together with welfare concerns have prompted regulatory action (Costello, 2009b; Heuch et al., 2005; Krkosek et al., 2007; Krkošek et al., 2005, 2013; Thorstad et al., 2015; Torrissen et al., 2013; Vollset et al., 2018).

The traffic light system implemented by the Norwegian government in 2017 codifies the importance of *L. salmonis* to regulatory decisions, by linking salmon aquaculture production to the risk of infestation-induced mortality in wild salmonid populations (Vollset et al., 2018). A key component of the risk assessment is the operational salmon lice model, which calculates the infection pressure through the coupling of a hydrodynamic model with a salmon lice particle tracking model. The particle model incorporates knowledge of *L. salmonis* biology and behaviour, such as development and vertical position, while the hydrodynamic model forecasts the distribution and abundance of those larval particles originating from salmon farms (Myksvoll et al., 2018, 2020; Sandvik et al., 2020). Ostensibly the operational salmon lice model describes the density of infectious copepodids, but the model output is not validated with data on planktonic stages. Rather, model validation relies on data from observation of infection pressure on wild-caught salmonids and sentinel cages (Myksvoll et al., 2018; Sandvik et al., 2016). The output of the operational lice dispersal model compares well with observed infection pressure, provides better coverage than reliance on observations alone and continues to be improved with updated information on *L. salmonis* biology (Myksvoll et al., 2018; Sandvik et al., 2016, 2020). Nevertheless, distribution and abundance of *L. salmonis* planktonic stages remain a source of uncertainty in the model, and key aspects of their biology in the planktonic stages, such as mortality, fecundity and fine scale distribution in the field, remain underparameterized (Brooker et al., 2018; Nelson et al., 2017; Skarðhamar et al., 2019).

Lepeophtheirus salmonis hatch from eggs strings carried by females and develop through three non-feeding planktonic stages: nauplius 1 and 2 (N1 and N2), and the infective copepodid stage. After the copepodid finds and attaches to a host, it develops through 5 more stages concluding with the adult stage (Hamre et al., 2013). While the parasitic stages can be readily observed and enumerated on the host fish, the free-living planktonic stages can only be identified within a zooplankton sample. However, finding and enumerating planktonic *L. salmonis* are challenging due to their relative low abundance in comparison with other species typically collected in a sample. Previous studies suggest a mean abundance of planktonic stages ranging from 0.075 to 0.70 m^{-3} with numerous zero counts and a few outliers, which indicates a high degree of patchiness (á Norði et al., 2015; Byrne et al., 2018; Nelson et al., 2017; Nilsen, 2016; Penston et al., 2011; Salama et al., 2013; Skarðhamar et al., 2019). In comparison, the global mean density of copepods is estimated to be 1,000 m^{-3} (Box hall, 1998), and a planktonic tow from the west coast of Norway typically yields 5,000 m^{-3} or more animals (T. Falkenhaug, personal communication, 6 June 2020). Thus, one may have to sort through 1,400 to 66,000 animals before identifying a single *L. salmonis* in a plankton sample.

Since identifying and enumerating planktonic *L. salmonis* stages in a zooplankton sample are a laborious task, several methodologies have been employed to that effect, with mixed results (Bui et al., 2020). Amongst them, fluorescence microscopy has been shown to increase the visibility of *L. salmonis* copepodids in comparison with other species (Bui et al., 2020; Fordyce, 2017). However, the reliability of the fluorescence signal has not been investigated nor has the optimal method been described in detail. Ideally, a specific combination of excitation and emission filters would result in *L. salmonis* fluorescing, while non-target animals are unaffected. In fluorescence, a molecule is exposed to an incident light and photons are absorbed by the molecule raising its energy level (excitation), but rather than returning to the ground level immediately, the molecule steps down its energy state and releases photons at a lower energy level with longer wavelengths (emission). The fluorescing molecule, the fluorophore, is characterized by its excitation spectrum, its emission spectrum, and its quantum yield or the amount of energy emitted divided by the energy absorbed. Thus, fluorescence is a predictable phenomenon that can be harnessed by spectroscopy to identify and quantify fluorophores (Lakowicz, 2013).

In a mixed solution where it is not possible to purify the fluorophores, the relative contribution of various compounds can be described by an excitation and emission matrix (EEM), in which the fluorescence intensity is recorded for each pair of excitation and emission wavelengths. EEM measurements have been used to characterize the dissolved organic matter in sea water, terrestrial water and waste water; classify phytoplankton communities; and identify the origin of food products (Coble et al., 1996; SádeCká & TóThoVá, 2007; Hudson et al., 2007; Richardson et al., 2010; Andrade-Eiroa et al., 2013; Carstea et al., 2016). In the same manner, this study classifies *L. salmonis* and

non-target copepod spp. that are commonly present in the planktonic assemblage with *L. salmonis*, according to their fluorescence profiles as observed by EEM measurements. However, fluorescence spectroscopy alone would not be a solution for enumerating *L. salmonis* within a plankton sample because their relative low abundance would not produce a detectable fluorescence signal.

A sufficiently large and consistent difference in fluorescence intensity between planktonic *L. salmonis* and non-target animals may be used as a signal for rapid identification. In the present study, we used EEM measurements to explore the fluorescence profiles of the target sea lice species and non-target copepod spp., and identified the wavelengths where the greatest contrast in the fluorescence occurred. The fluorescence intensity exhibited by the animals was then quantified at those wavelengths through fluorescence microscopy and analysed for statistical differences. The reliability of those fluorescence signals was further examined by investigating factors that might influence them, including storage time in formalin, host fish origin, copepodid age and developmental stage.

2 | METHODS

2.1 | Sea lice and non-target copepod sampling

To address the question of host fish origin, *L. salmonis* were sourced from farmed Atlantic salmon (*Salmo salar*), wild Atlantic salmon and sea trout (*Salmo trutta*). Another sea louse of significance to salmon aquaculture in the Northern Hemisphere is *Caligus elongatus*. It has the same planktonic life-history stages and appears almost identical to *L. salmonis* under the microscope (Schram, 2004). Therefore, *C. elongatus* females with egg strings were collected from wild fish along with *L. salmonis*, and additional egg strings were sourced from a laboratory culture.

Salmon lice eggs were sourced primarily from three laboratory strains of *L. salmonis*: LsGulen, LsOslo and Ls1A (Hamre et al., 2009), cultured at the Institute of Marine Research (IMR) facility in Bergen, Norway. Laboratory-cultured *C. elongatus* were provided by the University of Bergen (UIB) Sea Lice Research Center. *L. salmonis* and *C. elongatus* were also collected from wild fish trapped with fyke nets in various fjords in Western Norway during the spring 2019 and 2020. The female lice with egg strings obtained from adult wild fish were placed in a container with sea water from their collection point (minimum 0.25 L per female) and transported to the Bergen facility in a cooler. Additional *L. salmonis* eggs were provided by salmon farms located in Austevoll, Norway, and the Faroe Islands.

During the collection, host fish were fully anaesthetized with tricaine methanesulphonate (Finquel: 10 g 100 L^{-1}), and female sea lice with egg strings were removed with forceps. Egg strings were detached from the female louse and placed into incubation chambers where they hatched and developed through the planktonic stages. While the farm strains were hatched and incubated at the respective local institutions of Fiskaaling Aquaculture Research Station in the Faroe Islands and Austevoll Research Station (IMR), all others were reared at the IMR Bergen facility. For all sources, the hatchery set-up followed that described by Hamre et al. (2009). In Bergen, the incubators were provided with flowing sea water with a salinity of 34.5 ppt and temperature of 9.5 ± 1°C. The water was pumped from the adjacent fjord at a depth of 120 m and passed through a sand filter and disc filter. Similarly, in Austevoll, the filtered sea water was pumped from a depth 165 m, with a salinity of 32.6 and a temperature of 8.4 ± 0.1°C. At Fiskaaling, the incubator was filled with filtered sea water having a salinity of 35.2 ppt. The sea water (~40 L) was recirculated between the incubator and a holding tank connected to a watercooler (BOYU L series water chiller), which kept the temperature at 10 ± 0.5°C.

During the hatching phase, unhatched egg strings were moved to a new incubation chamber every 24 hr, while hatched nauplii remained in the original chamber. This allowed the hatch time of lice in each chamber to be defined within a 12-hr error margin. N1 stage nauplii were sampled immediately, while N2 stage nauplii were sampled 3 days post-hatch (DPH), with young and old copepodids sampled at 6 DPH and 12 DPH, respectively. At a temperature of 9.5°C, the expected duration of naupliar stages is approximately 4 days, while the duration of the nauplius and infective copepodid stages together is 17 days (Stien et al., 2005; Samsing et al., 2016).

Egg strings were collected on 11 separate occasions from the Bergen *L. salmonis* culture between March 2019 and March 2020. Several cohorts of equivalent-aged nauplii and copepodids were sampled from each collection of egg strings and either measured immediately or fixed in either 70% saline ethanol (34 ppt) or 10% formalin buffered with 9% (w/v) sodium tetraborate. Each of the 34 *L. salmonis* cohorts from the 11 cultures was fixed and then divided into 5–7 separate glass containers to mitigate possible chamber effects.

Non-target copepod spp., for use as comparators with respect to target sea lice species, were collected with a vertical plankton net with a 0.5 m diameter frame and 140 µm mesh size. Repeated tows to a depth of 10–30 m were made from a Bergen pier on 24 June and 10 July 2019, and from a boat in Bjørnafjorden on 14 April and 19 November 2019, and 27 March 2020. The dominant non-target copepod spp. found in the tows and sorted for measurement included *Calanus finmarchicus*, *Acartia* spp., *Pseudocalanus* spp., *Temora* spp., *Oithona* spp. and *Centropages* spp. Apart from *Centropages* spp., those copepod species have been reported as occurring in high numbers in the North Sea, Norwegian Sea, Faroe Islands and Northern Norway (Falkenhaug et al., 1997; Gundersen, 1953; Nielsen & Andersen, 2002; O'Brien et al., 2013). Thus, they occur in regions where salmon farming is prevalent and are commonly found in the zooplankton assemblage along with *L. salmonis* and *C. elongatus*.

2.2 | Fluorescence fingerprinting, and excitation and emission matrix (EEM) measurements

Fluorescence intensity was measured with a Shimadzu RF-6000 Spectrofluorophotometer using the 3D analysis application. The

instrument has monochromatic filters for excitation and emission spectra. Sequentially changing the filter wavelengths along both spectra and measuring emitted light intensity produces a matrix of fluorescence data termed an excitation and emission matrix (EEM). The instrument was set to measure fluorescence intensity between 200 and 600 nm, with filter bandwidths of 10 nm for excitation and 5 nm for emission. Filter scan rate was set to 60,000 nm/min, sensitivity was set to low, and the data interval was set to 2 nm.

The RF-6000 was fitted with Shimadzu's Constant Temperature Single-Cell Holder with Stirrer and Starna's Type 18-F/MS/Q/10-Micro Cell Cuvette, which has a nominal volume of 0.9 ml. Water at an approximate temperature of 12°C flowed through the cell holder during all measurements, which prevented the sample from overheating and killing live animals. The stirrer maintained a suspension of the animals in the cuvette, where only a small proportion of the volume was in the path of the excitation light, such that a random assortment of animals was measured for each sequential step of the 3D analysis. The number of animals in the sample influenced the stability of the measurement, while too many animals would disrupt the suspension, too few animals would not provide a homogenous mixture. The number of animals needed for a stable measurement depended on the species, stage and ultimately the body size of the animals.

Lepeophtheirus salmonis samples contained a mean of 150 animals, while non-target copepod samples contained between 25 (late stage *Centropages* spp.) and 250 (*Oithona* spp.) animals. Metadata for each formalin and live sample measured can be found in the supplementary material (Table S1). All *L. salmonis* and *C. elongatus* samples comprised animals from a single stage and age. Non-target copepod spp. were sorted to genus, and samples contained a mix of copepodid and adult stages. Less than an hour prior to measurement, animals stored in formalin were removed from preservation with a sieve, transferred through two filtered salt water rinses using a pipette and then transferred to the cuvette.

Fluorescence intensity was influenced by the fluctuating number of animals in the path of the excitation beam during the 5-min measurement. We compensated for this artefact by repeating measurements of each sample five times, calculating the mean and applying a smoothing function, which found the median value within 10 nm. The fluorescence intensity was further normalized on a 0- to 1-point scale by dividing intensity by the maximum fluorescence within each EEM measurement. EEM measurements made on animals stored in ethanol were highly variable between samples of the same species. Further examination suggested that the fluorophore, which originates from *L. salmonis*, leaches into the ethanol solution, separating the fluorescence signal from the animal (Figure S1). Thus, ethanol preservation hinders the identification and enumeration of the animals and ethanol EEM measurements were therefore excluded from further analysis. EEM data were processed in MATLAB using the *drEEM* toolbox to assemble the data set, apply scale transformations, remove Rayleigh and Raman scatter, and produce figures (Murphy et al., 2013).

2.3 | Spectrum section analysis of EEM measurements

Fluorescence peaks (uniquely high fluorescence intensity at a specific conjunction of an excitation (Ex.) and emission (Em.) wavelength) were identified from the EEM data set through a systematic sectioning of the excitation wavelengths into 20-nm wide bands centred on the focus wavelength. Fluorescence intensity within each section was normalized to the maximum intensity and evaluated for the relative fluorescence intensity difference between a target group of lice samples and non-target copepod samples, which served as comparators. The lice samples were divided into 4 target groups: “Nauplii” (N1 and N2 stages combined), “Young Copepodid” (sampled 6 DPH) and “Old Copepodid” (sampled 12 DPH), which both originated from *L. salmonis* maintained on Atlantic salmon (*Salmo salar)*, and “Sea Trout Copepodid” (sampled 6 DPH), which were *L. salmonis* that originated from wild-caught *Salmo trutta*. Since the duration of formalin storage affects fluorescence intensity in target *L. salmonis* copepods [see section 3.2.2, Formalin storage and fluorescence intensity], the EEM data set was limited to those target copepod samples that had been in storage for more than 60 days. The non-target copepod samples included in the analysis were in formalin storage for 7 days or more prior to measurement, and the *C. elongatus* samples were 33 days in formalin storage when measured.

The young copepodid samples were chosen as the target group for identifying excitation section peaks (the maximum fluorescence intensity found within each excitation section, specified as a conjunction of Ex. and Em. wavelengths). At each excitation section peak (±10 nm), the mean difference in relative intensity between target group and non-target copepod comparators was calculated as the *peak intensity distance*. The peak intensity distances of each excitation section were then evaluated to manually select the fluorescence peaks where the greatest and most reliable fluorescence difference between the lice target groups and non-target copepod comparators occurred. The fluorescence peaks that appeared to best differentiate target from non-target copepods were identified and selected for further analysis.

2.4 | Fluorescence microscopy

Fluorescent images of individual animals were taken with a Nikon DS-Fi3 on an inverted Nikon Eclipse Ti Microscope using three CHROMA filter sets: DAPI, Ex. 350 ± 50 nm and Em. 460 ± 50 nm; EGFP (FITC/Cy2), Ex. 470 ± 40 nm and Em. 525 ± 50 nm; and CY3/TRITC, Ex. 545 ± 25 nm and Em. 605 ± 70 nm (illustrated in Figure 2). The Nikon software NIS-Elements controlled operations and settings for the camera and microscope. Fluorescence saturation was avoided by setting the power of the Lumencor SOLA Light Engine to 25% for images taken with the DAPI and CY3/TRITC filter sets, while it was set to 5% for the EGFP filter set, and all other settings were kept the same. Observations were made using a glass-bottomed dish with a 0.16- to 0.19-mm-thick borosilicate glass base. Animals from

formalin samples were handled in the same manner as during the EEM measurements, that is removed from preservative, rinsed and placed in filtered sea water. Live animals were placed in a solution of filtered sea water and methyl cellulose, which inhibited their movement but did not produce any fluorescence in the spectra measured.

Image processing and analysis were conducted with Java and R, respectively. Fluorescence was recorded as an RGB image value with a 0 (black) to 255 (white) greyscale serving as a proxy for intensity (Figure 1). In each channel, the pixels with a value below a threshold set to 5 were disregarded and the number of pixels above the threshold was counted, and their greyscale values were summed. Across the three channels, the number of pixels and their total value were recorded, which gave a measure of total fluorescence intensity and the area fluorescing for each animal. The fluorescence was then analysed according to storage duration, animal size, sea louse species, origin and host fish, development stage, storage temperature and animal age. Linear regression and statistical tests including ANOVA and paired *t* tests specified in the results section were carried out using packages in the R software environment (R Core Team, 2018).

3 | RESULTS

3.1 | Spectrum section analysis of EEM measurements

EEM measurements showed that the fluorescence profile of *L. salmonis* differed broadly from non-target copepod spp. examined (Figure 2). Although all samples exhibited a wide fluorescence peak near Ex. 290 nm and Em. 320 nm, *L. salmonis* EEM measurements also featured increased fluorescence at higher wavelengths that was absent in other included species. Most non-target copepod measurements, including those of *Acartia* spp. (Figure 2c,f), lacked fluorescence in those higher wavelengths, while some species, such as *Temora* spp. (Figure 2b,e), exhibited fluorescence in this area, but at lower intensities. The fluorescence profiles of the live samples were less dynamic and of lower intensity than those stored in formalin, and the fluorescence profile of *L. salmonis* further varied in relation to stage, age of the animal, host fish and duration of formalin preservation. Those patterns of fluorescence were examined through spectrum section analysis of EEM measurements and fluorescence microscopy [section 3.2]. The spectrum section analysis data set included EEM measurements of live samples (Figures 3a and 4), target *L. salmonis* samples that had been in formalin storage for 60 days or longer, and non-target copepod samples that had been in formalin storage for more than 7 days (Figures 3b and 5). Spectrum section analysis found that peak intensity distance was greatest in excitation sections higher than 300 nm in both live and formalin samples. In excitation sections below 290 nm peak intensity, distance between target groups and the non-target copepod comparators was inconsistent and occasionally turned negative with the lice target group having the lower intensity.

3.1.1 | Spectrum section analysis of live samples

Through the spectrum section analysis, Ex. 330 nm and Em. 418 nm were identified as the fluorescence peak best suited for distinguishing live *L. salmonis* from non-target copepod comparators (Figure 3a). The peak intensity distance of the young copepodid target group remained high between Ex. 310 and 420 nm, but fluorescence

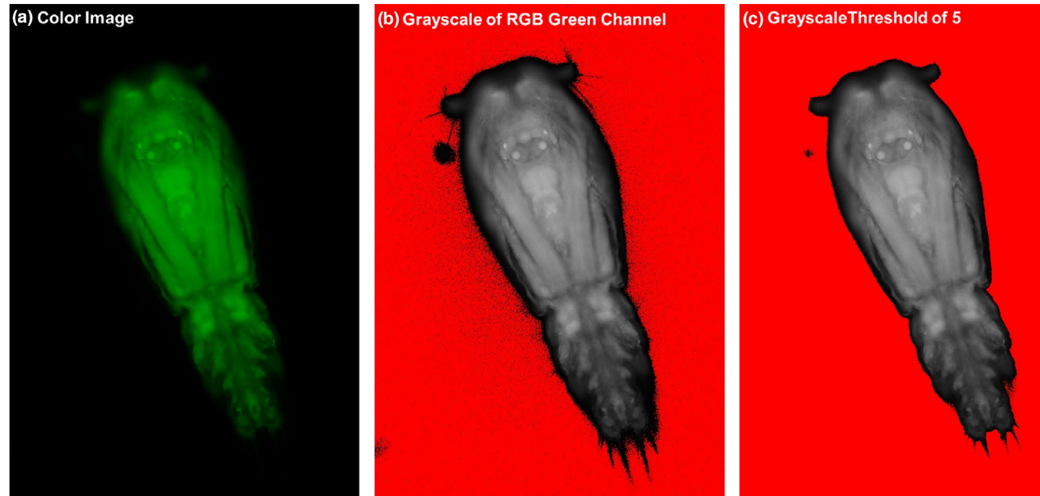

FIGURE 1 Fluorescent image processing of *Lepeophtheirus salmonis* copepodid. (a) Colour image (cropped) taken with the GFP filter set. (b) The green channel of the colour image in greyscale with red colour having an RGB value of 0, black and dark grey have low RGB values, and whites have a high value. (c) Thresholding removes all pixels below a value of 5

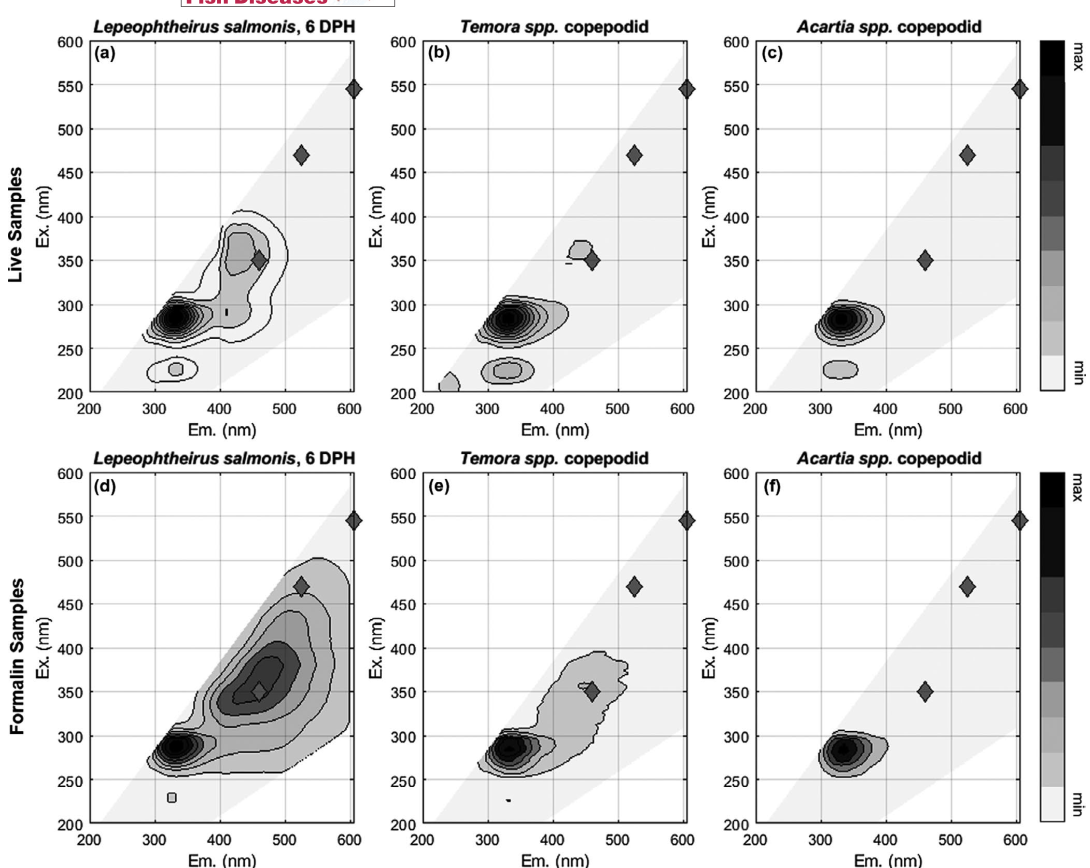

FIGURE 2 EEM measurements of live and formalin-preserved copepods. The displayed EEM measurements are means of measurements taken for the listed species (a:f). *L. salmonis* copepodid samples were in formalin storage for 60 days prior to measurement. Emission and excitation wavelengths (nm) are indicated on the *x*- and *y*-axes, respectively. Normalized fluorescence intensity is depicted through the greyscale contouring with the darkest shade representing maximum fluorescence in the EEM for each species. The diamonds mark the centre wavelength of the filter sets: Ex. 350 ± 50 nm and Em. 460 ± 50 nm; Ex. 470 ± 40 nm and Em. 525 ± 50 nm; and Ex. 545 ± 25 nm and Em. 605 ± 70 nm [see section 3.2, Fluorescence Microscopy]

intensity decreased rapidly after Ex. 380 nm and the peak distance of the nauplii group became negative after 350 nm.

At the identified excitation section and fluorescence peak (Ex. 330 nm and Em. 418), the greatest fluorescence intensity was found in the young copepodids with a mean of 0.21, and the lowest was found in the non-target copepods and N1 *L. salmonis* with means of 0.037 and 0.043, respectively (Figure 4, Table S2). The mean intensity at the peak for sea trout copepodids was 0.17, 0.12 for old copepodids and 0.11 for N2 *L. salmonis* samples. The sea trout copepodid intensities were less than young *L. salmonis* copepodids but >4 times greater than the mean of non-target copepod samples, which was 0.04. The *C. elongatus* samples also exhibited greater fluorescence than the non-target samples with a mean intensity of 0.12 but had a lower intensity than the young *L. salmonis* copepodids.

3.1.2 | Spectrum section analysis of formalin samples

Spectrum section analysis of formalin samples indicated that there were many sections between 310 and 510 nm where target groups of *L. salmonis* could be distinguished from non-target copepods (Figure 3b). Along those wavelengths, the fluorescence intensity of the young copepodid group increased to a peak at 380 nm and then decreased through higher wavelengths. The peak intensity distance was lowest in the sea trout copepodid group, while it was the highest in the nauplii and young copepodid target groups. The peak intensity distance calculated for the old copepodid target group was lower than that of the highest groups, but followed the same pattern.

Closer inspection of two local peaks, Ex. 380 nm and Em. 474 nm and Ex. 450 nm and Em. 516 nm, exhibited the pattern in

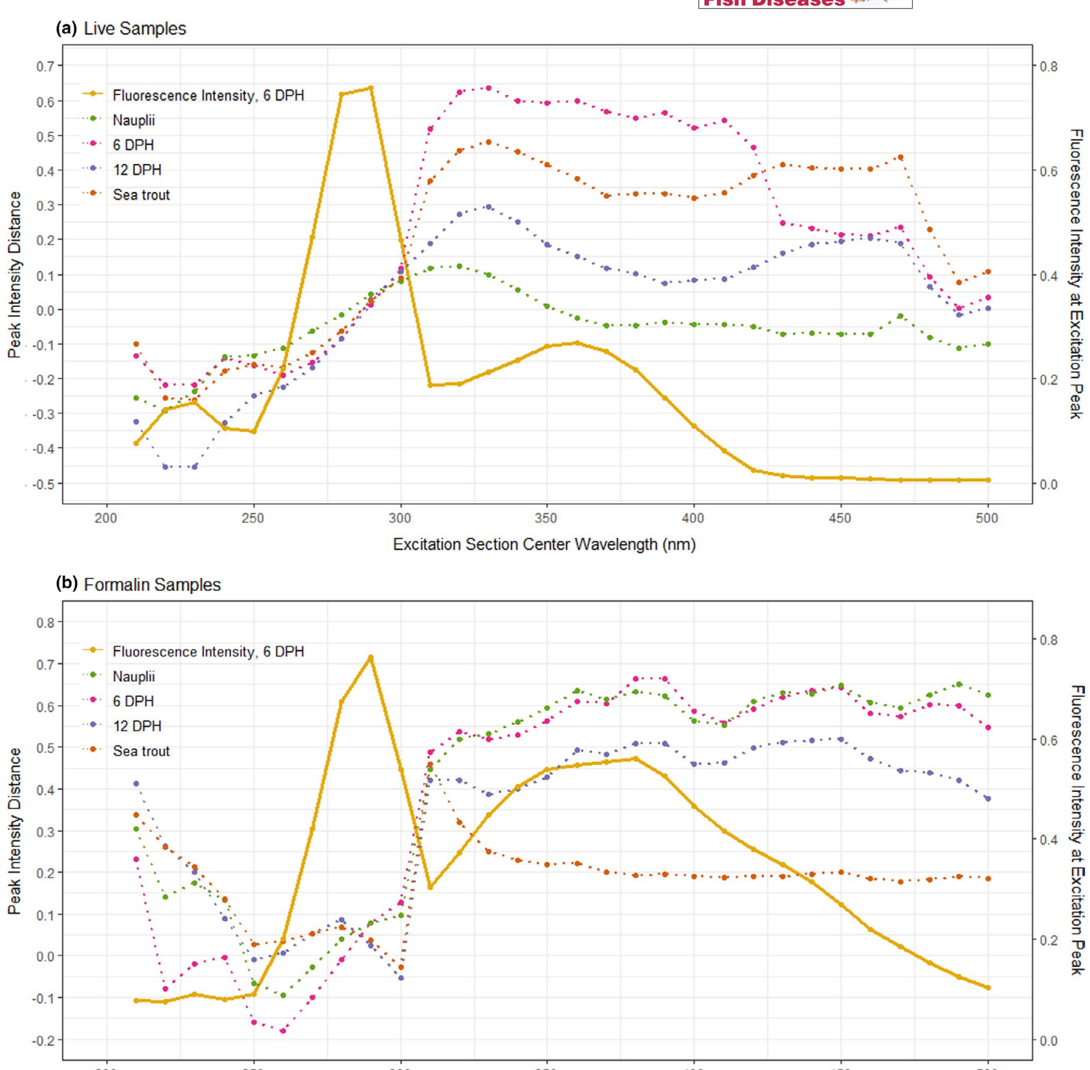

FIGURE 3 Fluorescence peaks identified through section analysis for (a) live samples and (b) formalin samples. The centre wavelength of the excitation section (±10 nm) is shown on the *x*-axis and the peak intensity distance on the y-axis. Peak distance is a calculation of the fluorescence intensity difference between the target group and non-target copepod comparators for the indicated excitation section peak. A second *y*-axis is included on the right side of graphs A and B, and the variable is depicted by the solid yellow line showing maximum fluorescence intensity of the young copepodid target group for the excitation section. Target groups "Nauplii, N1 and N2," "Young Copepodid" (sampled 6 DPH) and "Old Copepodid" (sampled 12 DPH) are *Lepeophtheirus salmonis* samples taken from *Salmo salar* host fish ,while "Sea Trout Copepodids" are 6 DPH *L. salmonis* samples from *Salmo trutta* host fish. All samples included in the Figure 3a and b graphs are also included in Figures 4 and 5, respectively

greater detail (Figure 5a,b, Table S2). Along both sections, the fluorescence intensities of nauplii (N1 and N2), young copepodid and old copepodid samples were greater than those of the non-target copepod samples. The highest mean intensities were found in the young copepodids at their respective fluorescence peaks, 0.61 (Ex. 380 ± 10 nm and Em. 474 ± 10 nm) and 0.29 (Ex. 450 ± 10 nm and Em. 516 ± 10 nm), while the means for non-target copepod samples were 0.09 (Ex. 380 ± 10 nm and Em. 474 ± 10 nm) and 0.02 (Ex. 450 ± 10 nm and Em. 516 ± 10 nm). The old copepodids had lower mean fluorescence intensities than the other *L. salmonis* from

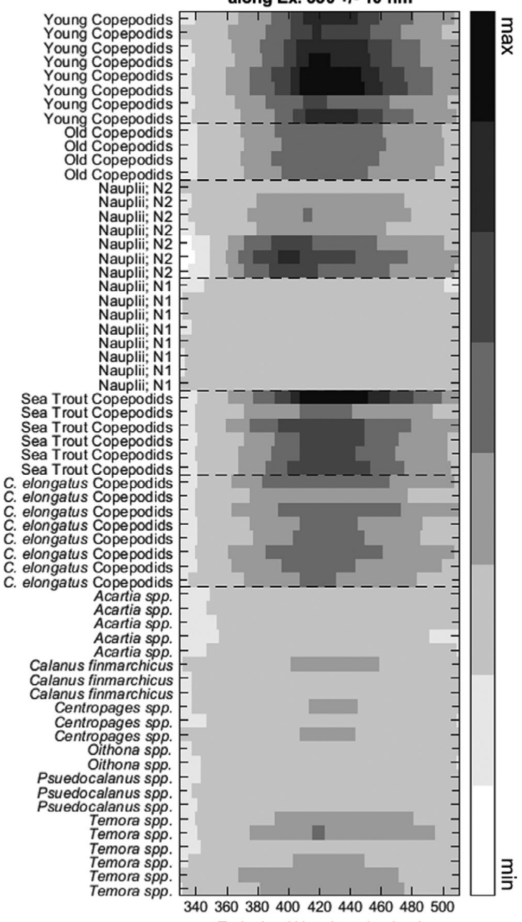

FIGURE 4 Relative fluorescence intensity of live samples along 330 ± 10 nm excitation spectrum with the darkest shades indicating maximum intensity. Target groups "Young Copepodids" (sampled 6 DPH), "Old Copepodids" (sampled 12 DPH) and "Nauplii, N1 and N2" are *Lepeophtheirus salmonis* samples taken from *Salmo salar* host fish, while "Sea Trout Copepodids" are 6 DPH *L. salmonis* samples from *Salmo trutta* host fish. "*C. elongatus* Copepodids" (sampled 6 DPH) were taken from *Salmo salar* host fish. Apart from *Calanus finmarchicus*, non-target copepod samples were identified to genus

Salmo salar hosts, with 0.49 (Ex. 380 ± 10 nm and Em. 474 ± 10 nm) and 0.24 (Ex. 450 ± 10 nm and Em. 516 ± 10 nm), but those means were distinctly higher than the non-target copepod samples. The sea trout copepodid samples were occasionally indistinguishable from the non-target copepod samples with some having lower intensity than the *Temora* spp. measurements. Likewise, *C. elongatus* fluorescence was not distinguishable from the non-target copepod fluorescence.

3.2 | Fluorescence microscopy

While each animal was imaged with all three filter sets, the following analysis focused on the formalin samples with the EGFP measurements (Ex. 470 ± 40 nm and Em. 525 ± 50 nm). The EGFP filter set includes the best performing fluorescence peak identified by the spectrum sectioning analysis: Ex. 450 ± 10 nm and Em. 516 ± 10 nm (Figure 3). Images of *L. salmonis* showed that fluorescence was widespread in the louse tissue with increased concentration in the eyespots and the maxillae (Figure 2). Non-target copepods similarly displayed widespread, but weaker, fluorescence with occasional areas in the gut or lipid sacs where fluorescence intensity was elevated.

3.2.1 | Total fluorescence and animal size

Total fluorescence intensity increased with animal size as measured by the number of fluorescent pixels above the threshold (Figure 6). The *L. salmonis* were raised under controlled laboratory conditions and were all at the same developmental stage and age, resulting in little variation in size. Meanwhile, the animals in the non-target copepod measurements were more variable in size due to the inclusion of several species in various developmental stages. Fitting the non-target copepod data to a linear regression, with total pixels as the independent variable and total fluorescence intensity as the dependent variable, resulted in the formula $f(x) = -6.29*10^6 + 38.1x$ ($R^2 = 0.77$). A linear regression fit to measurements of laboratory-cultured *L. salmonis* copepodids that had been in storage for over 30 days resulted in the formula $f(x) = -1.02*10^{68} + 186x$ ($R^2 = 0.47$). The number of fluorescing pixels did not change in relation to storage duration according to an ANOVA performed on the data set of *L. salmonis* measurements in storage for greater than 30 days ($F_{1,116} = 0.724$, $p = .396$). Thus, mean fluorescence intensity can be calculated from total fluorescence intensity and total number of fluorescing pixels for each animal imaged in order that further analysis be conducted.

3.2.2 | Formalin storage and fluorescence intensity

The mean fluorescence intensity of laboratory-grown salmon lice increased with storage duration in formalin (Figure 7). A saturation curve with the formula $f(t) = 84.09t/6.57+t$ was found to have the best fit with an R-squared of 0.867 (RMSE = 8.40). According to the formula, the mean intensity is 69.1 at day 30 and 75.8 at day 60, 82.0% and 90.1% of the asymptote maximum, respectively. Since the intensity rapidly increases in the first 30 days of storage and more slowly thereafter, significance tests were only performed on lice data sets from equivalent timeframes greater than 30 days. *C. elongatus* were measured at two formalin storage duration time points, day 33 and day 133, and their mean fluorescence intensities were 50.2 and 54.7, respectively. While the

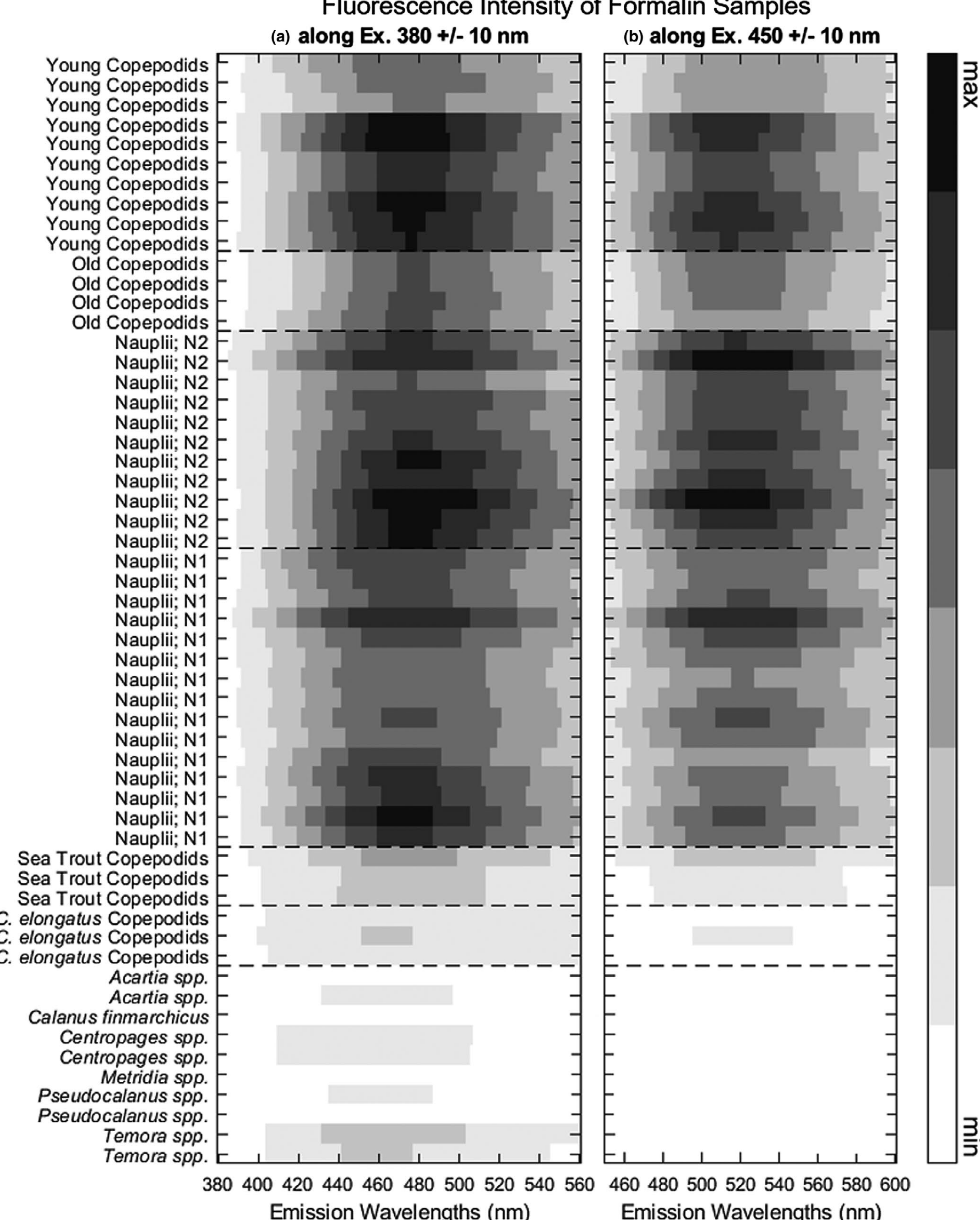

FIGURE 5 Relative fluorescence intensity of formalin samples along (a) 380 ± 10 nm and (b) 450 ± 10 nm excitation spectra with the darkest shades indicating maximum intensity. Target groups "Young Copepodids" (sampled 6 DPH), "Old Copepodids" (sampled 12 DPH) and "Nauplii, N1 and N2" are *Lepeophtheirus salmonis* samples taken from *Salmo salar* host fish, while "Sea Trout Copepodids" are 6 DPH *L. salmonis* samples from *Salmo trutta* host fish. "*C. elongatus* Copepodids" (sampled 6 DPH) were taken from *Salmo salar* host fish. All target group samples were in formalin storage for greater than 60 days, except *C. elongatus*, which was in storage 33 days. Apart from *Calanus finmarchicus*, non-target copepod samples were identified to genus. Non-target samples were in formalin storage for more than 7 days

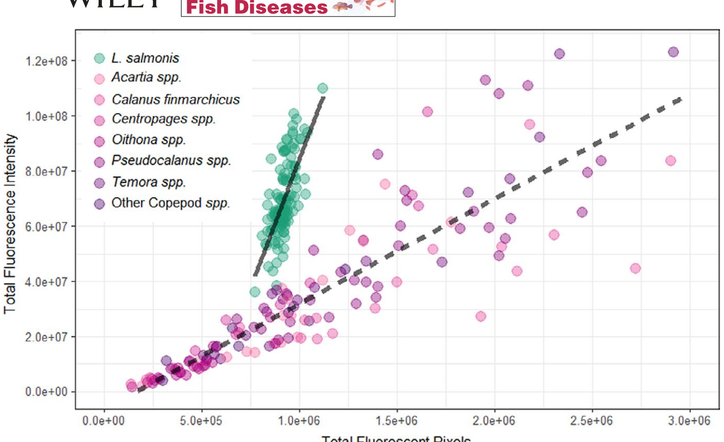

FIGURE 6 Relationship between fluorescence intensity and number of fluorescing pixels, measured with the EGFP filter set (FITC/Cy2), Ex. 470 ± 40 nm and Em. 525 ± 50 nm. Lines for linear regressions of *Lepeophtheirus salmonis* data (solid) and non-target copepod species data (dashed). Points depict single measurements, and colours indicate species. All *L. salmonis* data are from 6 DPH copepodids from laboratory-cultured salmon (IMR, Norway)

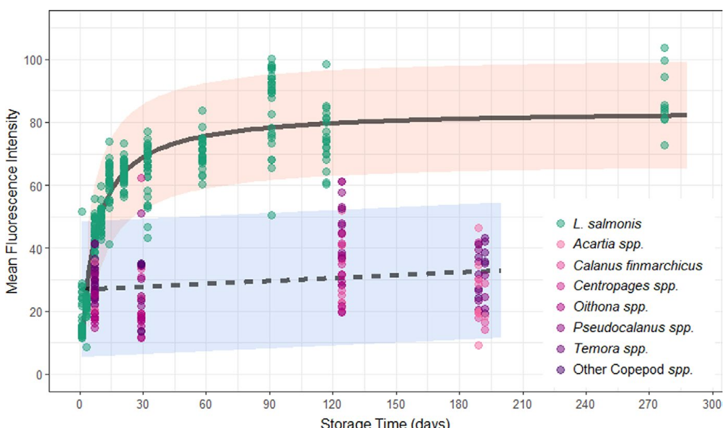

FIGURE 7 Relationship between mean fluorescence intensity (total intensity / pixels counted) and storage time in formalin, measured with the EGFP filter set (FITC/Cy2), Ex. 470 ± 40 nm and Em. 525 ± 50 nm. All *Lepeophtheirus salmonis* data are for 6 DPH copepodids from laboratory-cultured salmon (IMR, Norway). Points depict single measurements, and colours indicate species. 95% prediction intervals are indicated with shaded regions for the saturation curve fit to *L. salmonis* data and linear regression fit to non-target copepod spp. data

mean fluorescence intensity of *C. elongatus* measured on the latter date was greater, there were not enough storage duration data points to perform any further analysis in relation to formalin storage duration.

The fluorescence intensity of non-target copepods exhibited a significant, but weak linear relationship with storage duration in formalin (R^2 = 0.039, p = .0134) (Figure 7). Since storage duration of non-target copepods did not explain much of the variability in fluorescence intensity, we performed an ANOVA including the entire non-target copepod spp. data set (mean ± SE = 29.5 ± 0.93, n = 139) and the measurements of laboratory-grown salmon lice that had been in storage for over 30 days (mean ± SE = 75.3 ± 1.14, n = 118). The fluorescence intensity of non-target copepods was significantly different from the salmon lice ($F_{1,256}$ = 982, p < .001). However, the variance in mean fluorescence intensity may result in the occasional measurement of a *L. salmonis* copepodid below that of a non-target copepod. At 30 days in formalin, the lower bound of the 95% prediction interval for salmon lice copepodids was 52.2, while the upper bound for the non-target copepods was 49.3. Although the 95% prediction intervals do not overlap, 4.3% (6/139) of the non-target copepods had a mean fluorescence intensity over 52.2, and 1.7% (2/118) of the *L. salmonis* copepodid measurements were below 49.3.

3.2.3 | Factors influencing the fluorescence profile

Several factors were investigated to determine their relationship to the measured fluorescence intensity of the animal, including sea louse species, origin and host fish; development stage; storage temperature; and age (Figure 8). In all cases, the pattern of intensity, as influenced by the factor, is specific to the spectrum of fluorescence examined. The following analysis focused on the data set derived from the EGFP filter set. Within each factor, t tests were performed between a reference group and the various other categories presented, except for the age comparison in which the t test was performed for each filter set. Descriptive statistics and the Bonferroni-adjusted p-values for each significance test are provided in full in Table S3.

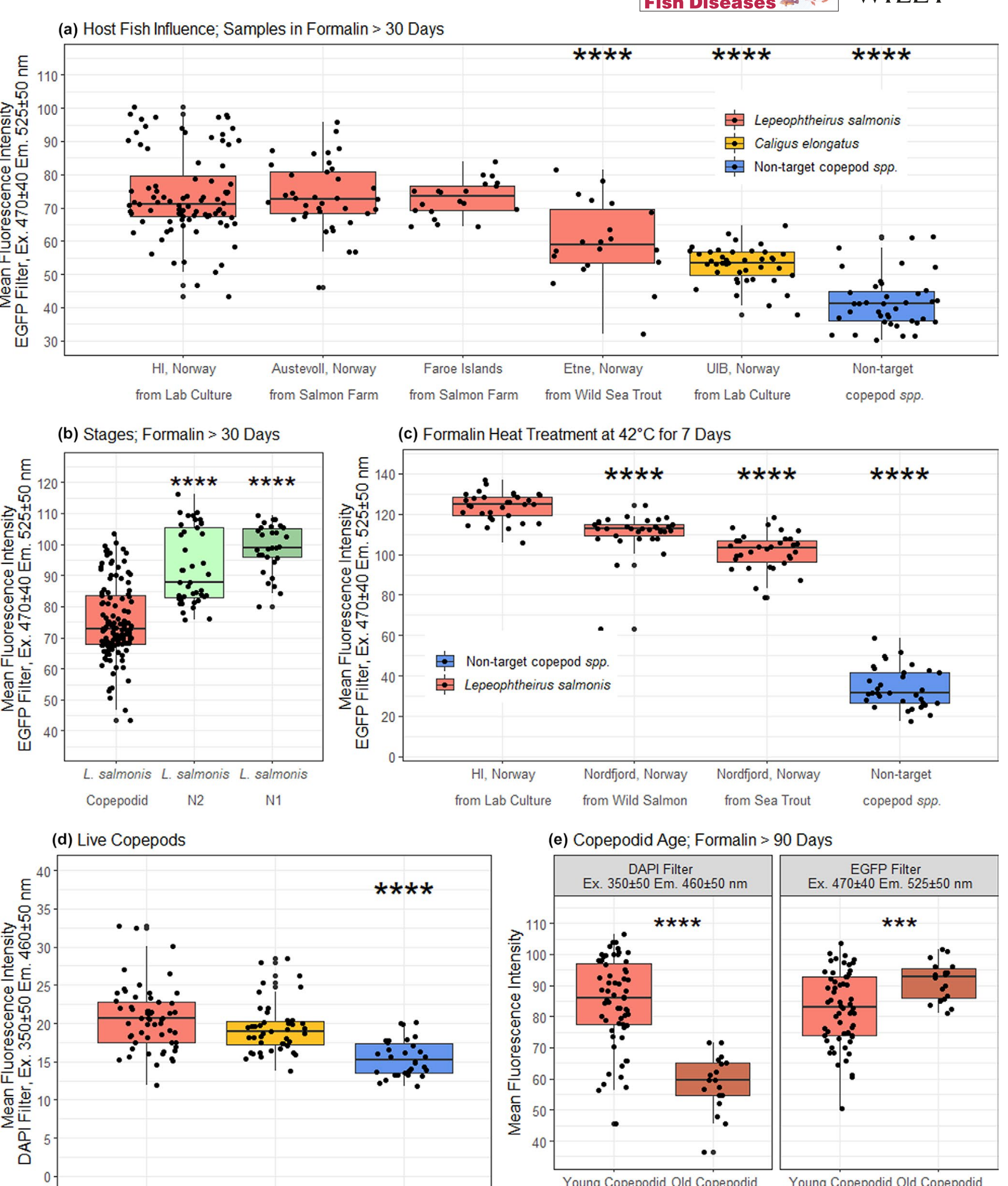

FIGURE 8 Factors influencing the mean fluorescence intensity (total fluorescence intensity / total number of fluorescing pixels) analysed by factor category (a–e) with selected reference category for *t* tests placed on the left side of each box plot, except for (e) in which the *t* test was performed for each filter set. Asterisks indicate Bonferroni-adjusted *p*-values (****<.00001 and ***<.0001)

Host fish (Figure 8a): Measurements of *L. salmonis* copepodids, which had been in formalin storage more than 30 days, were analysed according to their host fish origin. No significant differences were found between copepodids originating from Atlantic salmon hosts raised under laboratory conditions or taken from a farm. *C. elongatus* taken from laboratory-raised salmon and *L. salmonis* found on wild-caught sea trout were significantly different from the laboratory-cultured *L. salmonis* (p-value < .00001). Although they exhibited lower mean fluorescence intensity, they were both significantly greater than the non-target copepods (t test, $p < .00001$). Specifically, laboratory-reared *L. salmonis* mean fluorescence intensity is 1.4 times greater than *C. elongatus*, while *C. elongatus* mean fluorescence is 1.6 times greater than the non-target copepods.

Stages (Figure 8b): Measurements of samples, which had been in formalin storage more than 90 days, showed developmental stage of *L. salmonis* significantly affected the mean fluorescence intensity ($p < .00001$). Nauplius stages N1 and N2 exhibited greater fluorescence per pixel than the copepodid stage, with 98.7, 91.3 and 75.3, respectively. However, no significant difference was found between the stages when examining the total fluorescence intensity (ANOVA: $F_{2,189} = 1.86$, $p = .158$).

Heat treatment (Figure 8c): Laboratory-cultured 6 DPH *L. salmonis* copepodids stored at 22°C for 7 days had a mean fluorescence intensity of 44.6, while those stored at 42°C for 7 days had 124, a 2.8 factor increase (Table S3). The heat-treated copepodids from the laboratory culture had significantly greater mean fluorescence intensity than heat-treated copepodids from both wild-caught *Salmo salar* and *Salmo trutta* hosts ($p < .00001$). Their mean fluorescence was also found to be significantly greater than heat-treated non-target copepods by a factor of 3.6 ($p < .00001$). Meanwhile, the mean fluorescence intensity of *L. salmonis* copepodids from wild-caught *Salmo salar* and *Salmo trutta* was 3.3 and 3.0 times greater than the heat-treated non-target copepods ($p < .00001$).

Live copepods (Figure 8d): The fluorescence peak at Ex. 330 nm and Em. 418 nm was identified by the spectrum sectioning analysis as the best peak for distinguishing live *L. salmonis* from non-target copepod spp. The peak is located within the spectrum covered by the DAPI filter set (Ex. 350 ± 50 nm and Em. 460 ± 50 nm). Measurements taken with the DAPI filter set showed that mean fluorescence intensity of 6 DPH *L. salmonis* copepodids was significantly different from the non-target copepods. ($p < .00001$), but they were not significantly different from *C. elongatus* copepodids ($p = .0977$). The mean fluorescence intensity of the live *L. salmonis* copepodids was 1.06 times greater than live measurements of non-target copepods. Meanwhile in 30-day-old formalin samples measured with the EGFP filter, the mean fluorescence intensity of *L. salmonis* measurements was 2.25 times greater than the mean of non-target copepod samples (Figure 7). Thus, the relative difference between salmon lice and non-target copepod spp. in live samples is much less than that found in formalin samples.

Copepodid age (Figure 8e): Amongst samples that had been in formalin storage for more than 90 days, the DPH of *L. salmonis* copepodids significantly affected the fluorescence intensity in both the DAPI and EGFP filter data sets ($p < .0001$), but in opposite directions. In the DAPI data set, the mean fluorescence intensity of 6 DPH copepodids was 1.45 times greater than the 12 DPH mean fluorescence intensity. In the EGFP data set, 6 DPH copepodids differed from 12 DPH copepodids by a factor of 0.9.

4 | DISCUSSION

Collection of EEM data facilitated the identification of fluorescence peaks where intensity differences could be used to differentiate between target sea lice species and non-target copepod spp. Once those peaks were identified, further work employing fluorescence microscopy assessed the strength of the fluorescence signal and its reliability in response to influencing factors. The EEM measurements could not be utilized for that analysis or to calculate the absolute differences in fluorescence between samples because the intensity was normalized to the maximum, since the exact number of individuals measured could not be practically controlled. However, the EEM measurements efficiently resolved the fluorescence profiles of the various species and treatments examined, whereas fluorescence microscopy is limited to wavelength combinations defined by the filters used. Together, the exploration of fluorescence profiles with EEM measurements and the assessment of signal strength with fluorescence microscopy demonstrated that *L. salmonis* can be distinguished from non-target copepod spp. using fluorescence.

4.1 | Spectrum section analysis of EEM measurements

The spectrum section analysis provided a means of systematically processing the EEM measurements in a manner that mimicked the use of microscopy excitation filters and emission filters. Evaluating the intensity difference between EEM samples at each conjunction of excitation and emission wavelengths is also more economical than doing the same with many different filters. Rather than examining all possible combinations, the analysis focused on the points at which *L. salmonis* copepodids exhibited the greatest fluorescence and where their fluorescence would be greater than other animals. The target *L. salmonis* nauplii and copepodids could also be distinguished from other zooplankton where they had a lower fluorescence intensity, as seen with the negative peak intensity distances in wavelengths below 290 nm. Nielsen et al. (2019) similarly demonstrated that when using a 410-nm excitation light several zooplankton species exhibit a fluorescence peak at an emission wavelength of 686 nm, but not *L. salmonis*. The negative signal was due to fluorescence of chlorophyll consumed by the grazing zooplankters in contrast to the non-feeding *L. salmonis*. A negative signal might be useful in an automated process as argued by Nielsen et al. (2019), but it would not be useful in enumerating animals via a fluorescence modification of traditional light microscopy methods in which the animal must standout against the darkfield (as described by Bui

et al., 2020). Thus, wavelengths above 600 nm, where chlorophyll fluoresces, were not examined here and the spectrum section analysis identified the wavelengths with greatest positive peak intensity distance.

The EEM measurements characterized the fluorescence profiles of each copepod species and indicated the difference in fluorescence intensities between them. However, the relative difference observed in the EEM measurements between two species was occasionally contradicted by the fluorescence microscopy data. The mean fluorescence intensity of the live 6 DPH *L. salmonis* copepodids (*Salmo salar* host) was 4.8 times greater than the non-target copepods in the EEM measurements, but only 1.06 times greater in the fluorescence microscopy data using the EGFP filter set (Ex. 470 ± 40 nm and Em. 525 ± 50 nm). Likewise, in the formalin-fixed samples the mean fluorescence intensity of 12 DPH *L. salmonis* copepodids was higher than that of the 6 DPH group when measured by fluorescence microscopy (Ex. 470 ± 40 nm and Em. 525 ± 50 nm), but the relationship was reversed when looking at the same groups using the EEM measurements. Since the EEMs are normalized to the maximum fluorescence peak, usually near Ex. 290 nm and Em. 320 nm, an increased or decreased intensity, there would decrease or increase the normalized amount elsewhere. Thus, the EEMs were not direct measures of quantitative differences in fluorescence, so further fluorescence microscopy was required to validate these differences.

4.2 | Fluorescence microscopy

Mean fluorescence intensity was calculated for each animal so that comparisons could be made across species and treatments, but the total number of fluorescing pixels is also a useful signal. In automated processing of images, particle size (total pixels) could help distinguish *L. salmonis* from other species, which have comparable mean fluorescence intensities. Otherwise, false-positive identifications could occur if only using the mean intensity. Despite the large overall difference in mean intensities between formalin-preserved *L. salmonis* and non-target copepod species, overlap did occur between the animals imaged with the EGFP filter set (Ex. 470 ± 40 nm and Em. 525 ± 50 nm). Considering the relative rarity of *L. salmonis* in the water column, false positives could quickly become problematic, and using both mean fluorescence and total size may not be sufficient to prevent such occurrences. A second fluorescence signal, such as the negative chlorophyll signal suggested by Nielsen et al. (2019), might provide enough additional information to facilitate a fully automated detection system. Alternatively, nominal detection could be confirmed through morphological inspection of the animal. As *L. salmonis* are relatively unique in their appearance, positive identification could be quickly accomplished. Similarly, simple shape analysis / classification would likely prove sufficient to discriminate between problem specimens. Although only one fluorescence filter set can be used as an aid to traditional taxonomic methods at any time, the unique morphology of *L. salmonis* could thus be used for positive identification after locating the illuminated animal.

4.2.1 | Formalin fixation: storage time and temperature

The strong fluorescence signal in the formalin samples indicates that chemical reactions occur during fixation between the tissues and the formaldehyde to create fluorophores. The fluorophores generated are unknown, but the fluorescence signal shows that some of them are unique to *L. salmonis*. Formaldehyde fixation is a complex process that occurs in three steps: the initial penetration of the tissue, followed by covalent bonding of the formaldehyde with the tissue, and then cross-linking (Buesa, 2008). Penetration is rapid, while the binding may take 24 hr or more depending on the thickness of the tissue and the storage temperature (Fox et al., 1985). The formaldehyde binding can occur with any group containing a reactive hydrogen atom, but the rate varies considerably with amine reactivity being fastest. Cross-linking then occurs progressively with potential functional groups forming methylene bridges in a process that can continue for months or years (Dapson, 2010). Thus, the increase in fluorescence intensity of *L. salmonis* samples with storage time can be explained by the slow process of cross-linking. The heat treatment increases the rate of this reaction and the total number of fluorophores as shown by the greater fluorescence intensity. Although evaluating the mechanism is beyond this study, heating apparently changes the reaction equilibrium towards creation of a greater number of fluorophores. Rather than increasing the number of fluorophores, an alternative explanation could conclude that over time or through the heat treatment, new highly fluorescent fluorophores are created. Future studies that seek to enumerate *L. salmonis* through fluorescence should only process formalin samples, which have been stored at room temperature for greater than 3 months or should apply a heat treatment prior to examination.

4.2.2 | Factors influencing the fluorescence profile

While storage temperature affects the reactions occurring during fixation, the other factors examined relate to the endogenous macromolecules, which constitute the compounds forming fluorophores. Since the planktonic stages are non-feeding, they are dependent upon their maternally derived storage lipids for energy, which decrease in volume over time, as does carbon mass (Brooker et al., 2018; Gravil, 1996; Thompson et al., 2019). The fatty acid composition of storage lipids varies with maternal origin (Tocher et al., 2010), the incubation temperature and age (Skern-Mauritzen et al., 2020), and development stage (Thompson et al., 2019). Furthermore, at least three proteins in nauplii have been demonstrated to be of maternal origin (Dalvin et al., 2009, 2011). Thus, the composition of proteins and lipids in the animal is dependent on several factors. Some of those factors have been categorized and examined here, and their influence was reflected in the fluorescence patterns observed.

Old *L. salmonis* copepodids fluoresced at lower intensities than younger copepodids when using the DAPI filter set (Ex. 350 ± 50 nm and Em. 460 ± 50 nm), which suggests that the responsible

fluorophore is related to energy stores or an otherwise decreasing entity. Meanwhile, the same comparison made with the EGFP filter set (Ex. 470 ± 40 nm and Em. 525 ± 50 nm) yields a small increase in fluorescence and suggests the opposite. Therefore, the responsible fluorophore in the latter case is a robust fluorescence signal for detecting *L. salmonis* in samples where age cannot be controlled. When examining stage differences, the stability of the fluorophore is further demonstrated by N2 and N1 having progressively greater mean fluorescence intensity than the copepodids. The decrease in mean fluorescence intensity from N1 to copepodid can be explained by an increase in size with no change in total intensity, which indicates that there is no substantive change in the amount of fluorophore present.

No difference in fluorescence intensity was found between *L. salmonis* originating from laboratory cultures or farmed fish, but those from wild fish fluoresced less. The heat treatment further emphasized the trend, with laboratory-cultured *L. salmonis* fluorescing the most, followed by wild *Salmo salar* hosts and then wild *Salmo trutta* hosts. Following the maternal origin of lipids and proteins previously discussed, host fish diet is a possible influencing factor on fluorescence, but the intensity trend does not follow the gross dietary sources for each host fish category. Adult wild *Salmo salar* returning from the sea have a diet of wholly marine origin, while marine sources comprise 34%–89% of *Salmo trutta* diets (Davidsen et al., 2017), and the feed of farm raised and cultured *Salmo salar* is just 25% marine (Aas et al., 2019). A specific dietary component may still be responsible, or population-level differences between the host fish may be the cause of the fluorescence intensity differences. The lack of difference between the laboratory culture and the farm sites in Norway and the Faroe Islands indicates that genetic and temperature differences amongst the *L. salmonis* are not likely to be responsible. However, genetic variation within the Atlantic populations of *L. salmonis* is low (Glover et al., 2011), and further examination would be needed prior to application to a different population such as the Pacific subspecies (Skern-Mauritzen et al., 2014). Furthermore, the fluorescence signal exhibited by *L. salmonis* is not commonly shared by sea lice, as evidenced by the much lower fluorescence intensity in *C. elongatus*.

5 | CONCLUSION

The fluorescence signal induced by formalin fixation appears to be a reliable differentiator of planktonic *L. salmonis* in mixed zooplankton samples. A statistical difference was also observed between *L. salmonis* and non-target copepod spp. in live samples, but the small increase in fluorescence is not likely to be sufficient for routine identification. A modification of traditional taxonomic methods with fluorescence would aid in the locating and identifying of *L. salmonis* in formalin samples, greatly reducing processing times. Automated identification is also possible through the use of fluorescence, but multiple filter sets would be needed along with copious training of machine learning algorithms. While the development of a rapid identification method using fluorescence is motivated by the specific problem of *L. salmonis*, the work exemplified here could be replicated for other purposes.

The non-target copepod spp. examined here are commonly found in the zooplankton assemblage along with the relatively rare *L. salmonis*. However, not all common copepod species were examined, such as *Metridia* spp. and *Microcalanus* spp., nor were the many other less common copepod species, cryptic species and non-copepod zooplankton examined. Any number of species with an unknown fluorescence profile could be found in a mixed zooplankton sample along with *L. salmonis*. While the non-target copepod spp. herein provided useful comparators for examining the differences in fluorescence to *L. salmonis*, they did not constitute an exhaustive survey. Considering the number and variability of species present in any given zooplankton sample, it would be impractical to individually assess the fluorescence profiles of all species. Instead, we suggest that the reliability of a fluorescence identification method could be assessed with trials on a variety of zooplankton samples spiked with a known number of salmon lice.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ETHICAL APPROVAL

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. *Lepeophtheirus salmonis* is a copepod and unregulated by the animal use in research regulations in Norway or the European Union. Animal welfare was supervised by the Norwegian Food Safety Authority (Mattilsynet), with the Bergen salmon louse culture covered by approval number 11912 and NALO salmonid monitoring work covered by approval number 14809.

DATA AVAILABILITY STATEMENT

Summary data tables corresponding to data presented in figures are included in the supplementary material of this article. The excitation and emission matrix (EEM) measurements were taken with the proprietary software "LabSolutions RF" and processed in MATLAB to produce the figures and data presented. EEM metadata are included in Table S1, and the corresponding EEM measurements can be found in Zenodo at https://doi.org/10.5281/zenodo.4157041 (Thompson et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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ORIGINAL ARTICLE

WILEY

A novel method for the rapid enumeration of planktonic salmon lice in a mixed zooplankton assemblage using fluorescence

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Abstract

The relative rarity of the planktonic larval stages of salmon lice in comparison to other animals captured in a zooplankton assemblage is an obstacle to estimating their abundance and distribution. Due to the labour intensiveness of standard plankton sorting approaches, the planktonic stages of salmon lice remain understudied and unmonitored despite their importance to the spread of the parasite between salmon farms and to wild salmonids. Alternative methods of identification have been investigated and in a previous study a fluorescence signal was identified. Using filters to target that signal with fluorescence microscopy (excitation/emission wavelengths of 470/525 nm), the salmon louse has a fluorescence intensity 2.4 times greater than non-target animals, which distinguishes it from the zooplankton assemblage and enables rapid enumeration. Here, we present a novel method for the enumeration of planktonic salmon lice larvae, nauplius and copepodid stages, in a mixed zooplankton sample using fluorescence-aided microscopy. Performance of the method was evaluated with a blind trial which found a median accuracy of 81.8% and a mean sample processing time of 31 min. Compared with previously published findings, the novel method provides satisfactory accuracy and enumeration that is more than 20 times faster than traditional light microscopy approaches. Factors influencing the performance of the method are identified and recommendations are made for targeted sampling and automated enumeration.

KEYWORDS
Atlantic Salmon, Caligidae, Fluorescence, *Lepeophtheirus salmonis*, Zooplankton

1 | INTRODUCTION

Salmon lice *Lepeophtheirus salmonis salmonis* (Krøyer 1837; Skern-Mauritzen et al., 2014) are copepod ectoparasites that represent a pervasive problem for the salmonid aquaculture industry due to the welfare impacts on host fish, the economic impact for the industry and the potential for downstream environmental threats to wild salmonids (Torrissen et al., 2013; Vollset et al., 2018). Over 2.6

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billion tonnes of Atlantic salmon were harvested globally in 2019 (FAO-FIGIS, 2021), and in Norway, the production leader, there were 450 million animals Atlantic salmon stocked in open net pens across 650 actively farmed sites (Aquaculture Statistics, 2021). Due to the patchy distribution of their hosts and the scale of the marine environment, marine parasites typically have a low probability of transmission to their next hosts (Marcogliese, 2005). In the case of salmon lice, however, farmed salmonid hosts are readily available throughout the year, and account for more than 99% of the available hosts (Dempster et al., 2021). Thus, epidemic outbreaks of lice can originate on farms and subsequently spread to wild salmonid populations (Heuch & Mo, 2001; Pike & Wadsworth, 1999). Once attached, the parasitic stages of the lice feed on the mucus, blood and skin of the host fish (Mordue Luntz & Birkett, 2009), which may cause complications such as osmoregulatory failure and immunosuppression, and increased risk of mortality (Bowers et al., 2000; Wagner et al., 2008). Thus, the increased population of salmon lice on farms and the potential for increased infection pressure on wild fish is considered to be an environmental impact of salmon farming and an obstacle to sustainable growth (Anon, 2015; Taranger et al., 2015; Vollset et al., 2018).

The continued growth of salmonid production in Norway has been linked directly to the risk of salmon lice-induced mortality for wild populations of Atlantic salmon through the implementation of the 'traffic light system'. Under the current management framework, the Norwegian coast is divided into 13 production zones and an expert group evaluates numerous data sources to make an assessment of the lice-induced mortality in each zone. The Ministry of Trade and Industry, as the regulatory authority, then makes a decision based on the assessment of whether production capacity should be adjusted. In designated green zones, the production capacity can increase by 6%, in red zones it must decrease by 6% and in yellow zones there is no change (Anon, 2020). Among the sources of information, the expert group relies upon are several models (Sandvik et al., 2016), and monitoring data from farms (Jansen et al., 2012), sentinel cages with fish (Bjørn et al., 2011) and wild caught salmonids (Serra-Llinares et al., 2014). Notably, all the monitoring data relate to the parasitic stages attached to fish, and the models which forecast the spread of the infectious copepodid stages are reliant on those same data (Myksvoll et al., 2018; Sandvik et al., 2021).

Despite their importance in the infection pathway, the planktonic stages of salmon lice are not directly monitored and many aspects of their in-situ biology is under-parameterized due to the difficulty in measuring them (Brooker et al., 2018). Depending on temperature and origin of the host fish, the female louse can carry from less than 300 to nearly 1000 eggs in paired egg strings (Brooker et al., 2018). After hatching, the louse develops through two planktonic nauplius stages to an infectious copepodid stage, which may drift on the currents looking for a host for an estimated 14 days at 10°C (Hamre et al., 2013; Samsing et al., 2016). The planktonic stages of salmon lice in the water column are relatively rare in comparison to the numerous other species that comprise the zooplankton community. Previous work has found mean abundances of planktonic salmon lice ranging from 0.075 to 0.70 m^{-3} (á Norði et al., 2015; Byrne et al., 2018; Nelson et al., 2018; Nilsen, 2016; Penston et al., 2011; Salama & Rabe, 2013; Skarðhamar et al., 2019). Meanwhile, a typical plankton tow from the west coast of Norway can be expected to yield 5000 m^{-3} or more animals (T. Falkenhaug, personal communication, 6 June 2020) and globally the mean density of free-living copepods is estimated to be 1000 m^{-3} (Boxshall, 1998). Using traditional taxonomic identification and enumeration it may be necessary to sort through 1400 to 66,000 animals under a microscope before identifying a single *L. salmonis*.

Finding planktonic salmon lice within a zooplankton assemblage is a needle in a haystack problem and the traditional method is too labour-intensive for most endeavours. Bui et al. (2021) explored several alternative methods for identification and enumeration including some molecular techniques but found limited success in terms of throughput, accuracy and cost. Although one of the attempted methods utilized fluorescence, the filter wavelengths chosen followed work done by Fordyce (2017) for which the range of filters available was limited. In contrast, Thompson et al. (2021) examined the fluorescence profiles of salmon lice and non-target copepods under various conditions between the wavelengths of 200 and 600 nm to identify unique fluorescence signals. They found that formalin stored salmon lice copepodids had a mean fluorescence intensity that was 2.4 times greater than non-target copepods (excitation/emission wavelengths of 470/525 nm). This study follows the work by Thompson et al. (2021) by validating the methodology with a blind trial of the novel fluorescence aided method for the rapid enumeration of salmon lice in a mixed zooplankton assemblage. To this end, plankton samples from the Faroe Islands, Scotland and Norway were spiked with a known number of salmon louse copepodids and the participants attempted to quickly enumerate them. Enumeration time and accuracy were examined in relation to the characteristics of the sample and the zooplankton assemblage therein. Since extraneous fluorescence has the potential to disrupt the ability of the participants to find and enumerate the target lice, background fluorescence was measured through imaging. Background fluorescence and other variables were evaluated through statistical models and factors influencing the results are identified.

2 | METHODS

2.1 | Zooplankton community sampling

Zooplankton samples were collected from 5 sites in Norway, Scotland and the Faroe Islands (Figure 1). The Norway sites in Masfjorden and Boknafjorden were adjacent to active salmon farms and were sampled twice in the spring and twice in the autumn of 2019. Access to the Faroe Island sites was limited by seasonal weather patterns and one or the other site was visited twice in the spring and autumn. The site in Scotland was visited 10 separate times between May 7th and November 13th 2019, two of the dates were in June and thus in the summer season (Table 1). At the 4 sites in Norway

FIGURE 1 Location of sampling. Black triangles indicate sampling locations: Stonehaven in Scotland, Masfjorden and Boknafjorden in Norway, and two locations in the Faroe Islands

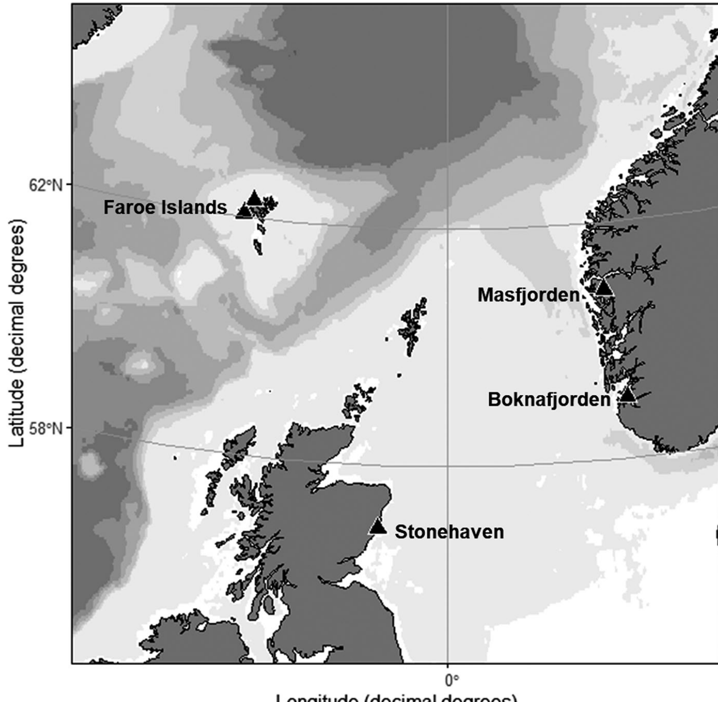


TABLE 1 Summary of sampling events, and number of replicates collected and enumerated

Site	Sampling events	Replicates (ETOH & Form.)	Total formalin	Enumerated by participant		
				A	B	C
Masfjorden, Norway	4	10	20	16	16	12
Boknafjorden, Norway	4	10	20	16	16	12
Spring site, Faroe Islands	2	10	10	9	9	7
Autumn site, Faroe Islands	2	10	10	8	8	6
Stonehaven, Scotland	10	4	10	10	10	10

and Faroe Islands, 10 replicate vertical tows were made to a depth of 40 m using a 50 cm diameter ring net equipped with either a 120 or 140 μm mesh. The Norwegian ring-nets were not equipped with flow-meters which would enable accurate measurements of the water volume filtered for each replicate tow, only the net depth was measured. Feed particles from the adjacent farms were collected in the Norwegian samples and were occasionally observed to fluoresce under microscopy. Samples from Scotland were collected as part the Scottish Coastal Observatory (SCObs) monitoring effort at the Stonehaven site (see Bresnan et al., 2015, 2016). Water depth at the Stonehaven site was 48 m and 2 vertical hauls were made to a depth 45 m using a 40 cm diameter bongo net fitted with a 68 and 200 μm mesh net. The Scottish samples collected with the 200 μm mesh net were taxonomically enumerated by Scotland Marine Science, and the 68 μm samples were transported to IMR facilities in Bergen Norway for fluorescence enumeration in the blind trial. Half of the replicate tows taken during each sampling event were preserved with 10% buffered formalin while the other half were preserved in 70% saline ethanol. However, only the formalin samples were used for rapid fluorescence enumeration in the trial since ethanol preservation had proven unsuitable for the fluorescence-aided method (Thompson et al., 2021).

2.2 | Zooplankton community composition and dry weights

The Scottish samples collected with the 200 μm net from the paired bongo tow were taxonomically enumerated as part of that ongoing monitoring programme. One ethanol sample from each set of

replicates from the other stations was taxonomically enumerated at the Fiskaaling Aquaculture Research Station in the Faroe Islands. In all cases, a subsample was taken and all animals in the subsample were identified to the lowest achievable taxonomical level. The zooplankton community data were then harmonized between the two laboratories by combining animal counts from lower taxonomic levels into higher taxonomic groupings that were shared across analyses. The data set was harmonized to 23 taxa with uncommon copepod species placed under the grouping of 'other copepod spp'. As a measure of zooplankton density in the fluorescence test samples, all formalin samples were sent to Fiskaaling after completion of the fluorescence trial, for total dry weight measurement taken after 24 h at 60°C.

2.3 | Lice spiking and enumeration

The zooplankton samples were spiked with formalin-preserved, 6 day post-hatch salmon lice copepodids, which were sourced from a mixed cohort of three laboratory strains of *L. salmonis*: LsGulen, LsOslo and Ls1A (Hamre et al., 2009), and cultured at the Institute of Marine Research (IMR) facility in Bergen, Norway in May 2019. The number of copepodids added to each sample was determined by randomly generating numbers following a Poisson distribution with a lambda of 15 (spike numbers in Supporting Data). The participating salmon lice enumerators in the trial were blind to this portion of the experiment until after they submitted their count numbers. The enumeration order of the samples was determined using random selection.

Samples were prepared for enumeration by first separating the zooplankton from the formalin preservation solution with a 90 μm mesh sieve. Using filtered sea water (FSW), the sample container was rinsed to flush out any remaining zooplankton on to the sieve, and then the sample was rinsed in the sieve to remove any excess formalin before transferring it to a glass beaker. The glass beaker was filled with FSW to dilute the sample and stirred in a figure of eight pattern before distributing the contents to 6-well plate dishes (Nunc). The wells had a diameter of 3.4 cm and a height of 2 cm and were each filled with approximately 6 ml of the sample solution. The number of well plate dishes used for each sample depended upon the density of the sample, with a minimum number used which allowed the sample contents to be distributed in a single layer in the wells. Late stage *Calanus* spp. and gelatinous zooplankton occurred in high abundance during five sampling events at the Norway sites which required those replicate samples, a total of 20, to be size fractioned prior to processing. The samples were first fractioned with a 2 mm mesh to remove the largest particles and then fractioned with a 1-mm sieve.

After enumeration, the well plates were emptied and the contents were rinsed with FSW into a single 20 × 30 cm tray, which was then emptied and rinsed onto a 90-μm sieve. The sample was then transferred back into the original sample container with the original formalin fixative plus additional fixative as needed to fill the container. The first round of counts was done concurrently by two participants ('A' & 'B'), and required a single sample handling. The third count (participant 'C') was done 2 to 4 weeks later and necessitated another sample preparation, for a total of three handling events. A subset of the replicate samples was enumerated from each of the sampling events by the participants, with participant 'C' enumerated one less sample from the Norway and Faroe Islands sites (Table 1).

The lice counters had differing levels of experience and expertise with microscopy and zooplankton taxonomy. All the participants had a Master of Science degree. Participant 'A' had 10 years of experience working with copepods and doing taxonomic enumeration of zooplankton samples with light microscopy. Participant 'B' had no previous experience with zooplankton taxonomic analysis, but had 5 years' experience using light microscopy and fluorescence for various biological assays. Participant 'C' had 4 years' experience identifying planktonic salmon lice using light microscopy. Prior to beginning the trial, a 5-h training session was conducted in which the participants were shown how to identify a salmon louse copepodid using the fluorescence-aided method. Under illumination, a subset of individual organisms in each sample will fluoresce. Using size and shape as supplementary cues, the participants selected potential targets highlighted by fluorescence and confirmed a positive identification through morphological examination, under high magnification if necessary (Figure 2; Supporting Demonstration Video S1 and Video S2). The time taken for each participant to enumerate samples was recorded via stopwatch, starting just prior to examination of the first well plate under the microscope and ending with the last well examined. Counters enumerated the spiked copepodid salmon lice and any wild sea lice which were unintentionally collected in

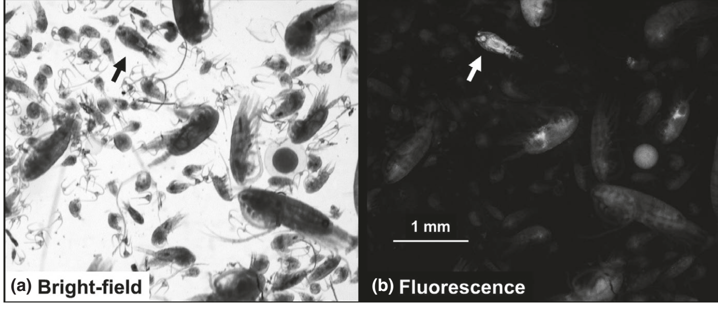


FIGURE 2 Rapid fluorescence enumeration. Under bright-field illumination (a) the target lice are not easily distinguished from the background zooplankton assemblage, but under fluorescence illumination (b) they have a greater fluorescence intensity and stand out, enabling rapid identification and enumeration

the sample. The count accuracy, or percentage of the count correct, was calculated from the number of salmon lice copepodids found, divided by the spike number in each sample.

2.4 | Fluorescence microscopy and imaging

Samples were examined with a Nikon SMZ18 stereomicroscope. Fluorescence illumination was provided by the Lumen 200 and a standard ET-GFP filter cube was used to specify an excitation wavelength of 470 ± 40 nm and an emission wavelength of 525 ± 50 nm. The microscope system was located in a darkroom, and examination and imaging was done without extraneous lighting. Nikon monochrome microscope camera, DS-Qi2, was used to take images with the software NIS Elements-F (e.g. Figure 2). After the first enumeration, every well that was examined was imaged with the same settings at the minimum zoom. The number of wells used for each sample ranged from 18 to 48. The image captured an area of 2.64 cm^2, 29.1% of the 9.07 cm^2 well area. Prior to taking the image, the well-plate dishes were gently swirled, which brought the majority of the sample towards the centre of each well and away from the edges. The centre of the well was placed in the middle of the image frame for image capture. The 14-bit images were recorded in grayscale as 8-bit tiff files and processed following Thompson et al. (2021). Intensity of each pixel was recorded as a grayscale value ranging from 0 to 16,383, and minimum threshold was used to remove all pixels below a set value. Thresholds were set at 3000, 5000, 7000, 9000 and 15,000, and the total fluorescence intensity and total number of pixels was measured for each image at that threshold. Mean and total fluorescence intensity and total fluorescing pixels were calculated for each sample.

Fluorescence intensity declined due to regular handling and enumeration, this bleaching effect was examined in a set of copepodids which came from the same cohort as the spike copepodids. Regular handling and enumeration was simulated by removing the 127 copepodids from formalin and placing them in seawater, imagining them immediately and examining them under illumination for 3 min. They were then left in the darkroom for 90 min before being imaged again. A subset of 48 were left under constant fluorescence illumination and imaged 6 times over 120 min. Mean fluorescence intensity was then calculated for each image by taking the total fluorescence intensity above the 3500 threshold divided by the number of fluorescing pixels above that threshold.

2.5 | Data analysis

Statistical models were used to evaluate both accuracy and enumeration time as they related to characteristics of the samples and blind trial. The percentage of *L. salmonis* correctly identified in each sample count was modelled using a generalized linear model (GLM) framework. Since the response variable, percentage correct of the count (accuracy) is proportional data, the GLM used a logistic regression. The response follows a binomial distribution and is weighted to the number of salmon lice copepodids included in each sample spike, whereby each louse functions as a trial for correctly or incorrectly observing its presence in the sample (see Zuur et al., 2009). The enumerating time of the counting portion of the sample processing was modelled with multiple linear regression (LM). The models were respectively fit using the 'glm' and 'lm' functions from the package *stats* within the R statistical software (R Core Team, 2020), and Figure 1 was produced using the R packages *ggplot2* (Wickham, 2011) and *ggOceanMaps* (Vihtakari, 2020).

Prior to model selection, the GLM of accuracy and LM of enumeration time initially contained the same explanatory variables, except where the response variable of one is included as an explanatory variable in the other (Table 2). Since the zooplankton community data is only available from a representative sample of each replicate set, it is not included in this analysis. Standard procedures for data exploration and model validation were used to identify statistical problems arising from outliers, heterogeneity of variance, collinearity, dependence and interactions (Zuur et al., 2010). During model selection, a stepwise goodness of fit approach utilized Akaike information criterion (AIC), an estimator of prediction error, to identify and remove the worst performing covariates in each iteration (see Zuur et al., 2009). After model selection, model assumptions were verified by plotting residuals versus fitted values, versus each covariate in the model and versus each covariate not in the model. The residuals were assessed for temporal and spatial dependency. The variable background fluorescence, was measured in terms of both mean and total fluorescence intensity, and fluorescent pixels above a set threshold. Total fluorescent pixels was chosen as the best explanatory variable among those highly collinear options, and the best performing threshold intensity was selected by AIC between alternative models. Presentation of results from statistical analysis and the selected models follows standard protocols described by Zuur and Ieno (2016).

3 | RESULTS

3.1 | Blind trial: accuracy & enumerating time

A total of 159 counts were performed with participants 'A' and 'B' each enumerating 59 samples over 12 days while 'C' enumerated 47 in 5 days. The total number of salmon lice copepodids added during the spike was 875, each sample contained an average of 14.6 with a spike minimum of 1 and maximum of 24 copepodids. In four of the samples, a single wild salmon louse nauplius was found, and in three of the Stonehaven samples *Caligus* spp. copepodids were identified, these were removed from the total count.

Prior to rapid fluorescence enumeration in the trial, samples were prepared in batches of 4–5 which took an average of 11 min to process. Size fractioning was done on 20 of the samples, all of which were from the Norwegian sites. The processing step took additional time but since the samples were mixed in batches, the

preparation time cannot be calculated separately for fractioned and non-fractioned samples.

Since the dataset has a pronounced right skew, the median accuracy is presented as the measure of central tendency, but mean values are provided. Participant 'A' achieved the greatest median accuracy at 92.3%, followed by 'B' with 85.7% and then 'C' had a much lower median accuracy with 56.2% correct (Table 3). The overall median accuracy was 81.8%. If only considering the dataset from participants 'A' and 'B', the overall median percentage correct was 89%, and examined separately, the size fraction greatly reduced the percentage from 93.8% overall to 52.5%. Meanwhile, the mean enumerating time differed by less than 30 s across counters with an overall mean of 19.9 min. Factors influencing the count accuracy and those influencing enumerating time are examined with a generalized linear model and multiple linear regression analysis in later sections.

TABLE 2 List and description of covariates used in the statistical models

Covariate	Type	Description
Background Fluorescence	Continuous	Total number of pixels fluorescing above threshold, summed across all images and base 10 log transformed
Sample Process Order	Continuous	The order of sample processing was randomly selected and the same for each counter
Sampling Sites	Categorical 4 levels	see Figure 1, Faroe Islands (both locations together), Stonehaven in Scotland, Masfjorden and Boknafjorden
Season	Categorical 3 levels	Spring (April and May), summer (June), autumn (October and November)
Size Fraction	Categorical 2 levels	Yes or No. Processing step to remove gelatinous zooplankton and late stage *Calanus* spp.
Wells	Continuous	Number of wells each sample was distributed between
Fluorescent Feed	Categorical 2 levels	Yes or No. Was fluorescent feed from an active salmon farm observed in the sample
Counter	Categorical 3 levels	The participant which processed the sample
Dry Weight	Continuous	A measure of the total biomass in each sample (grams)
Accuracy	Continuous	Percentage of salmon lice correctly enumerated in the sample
Enumeration Time	Continuous	Time taken for counting portion of sample processing (min)

TABLE 3 Summary of blind trial

		Accuracy (%)			Enumerating time (min)		
Participant	***n***	**Median**	**Mean**	**s.e**	**Median**	**Mean**	**s.e**
All data							
A	59	92.3	81.7	0.03	18.5	19.9	1.21
B	59	85.7	74.4	0.03	17.0	19.7	1.03
C	47	56.2	52.6	0.04	18.5	20.1	1.17
No size fraction							
A	39	100.0	92.1	0.02	14.5	16.1	0.93
B	39	90.9	84.6	0.03	16.0	16.5	0.78
C	32	65.2	60.1	0.04	16.5	17.3	0.95
Size fractioned							
A	20	56.6	61.5	0.06	25.0	27.2	10.50
B	20	51.3	54.6	0.06	25.5	25.8	9.12
C	15	27.3	36.5	0.08	28.0	26.1	9.58

Note: Median, mean and standard error of the mean is presented for the percentage of the count correct and enumerating time by the participant and size fraction.

3.2 | Zooplankton community composition and dry weights

Zooplankton community data are available from a single ethanol replicate sample representative of each sampling event (replicate sample set) while every formalin sample was measured for dry weight. The mean coefficient of variance (CV; standard deviation (SD)/mean * 100%) for each set of replicate dry mass measurements ranged from a low of 41.5% for the spring Faroe Island samples to a high of 95.6% for the autumn Faroe Island samples. Here, variance in the percentage composition of a taxon in a replicate sample is assumed to be less than the variance in the total number of that taxon (following Thompson, 2012). Thus, the abundances of specific taxa are presented as percentages of the total zooplankton composition.

Copepods dominated the zooplankton assemblage in the enumerated samples with a mean composition of 71.2%. Of the 11 copepod taxa identified, two were much more abundant in the samples than the rest, *Oithona* spp. with mean of 23.6% and *Acartia* spp. with a mean of 17.1%, and after them *Paracalanus* spp. was the next most abundant at 8.9%. *Calanus* spp., which overwhelmed some samples and necessitated the size fractioning step, was the 6th most abundant copepod taxa in enumerated samples with a mean of just 2.6%. Barnacles with a mean composition of 8.4% and bivalves with a mean of 6.8% were the most prevalent non-copepod zooplankters in the samples.

Since the Stonehaven vertical hauls were made to near bottom, sediment was found in all samples and could not be separated from the dry weight measurement. Excluding Stonehaven samples, the greatest mean dry weight measured from replicate samples was 0.451 g from Masfjorden in the spring. The samples taken there in the spring also had the highest percentage of animals identified as *Calanus* spp. with a mean of 18.5%. Generally, the spring samples had higher dry weights, excluding Stonehaven, the overall mean for spring was 0.294 g compared with 0.142 g for autumn. Spring samples also had a greater mean total number of animals in the samples, 17,400 versus autumn samples with 4500 animals. Along with the greater amount of biomass and number of animals, the spring samples exhibited higher background fluorescence, with mean log total pixels of 6.21 compared with 5.36. The full data set of zooplankton counts and sample dry weights are available in the Supporting Material.

3.3 | Statistical model of *L. salmonis* enumeration accuracy

Prior to model selection and removal of terms, the full model of accuracy had an AIC of 628.9, and an AIC of 630.4 after removal of five terms. During stepwise model selection, the first variable removed was enumerating time, followed by season, process order, fluorescent feed and sampling site was last variable to be removed. The final model (Equation 1; Table 4) had an explained deviance of 70.6% on 156 degrees of freedom, and a dispersion statistic of 1.79. All remaining covariates in the model were significant with p-values less than 0.01, the parameter estimates and standard errors are listed in Table 3, along with the specified p-values.

TABLE 4 Estimated regression parameters, standard errors, *z*-values and p-values for the GLM presented in Equation 1

	Estimate	Std. error	z value	p-value
Intercept	6.64	0.55	12.1	<0.001
Counter (participant B)	−0.42	0.14	−3.1	0.002
Counter (participant C)	−1.89	0.14	−13.5	<0.001
Size Fraction (Fractioned)	−1.48	0.15	−10.0	<0.001
Background Fluorescence	−1.02	0.09	−11.3	<0.001
Wells	0.08	0.01	6.5	<0.001
Dry Weight	−2.25	0.47	−4.8	<0.001

$$\begin{aligned}\text{Accuracy}_i &= \alpha + \beta_1 \times \text{Counter}_i + \beta_2 \times \text{Size Fraction}_i + \beta_3 \times \text{Background Fluorescence}_i \\ &+ \beta_4 \times \text{Wells}_i + \beta_5 \times \text{Dry Weight}_i + \varepsilon_i\end{aligned} \tag{1}$$

Under the GLM framework, the counters' accuracies were compared with 'Participant A' as the reference, which showed that counter 'B' had a reduced likelihood of correctly identifying all the lice in the sample and 'C' more so, which was reflected by the estimates of their coefficients −0.42 and −1.89 respectively. The model further indicated that fractioning the sample had a negative impact on correctly enumerating all lice in the sample. The coefficient estimates of −1.02 and −2.25 for background fluorescence and dry weight signify that increases in either reduces the accuracy of the sample count. Increasing the number of wells increases the accuracy with a coefficient of 0.08. The best performing covariate of background fluorescence and the one included in the model was the total number of pixels above an intensity threshold of 7000. In the comparison of alternative models, 4 other thresholds were examined: 3000, 5000, 9000 and 15,000. In order, the AICs of those alternative models were 686.8, 640.9, 639.9 and 661, and their respective explained deviances were 64.9%, 69.5%, 69.6% and 67.4%.

3.4 | Statistical model of sample enumerating time

The full model of enumerating time had an AIC of 999.7, and after model selection and removal of six terms it had an AIC of 1004.6. The first variable removed was counter, followed by accuracy, fluorescent feed, size fraction, sampling sites and last variable removed was process order. The final model (Equation 2) had an adjusted R^2 of 0.652 with 159 degrees of freedom. All covariates in the model were significant with p-values less than 0.05, the parameter estimates

and standard errors are listed in Table 5 along with the specified p-values.

$$\text{Enumerating Time}_i = \alpha + \beta_1 \times \text{Background Fluorescence}_i + \beta_2 \times \text{Season}_i + \beta_3 \times \text{Wells}_i + \beta_3 \times \text{Dry Weight}_i + \varepsilon_i \quad (2)$$

The multiple linear regression model found that all variables in the model had a partial effect that increased the enumerating time. Since the reference season in the model is spring, the seasons of autumn and summer had the effect of increasing enumerating time by 2.84 for the former and 4.56 for the latter. An increase in background fluorescence increased enumeration time as did an increase in number of wells and dry weight, with coefficients of 3.40, 0.52 and 11.57 respectively.

3.5 | Factors influencing enumeration accuracy and time

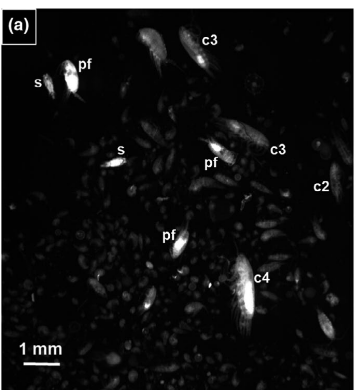
While participants 'A' and 'B' performed their counts on the same day after a single handling and processing, participant 'C' enumerated the samples on a later date after the samples were placed back in formalin and then processed again. Thus, there were additional handling steps that could have contributed to a loss of animals including some of the salmon lice in the spike. In addition to the handling effect, the added processing time exposed the samples to light which can cause photobleaching. The photobleaching could be due to ambient light in the laboratory or from the fluorescence excitation during the enumeration, and it results in a reduction in the fluorescence intensity. In the photobleaching trial, initial mean fluorescence intensity of salmon lice was found to be 6337 and following the simulated handling it decreased by 4.4% to 6061. In the subset exposed to constant illumination, mean fluorescence intensity decreased rapidly and then steadily. After 33 min it was 72.7% of the start intensity, then 68.6% at 53 min and finally 62.2% at 120 min.

TABLE 5 Estimated parameters, standard errors, *t*-values and p-values for the multiple linear regression presented in Equation 2

	Estimate	Std. error	*z* value	p-value
Intercept	−17.92	4.18	−4.3	<0.001
Background Fluorescence	3.40	0.64	5.3	<0.001
Season (Autumn)	2.84	0.94	3.0	0.003
Season (Summer)	4.56	2.15	2.1	0.036
Wells	0.52	0.08	6.7	<0.001
Dry Weight	11.57	3.40	3.4	0.001

Size fractioning may result in the unwanted loss of the spiked salmon lice from samples. The handling step was performed on 20 of the 59 samples enumerated (Table 3). Zooplankton community counts were performed on both the large (>1 mm) and small fraction (<1 mm) of four representative samples from those sample sets. In those cases, 0.7% to 10.2% of the total number of animals were retained in the larger size fraction. The most retained taxon was *Calanus* spp., with an average of 65.4% of their total number found in the larger fraction. However, smaller taxa such as *Acartia* spp. and *Paracalanus* spp. were also retained to a lesser extent in the large fraction, 1.1% and 2.9% of their total respectively. Salmon lice copepodids will pass through a 1 mm mesh in a controlled setting, but during the size fractioning, the larger sized sieve can get clogged by animals that do not pass through it, such as late stage *Calanus* spp. Furthermore, the samples contained Cnidarians and Appendicularians. These and other gelatinous zooplankton have been observed adhering to copepods in preserved samples which may cause the copepods to be retained during size fractioning.

Size fractioning was done to reduce the overall number of large zooplankters in the sample and to specifically remove late stage *Calanus* spp. The non-target copepods examined by Thompson et al. (2021) included early copepodid stage *Calanus* spp. (C1 & C2), which fluoresced at a lower intensity than the salmon lice. The samples in the blind trial included later stages which feature lipid sacs that fluoresced with an intensity comparable to salmon lice (Figure 3a). When the late stage *Calanus* spp. occurred in large numbers, they overwhelmed the sample with fluorescence and that necessitated the size fractioning.



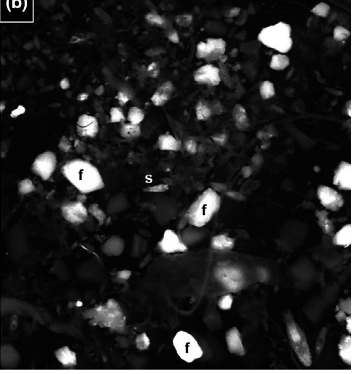


FIGURE 3 Fluorescent image of animals and particles in samples. Sample collected from Masfjorden, Norway in the Autumn (a), and Boknafjorden, Norway in the Spring (b). 's' marks the location of salmon lice copepodids, 'pf' are *Pseudocalanus* spp. females, 'c (2–4)' are *Calanus* spp. copepodid stages 2–4, and 'f' are feed particles from adjacent salmon farms

Fluorescing feed particles from salmon farms had the potential to overwhelm the sample with fluorescence (Figure 3b). Half of the samples from the Norwegian sites contained the fluorescing particles ($n = 16$). It was noted when the particles occurred in the samples and when they were present counters had a median percentage count correct of 42.3%. The median accuracy more than doubled to 89.5% when they were not present.

4 | DISCUSSION

4.1 | Accuracy and enumeration time

The fluorescence-aided method for rapid identification of salmon lice can provide results at 82% accuracy, using far less time, effort and resources than alternative methods. In a comparison of lice enumerating methods, Bui et al. (2021) found light microscopy to be 86% accurate and it took 87 min to process each sample which contained no more than 1531 total animals. Here, the samples contained an average of 11,600 animals and if including the processing time took 31 min to enumerate. Caution should be exercised when making a comparison between these differing datasets, but contrasting the number of animals processed per minute suggests that this novel method is more than 20 times faster. Nevertheless, the statistical model indicates that the more biomass and fluorescent material in a sample, the longer the enumeration will take. In general, the results suggest that enumeration time can be reduced by eliminating extraneous material from the sample. Regardless of whether that is possible, using this method the enumeration of planktonic salmon lice in a mixed zooplankton sample is no longer such a labour-intensive task.

Among the factors affecting accuracy, inter-operator variability was the most concerning, especially the substantial differences between participant 'C' and the other participants. While photobleaching and handling loss are possible contributing factors, inadequate experience and differing operator ability could also contribute to the inaccuracies. Using traditional light microscopy for zooplankton identification and enumeration is a challenging, time-consuming effort that requires considerable experience (Postel et al., 2000). Workers must be given extensive training and quality control should be assured through ring-tests that examine consistency between analysts and laboratories. In a series of ring-tests administered by the NE Atlantic Marine Biological Analytic Quality Control Scheme, 12 to 19 participants were given 10 animals and asked to identify them. Mean accuracies for the three tests were 83%, 84% and 78% with the individual participants' accuracy ranging from 40% to 100% correct (Fischer et al., 2015, 2017; Wootten & Johns, 2019). The fluorescence method described here does not require the same degree of training or expertise as a zooplankton taxonomist since the analyst merely needs to identify only three stages of a single species rather than multiple developmental stages of hundreds of species. However, sorting through the sample and finding the louse can still be a challenging task. In the GLM, the explanatory variables of background fluorescence, dry weight and wells all have coefficients which indicate that accuracy decreases with increasing material in the sample, especially when that material is highly fluorescent. The findings show that these factors can significantly influence the results and should be considered prior to application of the method.

4.2 | Challenges to fluorescence aided enumeration

Along with identification errors, the quantification of zooplankton encounters three more sources of error at the point of the sampling event: the planktonic animals exhibiting avoidance of the sampling gear, escapement from that gear and patchiness (Skjoldal et al., 2013). Some animals are able to avoid plankton nets and so the net must be big enough or towed fast enough to reduce this source of error. It has also been observed that 50% of animals will be extruded through a net's mesh that is equal to its size, thus the size of the mesh must be adjusted to the target animal. The first two sources of error are largely addressed by choosing the sampling equipment which is best suited to addressing the research question (Skjoldal et al., 2000; Wiebe & Benfield, 2003). The third challenge of patchiness, the heterogeneous distribution of plankton in time and space, is a fundamental aspect of the structure and dynamics of ecosystems (Levin, 1992), which has been empirically recognized in plankton since the 1950s (e.g. Barnes & Marshall, 1951). Broadly, it is recognized that both physical and biological mechanisms are responsible for the observed patchiness of zooplankton (Pinel-Alloul, 1995). The forces which drive patchiness will depend on the nested scale the organism exists within. At larger spatial scales, the physical effects predominate and at the smaller spatial scales, zooplankters' habitat preference, food searching and mate searching behaviours will matter more (Pinel-Alloul & Ghadouani, 2007). While, salmon louse ecology is fundamentally different from that of free-living copepods, the chemotactic and phototactic behaviours exhibited by the infectious copepodids (Fields et al., 2018) will also produce patterns of heterogeneous distribution (e.g. Johnsen et al., 2014; Nelson et al., 2018). Regardless, increasing the sample volume and the number of animals counted will counteract the impact of patchiness by reducing the variance and increasing the precision of the estimate (Downing et al., 1987; Postel et al., 2000; Wiebe & Wiebe, 1968). The low abundance of salmon lice and their patchiness suggests that sampled volumes should be very large (i.e. many m^3), which presents challenges due to the factors reported on here; however, targeted sampling can help to mitigate these difficulties.

High abundances of late stage *Calanus* spp. necessitated the additional size fractioning step because the lipid sacs of the *Calanus* spp. were observed to fluoresce, which could obscure the salmon lice. Unfortunately, that handling step had a negative effect on the salmon lice count accuracy and it nonetheless allowed some smaller *Calanus* spp. through. However, the life history of *Calanus* spp. is well studied and that knowledge could be used to avoid capturing the late stage copepodids with fluorescent lipid sacs. They are a pelagic species that are abundant in large numbers in the surface

waters through the spring and summer, but then overwinter at depth (Kaartvedt, 2000). Like many zooplankters, they also exhibit diel vertical migration and depth preferences related to their developmental stage, with later stages preferring deeper waters (Dale & Kaartvedt, 2000; Ji et al., 2017; Kaartvedt, 2000). Thus, late stage *Calanus* spp. could be avoided by sampling shallower depths when their abundance is high. Otherwise, the sampling regime should include a size fractionation step prior to the formalin fixation that would remove large zooplankters including late stage *Calanus* spp. and gelatinous zooplankton. Similarly, fluorescent feed was not included as a variable, but where it occurred, the samples had high background fluorescence which reduced accuracy and increased enumeration time. Thus, if sampling near farms, the site should be upstream of the pens or conducted at a time when there is minimal feed debris in the water. A few specific recommendations can be made here based on the findings from this investigation, but in general, one should avoid sampling any animal or material that has a strong fluorescent signal. However, it may not be possible to implement targeted sampling and in those cases fluorescence-aided enumeration remains a robust method.

4.3 | Towards automation: sampling, fixing, imaging and classifying

The fluorescence-aided enumeration method is distinctly faster than the traditional method using light microscopy and even faster throughput is possible with automation. Here, automation refers to the classification of objects in images as salmon lice, and such automation is most advantageous if it follows a streamlining of the three previous steps: sampling, formalin fixation and imaging. As previously described, the volume of water sampled should be large, targeted to certain times, depths and locations, and processed in a manner that removes unwanted large animals and gelatinous zooplankton. A pumping system would be best capable of achieving these goals: it provides many opportunities for processing the sample prior to fixation, the depth can be specified since the pump samples at a point rather than merging depths/positions like a net, and the volume sampled can be precisely controlled (Wiebe & Benfield, 2003). In a study of planktonic salmon lice abundance, Nelson et al. (2018) found no difference between net samples and pump samples, but they remarked that the pump was more flexible in its deployment. After the sample is collected, it must be preserved in formalin and stored for 90 days at 22°C before the fluorescence signal is reliable. However, that preservation time can be reduced to a week and possibly less through a heat treatment at 42°C (Thompson et al., 2021). Usually, collecting a sample and fixing it in formalin is done by hand by a worker on site, but there are notable exceptions. The continuous plankton recorder is a semi-autonomous sampling device that was conceived by Alister Hardy in the 1920s and continues to be used today. Towed by ships of opportunity, the device collects and filters water continuously, plankton is captured on a silk mesh which is rolled onto a cassette and stored in a formalin solution (Reid et al., 2003). A similarly designed autonomous pump system was developed in 1990 and could be moored for 6 months and configured to take up to 80 samples (Garland, 2000). However, these devices collect the animals on mesh and automated imagining is typically done with flow-through devices that do not interfere with taking rapid unobstructed images of the animals (Benfield et al., 2007). However the sampling is accomplished, it must allow sampling of large volumes of water and facilitate the formalin heat treatment and subsequent imaging of the animals.

After the sampling and formalin preservation steps, automation may be achieved through the controlled capture of many images so that object features such as colour, shape and size can be consistently extracted and then passed on to machine learning algorithms. During a training step, the practitioner classifies objects within images, and the algorithms learn the values of associated features so that they can later independently find those objects which possess the same range of values in sample images. The classification of pelagic objects through machine learning has advanced considerably over the past few decades with the number of classifiable taxa growing from 5 to near 100, and deep learning algorithms replacing the necessity for training data sets (Irisson et al., 2021). The fluorescence signal described here and by Thompson et al. (2021) could be included as a feature for the automatic classification of salmon lice larvae in a sample. If the goal of the automated image classification is to identify salmon lice then the algorithm merely needs to decide if an object is or is not a louse, and the additional use of a fluorescence signal could enable the classification accuracy to be extremely high. Regardless, the primary challenge of enumerating lice in a sample is their relative low abundance and a classification algorithm could serve to select a few objects out of tens of thousands, with these subsequently being confirmed by an expert.

5 | CONCLUSION

The described trial has demonstrated that the novel method of fluorescence-aided enumeration of salmon lice in a mixed zooplankton sample is fast and reliable. However, thoughtful deployment of the method should be exercised following the recommendations described here, to prevent a sample from being overwhelmed with non-target fluorescence. In its current form, the novel method provides a significant advance over current practices and will enable workers to broaden the scope of research into the planktonic stages of salmon lice. After overcoming a number of engineering challenges, automation of the method could enable the widespread surveillance of salmon lice larvae and provide an invaluable additional tool for managing sea lice in aquaculture.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

JB, SB, SD, MF, GN, and RSM conceived of and developed the project. JB, GN, MF, and SB collected and prepared samples. CT designed and carried out the experimental trials of the method with blind control by RSM. CT and SB analyzed the data with input from RSM and SD. CT wrote the initial manuscript draft in consultation with RSM and SB. All authors discussed the results and contributed to editing and revising the final manuscript. RSM provided overall project administration.

ETHICAL APPROVAL

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. The zooplankton samples were collected and preserved in the field and not subjected to any experimental treatments. *Lepeophtheirus salmonis* is a copepod and unregulated by the animal use in research regulations in Norway or the European Union. Animal welfare was supervised by the Norwegian Food Safety Authority (Mattilsynet), with the Bergen salmon louse culture covered by approval number 11912.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available within the article and its Supplementary Materials.

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