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ORIGINAL ARTICLE

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

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

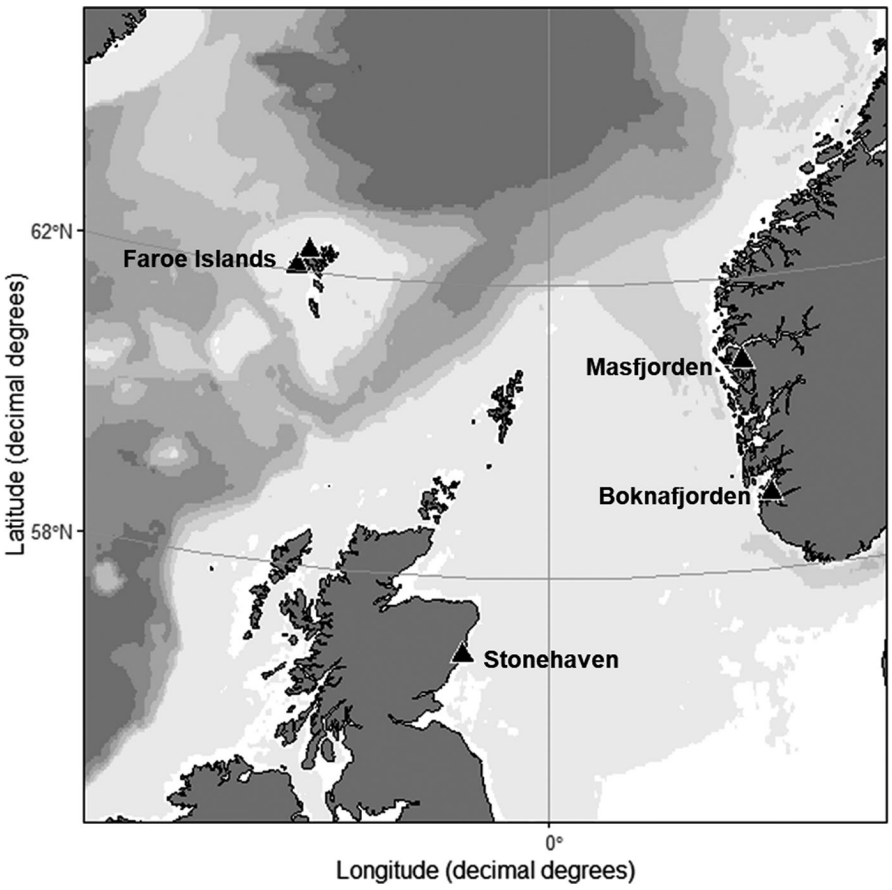

FIGURE 1 Location of sampling. Black triangles indicate sampling locations: Stonehaven in Scotland, Masfjorden and Boknafjorden in Norway, and two locations in the Faroe Islands

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

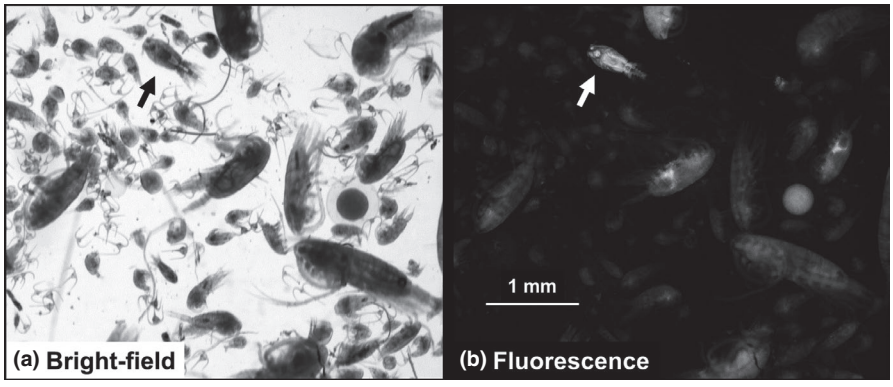

FIGURE 2 Rapid fluorescence enumeration. Under bright-field illumination (a) the target lice are not easily distinguished from the background zooplankton assemblage, but under fluorescence illumination (b) they have a greater fluorescence intensity and stand out, enabling rapid identification and enumeration

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

$$\text{Accuracy}_i = \alpha + \beta_1 \times \text{Counter}_i + \beta_2 \times \text{Size Fraction}_i + \beta_3 \times \text{Background Fluorescence}_i + \beta_4 \times \text{Wells}_i + \beta_5 \times \text{Dry Weight}_i + \varepsilon_i \quad (1)$$

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.

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.

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

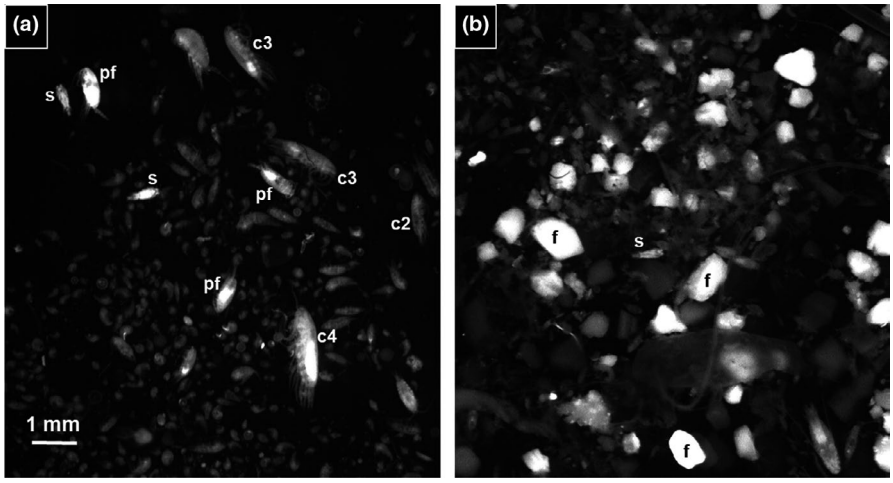

FIGURE 3 Fluorescent image of animals and particles in samples. Sample collected from Masfjorden, Norway in the Autumn (a), and Boknafjorden, Norway in the Spring (b). 's' marks the location of salmon lice copepodids, 'pf' are *Pseudocalanus* spp. females, 'c (2–4)' are *Calanus* spp. copepodid stages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