Received: 7 December 2020 | Revised: 17 February 2021 | Accepted: 20 February 2021

DOI: 10.1111/are.15202

Aquaculture Research WILEY

ORIGINAL ARTICLE

Finding the needle in the haystack: Comparison of methods for salmon louse enumeration in plankton samples

Samantha Bui[1] | Sussie Dalvin[2] | Tone Vågseth[1] | Frode Oppedal[1] | Frode Fossøy[3] | Hege Brandsegg[3] | Ása Jacobsen[4] | Gunnvør á Norði[4] | Mark John Fordyce[5] | Helena Kling Michelsen[2] | Bengt Finstad[6] | Rasmus Skern-Mauritzen[2]

[1]Institute of Marine Research, Matre, Norway

[2]Institute of Marine Research, Bergen, Norway

[3]Norwegian Institute for Nature Research, Trondheim, Norway

[4]Fiskaaling – Aquaculture Research Station of the Faroes, Hvalvík, Faroe Islands

[5]Marine Scotland Science, Aberdeen, UK

[6]Department of Biology, NTNU, Trondheim, Norway

Correspondence
Samantha Bui, Institute of Marine Research, Matre 5984, Norway.
Email: samantha.bui@hi.no

Funding information
This work was supported by the Norwegian Research Council (Grant numbers 244439/E40 for SB, RSM, SD, TV, FO, and 254718 for FF, HB, BF) and Granskingarráðið—the Research Council Faroe Islands (Grant No. 0436 for GáN and ÁJ).

Abstract

The economic and social implications of salmon louse (*Lepeophtheirus salmonis*) epidemics in salmon aquaculture drive focus of the dispersal dynamics of the planktonic larval stages. The vast spatial scale and high connectivity of the marine environment creates difficult conditions to monitor the infective planktonic louse stage, whereby the number of samples required for a representative description is bottlenecked by processing capacity. This study assessed five quantification methods for accuracy and precision in enumeration of lice in plankton samples, validated against the benchmark method of light microscopy. Visual-based (fluorescence microscopy and automated fluid imaging) and molecular-based (droplet digital PCR, quantitative fraction PCR and quantitative PCR) were tested using high- and low-density plankton samples spiked with louse copepodids, with spike numbers blind to assessors. We propose an approach to comparative assessment that uses the collective bias and deviation of a test method to determine whether it is acceptably similar to the benchmark method. Under this framework, no methods passed the comparative test, with only ddPCR comparable to light microscopy (87% mean accuracy and 74% precision). qfPCR and fluorescence microscopy were moderately efficient (88% and 67% accuracy, and 36% and 52% precision respectively). Molecular techniques are currently restricted in distinguishing between larval stages, which is an essential distinction for some research questions, but can be economical in processing numerous samples. Overall method suitability will depend on the research objectives and resources available. These results provide evidence for operational accuracy for the tested methods and highlight the direction for further development to optimize their use.

KEYWORDS
automated fluid imaging, fluorescence, method validation, microscopy, molecular methods

1 | INTRODUCTION

The ectoparasitic salmon louse (*Lepeophtheirus salmonis*) is the single most problematic species for salmon aquaculture in all farming countries in the Northern Hemisphere (Igboeli et al., 2013; Olaussen, 2018; Torrissen et al., 2013). The control and prevention of infestations constrains the sustainability of the industry, and negative interactions with wild populations is a controversial topic (Krkosek et al., 2007; Myksvoll et al., 2018). *Lepeophtheirus salmonis* has eight stages and a simple life cycle: when hatched from eggs, the planktonic louse larvae are lecithotrophic and exist as non-infective nauplii through two stages, followed by the infective copepodid stage (Johnson & Albright, 1991). The copepodid can survive without a host for up to 13 days in low temperatures (Samsing et al., 2016), but the duration of their existence in the plankton is directly influenced by the energy content of the yolk, the consumption of which is highly temperature-dependent (Brooker et al., 2018; Skern-Mauritzen et al., 2020). They have a body length of ~700 μm and width of ~250 μm (Schram, 2004) and a reasonably distinct morphology compared with other copepods, with obvious parasitic-type rostrum, mandibles and maxillipeds (Johnson & Albright, 1991). Their distribution is dictated by behavioural factors and hydrodynamic forces (Crosbie et al., 2019; Johnsen et al., 2016). After a host is found and successfully infested, the louse stays attached to the host and continues to moult through four more stages before reaching the reproductive adult stage (Hamre et al., 2013).

To describe the planktonic abundance, field behaviour and dispersal patterns of copepodids, extensive sampling regimes are required to find the 'needle in a haystack'. In Norwegian fjords, copepodids are predicted to be present between 0 and 5 individuals m^{-2} (www.imr.no/lakseluskart) and occasionally higher; field studies have found a maximum of ≈1 individual pr. 3 m^3 in horizontal tows (S. Bui, unpublished data). Several studies have been conducted in Atlantic salmon farming countries that have had some success in quantifying abundances of nauplii and copepodid in coastal waters using microscopy (á Norði et al., 2016; Costelloe et al., 1998; Nelson et al., 2018; Nilsen, 2016; Penston et al., 2008; Skarðhamar et al., 2019). However, although conventional microscopy has the advantage of being capable of assigning larvae to stage, it is labour-intensive and subject to inter-operator variability, requiring trained personnel.

The ability to map the planktonic distribution of salmon lice is constrained by the effort required to identify copepodids in a plankton sample using light microscopy—it is simply too resource demanding for routine observations to be feasible. To circumvent this, indirect methods of enumerating louse abundance in coastal waters have been utilized, including site-specific louse abundances in farms (Jansen et al., 2012), sentinel cages (Bjørn et al., 2011) and louse abundances on migrating wild salmonids (Serra-Llinares et al., 2014). Ultimately, these approaches do not fill the knowledge gap on actual planktonic abundances of lice.

An alternative approach to microscopy for direct monitoring of salmon louse larvae is the use of molecular methods. Recently, DNA-based identification methods are rising in prevalence as molecular techniques can be a more rapid and accurate approach, particularly in distinguishing species that are morphologically similar (Goffredi et al., 2006; Henzler et al., 2010; Komai et al., 2019; McManus & Katz, 2009; Rocha et al., 2019). Molecular methods reduce the operator dependency and potential error that is associated with manual counts, and are theoretically very precise (i.e. low variability), but accurate (i.e. low bias) quantification remains an obstacle to acceptable efficiency. Automated morphological plankton identification has been developed as a potential solution, through image-based processing whereby plankton are recorded or imaged, and described via human interpretation or automated software post processing (Benfield et al., 2007).

Collecting plankton samples that can provide robust results about *L. salmonis* necessitates a processing method that is more time- and cost-efficient. The issue has prompted the search for alternative methods for processing samples and stimulated the development of solutions from various disciplines. In the present study, we evaluated and compared a selection of methods that are currently in development, varying from microscopy-based to automated imaging, to genetic quantification. We propose a simple framework for assessing the accuracy and precision of the alternative methods as a means of validating their use; a method validated in a laboratory setting could then be applied in the field with a known level of error or imprecision.

2 | MATERIALS AND PROCEDURES

2.1 | Plankton collection and biodiversity description

Plankton for the background stock were collected using a 120-μm WP2 net (diameter = 55 cm; HydroBios GmbH). A combination of vertical and diagonal hauls (20–0 m deep) was conducted in September 2017 in southern (Sandnesfjorden) and south-western (Hjeltefjorden) Norway. Parallel environmental profiles were taken down to 10 m at collection sites using a CastAway-CTD™ profiler (SonTek), which reported water temperatures of ~15°C and salinity ranging from 9 to 29 ppt. At these localities, occurrence of salmon lice was not expected as indicated by the salmon louse dispersal model for the sampling period (www.imr.no/lakseluskart/) and low haul volume. A total of 18 hauls formed the background stock. After collection, samples were immediately rinsed on a 125-μm sieve and preserved using 80% saline ethanol, and soon after transferred to the laboratory for further processing.

Five samples that did not undergo method testing were analysed for a description of the planktonic species assemblage. Subsamples (see Section 2.3) were inspected, with subsample volume ranging from 0.5% to 4.9% of total sample volume. A total of 37 species were identified to at least the genus level, with the most commonly occurring organisms being *Oithona similis*, unidentified copepod nauplii, *Acartia longiremis* and *Pseudocalanus* sp. (Table S2).

2.2 | Target organism

Copepodids used to spike samples (see Section 2.3) were produced at a hatchery facility at the Institute of Marine Research, Bergen. Egg strings from adult females were collected and incubated using the protocol described by Hamre et al. (2009). Larvae were incubated at 10°C and collected after they had reached the copepodid stage.

2.3 | Sample preparation

Upon arrival of all plankton samples in the laboratory, the samples were merged, rinsed once again with 80% saline alcohol, suspended in 2 L 80% saline ethanol and thoroughly mixed using a magnetic stirrer. Two densities of samples were created to test method efficacies with low and high particle concentrations. Thus, a subsample of the zooplankton was removed for further dilution, resulting in a low-density and a high-density plankton sample stock (difference of ~3.8-fold plankton concentration). The low- and high-density stocks were further divided equally, to produce 32 replicate samples per density.

All replicate samples were spiked with either 1, 2, 6 or 11 laboratory-produced *L. salmonis* copepodids, with 11 samples left unmanipulated to serve as negative controls (Table 1). This abundance level is reflective of realistic samples targeting salmon lice (Nelson et al., 2018; Skarðhamar et al., 2019). Each test method received 10 high- and 10 low-density samples (of varying spike levels) to process whole; because of the limited number of samples, all underwent two enumeration tests—first processed by one of the visual methods, followed by one of the molecular methods (Table 1). To guard from the loss of plankton material between enumeration methods, samples were handled carefully to ensure all particles were recaptured and present for the subsequent test.

2.4 | Enumeration

In addition to light microscopy, five enumeration methods were tested for accuracy by comparing the number of lice found with the number of lice spiked into samples. These accuracies were compared to the accuracy of the benchmark method, light

TABLE 1 Organization of samples that were used by the enumeration methods. A paired set of high- and low-density plankton samples (total N = 20–22 per method) were processed by a visual enumeration method, followed by a molecular method. Samples were handled carefully during visual enumerations, to ensure no material was lost for the subsequent test

Biovolume density	Number of spiked copepodids	No. of samples	First method	Subsequent method
High	0	1	Light microscopy	qfPCR
	2	2		
	6	3		
	11	3		
Low	0	1		
	2	3		
	6	3		
	11	3		
High	0	2	Fluorescence microscopy	qPCR
	1	2		
	2	3		
	6	3		
	11	3		
Low	0	1		
	2	3		
	6	3		
	11	2		
High	0	1	Automated fluid imaging	ddPCR
	2	3		
	6	3		
	11	3		
Low	0	1		
	2	3		
	6	3		
	11	3		

microscopy. If any of the tested methods were to be adopted for more conventional use, it would need to be just as good or more accurate (and precise) than microscopy. See Section 2.5 for the validation approach.

In brief, 3 sets of 10 low- and 10 high-density samples were produced, and with six enumeration methods, all series were reused once. Firstly, the three sets of samples were analysed at the Institute of Marine Research, Norway, using one of the three visual-based methods—light microscopy, fluorescence microscopy and automated imaging. Secondly, three separate laboratories each received one of the sets of samples and conducted a molecular method as per the protocol developed in their laboratory. DNA was extracted and used for one of three PCR-based methods (droplet digital PCR, quantitative fraction PCR and quantitative PCR; see Table 1) following the individual laboratory in-house protocols. As each laboratory used specific protocols and equipment, the comparative test among molecular methods compared the complete protocols that were used rather than the individual steps in the protocols. Hereafter, we reference only the method tested, but this incorporates all factors associated with individual protocols of the different laboratories that conducted the test (i.e. purification protocols, primers used, instrument settings).

All personnel involved with enumeration tests were blind to the number of lice spiked in the samples and were unaware of the potential range of lice to be expected or replication of spike levels. For all methods, the number of lice found, and the time taken to process the sample were recorded. Crude cost per sample was estimated in parallel, with labour separated from expendables and equipment costs. The final reported values of estimated number of lice in each sample were used in method assessment.

2.4.1 | Light microscopy

A single person, previously trained in copepodid identification based on morphology, processed all samples using a stereomicroscope (Olympus SZX16). Samples were transferred to a Bogorov plankton counting chamber for enumeration.

2.4.2 | Fluorescence microscopy

Fluorescence microscopy involved the illumination of the sample by UV excitation via a modular LED UV excitation source (Fluorescence Adapter Kit, NightSea™), coupled with an emission filter attached to the same stereomicroscope used for the light microscopy method above (Fordyce, 2017). This approach caused *L. salmonis* copepodids to fluoresce a slightly brighter or different hue than other particles in the sample, which allowed for more rapid screening of the sample by colour/brightness, before closer assessment of the target individual's morphology. The operator was the same person who conducted the light microscopy test. In this method, only the fluorescence colour and intensity were used to distinguish unique individuals, rather than detection through morphology first. Apart from the fluorescence addition to the microscope, the processing method remained the same as for light microscopy.

2.4.3 | Automated fluid imaging

Digital imaging was achieved using a Flow Cytometer and Microscope (FlowCam VS®; Fluid Imaging Technologies), which essentially functions by drawing fluid through a glass flow cell and uses a camera behind a microscope objective lens to continuously image particles within the cell (detailed description in Sieracki et al., 1998). Samples were diluted with 80% saline ethanol to either 90–150 ml for low-density samples, or 500–550 ml for high-density samples. A beaker holding the fluid was placed on a magnetic stirrer and introduced into the machine through a tube, operated by an external peristaltic pump. Flow rate was 7 ml min^{-1} through a 2 × 4 mm flow cell, and images were taken through a 2× objective at a rate of 7 frames s^{-1}. With this set-up, particles were often imaged more than once.

Silhouette images are taken of every particle that flows past the camera and can be later sorted using the post-processing software that is provided with the instrument. The software requires numerous user-created libraries of target and non-target organisms. These libraries provide the basis for filters that are the foundation for automated classification of sample particles. For this study, all classified images and size-filtered unclassified imaged were checked to ensure that any copepodids in the sample were found. Particle measurements provided by the software, specifically length, was used to aid image analysis. All samples were processed and all images assessed by a single person with extensive *L. salmonis* research experience.

2.4.4 | Droplet digital PCR

The plankton samples were first homogenized in 50-ml tubes (1.4 mm Ceramic Matrix-D Beads; MP Biomedicals) at 4.0 rpm for 40 s using a FastPrep-24 homogenizer (MP Biomedicals), in a total volume of 45 ml 80% saline EtOH. From each sample, three subsamples of 500 µl were transferred to three 1.5-ml Eppendorf tubes and dried in a heating cabinet at 56°C. Subsequently, 560 µl ATL buffer (Qiagen) and 60 µl proteinase K (Qiagen) were added to each tube, vortexed and incubated at 56°C overnight. DNA was extracted from each of the subsamples using DNeasy Blood and Tissue Kit (Qiagen) and eluted in 100 µl AE buffer (Qiagen).

The DNA concentration of *L. salmonis* in each subsample was determined using a droplet digital PCR targeting COI (QX200 AutoDG Droplet Digital PCR System; Bio-Rad Laboratories). All subsamples were analysed using a species-specific assay for *L. salmonis* (McBeath et al., 2006) and included a VIC-labelled TaqMan MGB Probe. In a total reaction volume of 22 µl, droplet digital PCRs (ddPCRs) consisted of 3.64 µM of forward and reverse primers (McBeath et al., 2006), 0.86 µM probe (McBeath et al., 2006), dH_2O, ddPCR™ Supermix for Probes (No dUTP; Bio-Rad Laboratories) and 5 or 1 µl DNA template. All subsamples were analysed with both 5 and 1 µl

DNA template to investigate potential PCR inhibition. In each ddPCR run, DNA isolated from *L. salmonis* copepodids was used as a positive control and dH_2O a negative.

Droplets were generated using the AutoDG Instrument (Bio-Rad Laboratories), and PCR amplification was performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems). The following thermal cycling conditions were used: an initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, annealing and extension at 60°C for 1 min, a final step of denaturation at 98°C for 10 min and a final hold at 4°C. The PCR plates were transferred to a QX200 Droplet Reader (Bio-Rad Laboratories) for automatic detection of the fluorescent signal in the droplets. The QUANTASOFT software v.1.7.4 (Bio-Rad) was used to separate positive from negative droplets according to the manufacturer's instructions.

The total number of DNA copies in a sample was calculated based on the concentration estimated by QuantaSoft, template volume (1 or 5 µl), elution volume (100 µl), subsampled volume (500 µl) and total sample volume (4500 µl). As *L. salmonis* larvae were not available at the time of analyses, we could not run single larvae or spike the test samples (see Section 4 below), and the true numbers of lice were thus disclosed for two random samples, (one from the high-density samples containing 11 larvae and one from the low-density samples containing six larvae). Based on these numbers, we estimated that a single larvae contained ca. 3.25 million DNA copies, and this value was used to calculate the number of larvae in the remaining samples.

2.4.5 | Quantitative fraction PCR

Quantitative fraction PCR (qfPCR) derives abundances of organisms in samples based on frequencies of observed positive amplifications by PCR using fractions of a sample as templates. In practical terms, the samples to be analysed were divided into fractions that subsequently were subjected to DNA isolation and a template-specific PCR. The PCR yields a binomial result depending on the presence of the target in the fraction, and the frequency of positive amplifications is then used to calculate a range of probable initial concentration. In this study, the samples were divided into 11 fractions. The results were interpreted by comparison with simulated results (see Section qfPCR—simulated results).

qfPCR—Sample fractionation, DNA isolation and PCR

Samples were divided into 11 fractions by thoroughly mixing using a 50-ml pipette and subsequently dispensing the samples into 11 aliquots of equal volume, hereafter referred to as fractions. These were then centrifuged at room temperature at 300 *g* for 10 min; then, the storage solution containing alcohol was drained off, and the samples were left to evaporate residual ethanol. DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen). After ethanol evaporation, the fractions were resuspended in 540 µl buffer ATL containing 60 µl proteinase K, and transferred to 2 ml microtubes before spinning down and incubating with slight agitation at 56°C overnight. DNA from the fractions was subsequently isolated according to the supplier's protocols, and DNA quality and concentration was assessed using a NanoDrop 1000 instrument.

The fractions were subjected to quantitative PCR (qPCR) targeting COI to allow quantitative evaluation of amplifications. The amplifications were performed in 20 µl reactions with 4 µl (25 ng μl^{-1}) template, 10 µl Fast SYBR® Green Master Mix (2×), 1.6 µl forward primer (10 mM), 1.6 µl reverse primer (10 mM) and 2.8 µl water. The reactions were completed on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) with the 40 cycles of 95°C for 1 s (dissociation) and 20 cycles at 60°C (annealing and extension). The primers (McBeath et al., 2006) used were as follows: LsalmonisCOIfor (GACATAGCTTTCCCCCGCTTA) and LsalmonisCOIrevA (AGTTCCT GCACCACTTTCTACTAATG).

The specificity of the qPCR assay was evaluated by including the appropriate positive and negative controls: Negative reactions were performed on samples using distilled water (NTC controls) or gDNA from *Calanus finmarchicus* (Calanus controls) as template, whereas positive controls utilized gDNA from *L. salmonis* as template. Using automatically set Ct values, all NTC controls showed no amplification, all positive controls were detected before cycle 20, while 2 of 9 Calanus controls reached the threshold values at late cycles (cycles 36.5 and 37.5). It was therefore decided to interpret Ct values above 35 as no amplification and Ct values of 35 or less as positive amplifications. The number of fractions yielding positive amplifications was interpreted in terms of number of larvae according to Section qfPCR—simulated results.

qfPCR—simulated results

A theoretical simulation experiment was performed in R v. 3.5.3 (R Development Core Team, 2019) to predict the number of fractions expected to be positive given that the sample contained a given number of salmon lice. In simulations, larvae from samples containing 1–500 individuals (with an increment of 1) were randomly assigned to one of 11 fractions. The simulation was repeated 100 times for each number of larvae. In the simulations, fractions were given binary values whereby those that received larvae were given the value 1, whereas those that were empty were 0.

The resulting average number of larvae given a certain number of positive wells (0–11) is listed in Table S1, along with the upper and lower 95% confidence quartile limits. It may be argued that most samples will contain only few larvae (i.e. <10; Nelson et al., 2018; Skarðhamar et al., 2019) and that this may cause the simulation to yield misleading results. To assess the potential effect of this, we repeated the simulation with 1–30 lice and added simulations for 250 and 500 larvae to mimic the sporadic occurrences of samples with high larval loads. The results of this are shown in Table S1 and reveal that the potential effect is insignificant at levels regularly encountered (i.e. fewer than 10 larvae).

2.4.6 | Quantitative PCR

The plankton samples were centrifuged at 10,000 *g* for 10 min after which the supernatant was removed. The samples were since put

in a −80°C freezer for 30 min before being dried in a Christ Alpha 1-2 LDplus freeze dryer (Buch & Holm) for 18 h. DNA extraction was performed by the HotSHOT method (Truett et al., 2000), and DNA was used as the template in subsequent qPCRs. First, the lysis buffer was added, enough to ensure that the material could be completely dissolved in the liquid, and the samples were since homogenized using a Bio-Gen PRO200 homogenizer (PRO Scientific). They were since placed in an oven at 95°C for 30 min and then cooled in a refrigerator at 4°C for another 30 min, before the neutralization buffer was added. Ethanol precipitation was performed by adding 1/10 × sample volume of 3 M sodium acetate, pH 5.2, followed by 2.5 × total volume of 99% ethanol. The samples were incubated in a freezer at −18°C for 15 min and thereafter in a refrigerator at 4°C overnight. The following day, the samples were centrifuged at 13,000 *g* for 30 min at 4°C. The supernatants were discarded and the samples rinsed with 4 ml of 70% ethanol and gently mixed and centrifuged again for 15 min. The supernatants were again discarded and the pellets dissolved in 2 ml elution buffer.

The samples were diluted 1:10 for qPCR analysis using the *L. salmonis* primers and probe described in McBeath et al. (2006), and run in triplicates. The qPCR mix contained 4.0 µl of QuantiTect Probe PCR Master Mix (Qiagen), 0.4 µl of 10 µM forward and reverse primers, 0.25 µl of 10 µM sequence-specific FAM probe and 2.95 µl of H_2O. To the mix, 2 µl of DNA was added before being run on a StepOnePlus qPCR instrument (Thermo Fisher). The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Plankton samples collected previously at other locations with known content of *L. salmonis* copepodids were used as standards. The extraction method for these samples was similar as for the samples from this study, only using a Speed-Vacuum dryer instead of freeze drying the samples. The samples that were used as standards were run on qPCR, also in triplicates, along with the present samples. The quantity of copepodids in the test samples was calculated using a previously generated standard curve, based on 16 samples containing 1–12 copepodids.

2.5 | Method validation framework

In order to compare the quantitative efficiency of the utilized methods, we standardized their performance relative to the benchmark method according to Westgard et al. (1974) by assessing both precision and accuracy. Precision is the distribution of individual measurements around the *measured* sample mean (termed random analytical error by Westgard et al., 1974; Figure 1) and represents the variation in acquired results. Accuracy is the extent by which the measured sample mean deviates from the *true* mean (termed systematic analytical error; Figure 1). Westgard et al. (1974) proposed that the random and systematic errors combined are the total analytical error and that acceptable performance requires that the total analytical error must be smaller than the total allowable error.

In relation to plankton enumeration, the allowable error should reflect the aim of the study and statistical distribution of the target species.

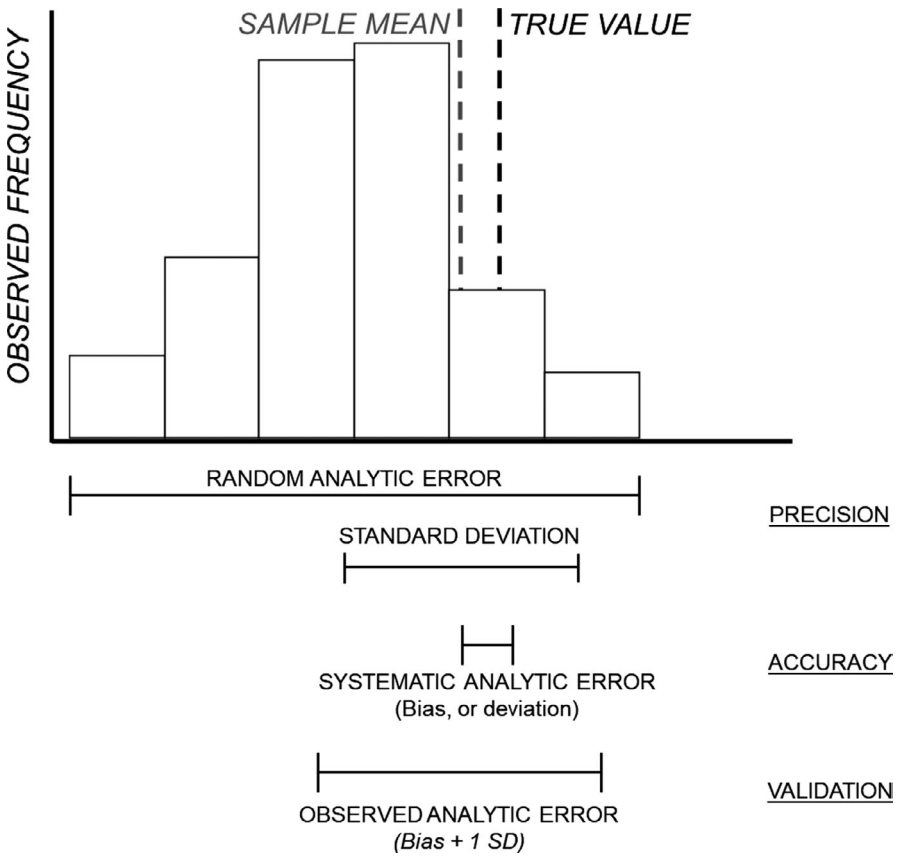

FIGURE 1 A theoretical frequency plot represents replicate samples from a population or larger sampling effort. The total variation around the mean is defined as *random analytic error* and indicates the level of precision (or repeatability). When the sample mean is not aligned with the true value, the discrepancy is termed the *systematic analytic error* (or bias) and reflects accuracy. We suggest that the *observed analytic error* (systematic analytic error + one standard deviation) of the benchmark method be deemed the *allowable analytic error*, providing the threshold when validating a test method. Thus, observed analytic error of test methods should be lower than the allowable limit as set by the benchmark method. In this example, observed analytical error (OAE) is relatively large relative to the spread of data values. Adapted from Westgard et al. (1974)

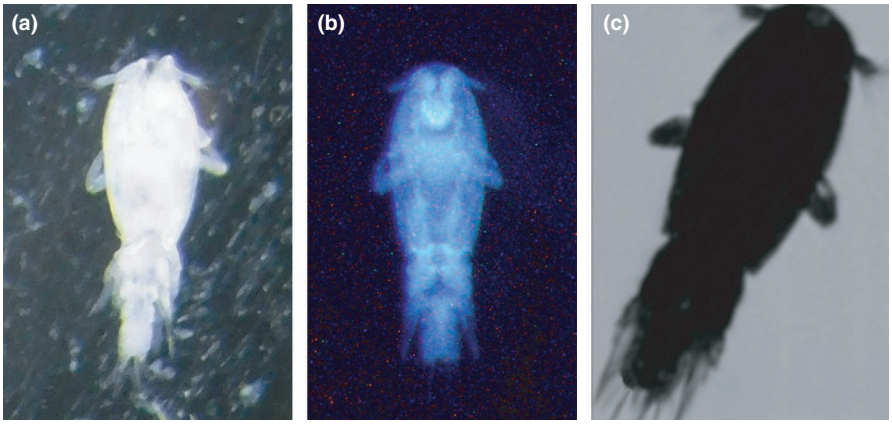

FIGURE 2 Images of salmon louse copepodids from the image-based methods (not on the same scale). Panels show the appearance of copepodids using standard microscopy (a) and fluorescence microscopy (b), and from the automated fluid imaging system (c)

Under numerous scenarios, identification using microscopy is the most accepted and reliable approach, occasionally followed by genetic verification of the target species. Microscopy is not always perfectly accurate and precise; however, the variability of this benchmark method can be used to define the minimum acceptable analytical error in alternative methods that could be an improvement with increased speed or efficiency. We suggest that the criteria for acceptance of a new method for identification and enumeration of a target species should be within the observed analytic error (*bias + 1 standard deviation*) of a benchmark method (Figure 1), in this case, light microscopy. Thus, the allowable analytic error is determined by the observed analytic error from microscopy, which sets the threshold by which other methods must have lower or comparable observed analytic errors to be considered valid.

2.6 | Data analysis

Each method delivered a single value of estimated abundance of copepodids per sample processed. The results from replicate samples were pooled across spike levels used to calculate estimates of accuracy and precision used for method validation. To test for the effect of sample density on method performance, a generalized linear mixed model was used in R (R Development Core Team, 2019) to compare the successful enumeration of copepodids between methods, with sample number as a random factor.

3 | RESULTS

3.1 | Method comparison

No difference in shape or size of copepodids was observed between visual methods (Figure 2). All methods were compared to the baseline method of light microscopy, which had a mean accuracy (sample density and spike level pooled; see Section 3.3) of 86%, and a precision of 77% (Table 2). This resulted in an observed analytic error (OAE) of 33.3 (Table 2), which became the threshold by which other methods would be assessed against. In terms of OAE, only ddPCR acquired a result (36.0) acceptably close to light microscopy (Table 2). Fluorescence microscopy, automated imaging and qfPCR were within the OAE range of 64–77, whereas qPCR was an outlier with eight times the OAE of microscopy (Table 2).

In terms of accuracy, the most efficient methods were qfPCR (87.7%) and ddPCR (mean 86.6%), followed by fluorescence microscopy (67.2%; Table 2, Figure 3). However, the two latter methods exhibited poorer precision, leading to a higher OAE score (Table 2). The automated imaging method had the third-highest precision after ddPCR, but this was not matched by accuracy (Figure 3). qPCR was a significant outlier in both accuracy and precision (Figure 3), largely due to overestimation in samples with low spike numbers (Figure 4).

TABLE 2 Key values evaluating performance of tested methods, pooled over all tested samples (density and spike level). Represented are the accuracy, coefficient of variation (CV), estimate of precision, bias (mean deviation from 100% accuracy) and observed analytical error (OAE) of each method

Method	Accuracy (%)	SD	CV[a]	Precision (%)[b]	Bias[c]	OAE
Light microscopy	86.1	19.4	22.6	77.4	−13.9	33.3
Fluorescence microscopy	67.2	32.1	47.8	52.2	−32.8	64.9
Auto. fluid imaging	51.4	28.1	54.6	45.4	−48.6	76.7
ddPCR	86.6	22.6	26.1	73.9	−13.4	36.0
qfPCR	87.7	55.9	63.8	36.2	−12.3	68.3
qPCR	212.1	154.2	72.7	27.3	112.1	266.3

[a]CV calculated as standard deviation divided by the mean, multiplied by 100.
[b]Percentage precision calculated as 100 minus CV.
[c]Bias calculated as exact accuracy (100%) minus mean observed accuracy.

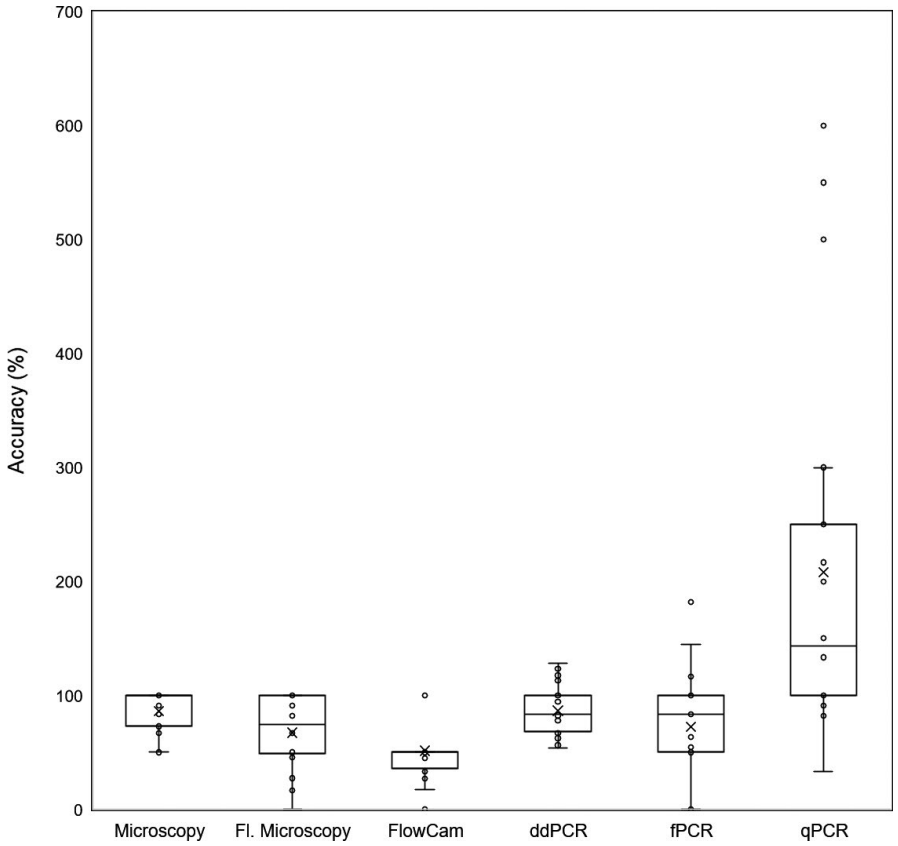

FIGURE 3 Boxplot of percentage accuracy in enumeration of salmon lice from the tested methods, with cross-markers indicating mean values. Methods represented are light microscopy (Microscopy), fluorescence microscopy (Fl. Microscopy), automated fluid imaging (FlowCam), ddPCR, qfPCR and qPCR. Data are pooled over sample densities and spike levels

3.2 | Sample density and number of target specimens

Of the tested methods, the qPCR analysis exhibited the greatest estimate variability between sample densities: results were more accurate with high-density samples (mean overestimate of 51% from 100% accuracy) than with low-density samples (estimates three times the real number), with the latter also displaying larger variation between replicates (Figure 5). Pooling the two densities in the results may mask the potential that qPCR has for high-density samples, and highlights the necessity for the standards to be of similar density as the samples. Thus, results from qPCR were excluded ad hoc from this analysis due to the exceedingly large variation and accuracy values that were not within the realistic range of other methods.

For the remaining methods, the GLMM showed that sample density did not affect method performance (z = 1.2, p = 0.2; Figure 5); hence, data were pooled between high- and low-density samples for OAE calculations (Table 3). Only one test method reported a false positive in the samples with no spiked lice (qfPCR, one sample; Table 3). Deviation and variance (bias) became larger when more copepodids were spiked into samples, particularly for the FlowCam and fluorescence microscopy (Figure 4). In contrast, genetic methods of ddPCR and qfPCR exhibited stable detection rates (after spike levels of 2 or more lice) compared with light microscopy (Figure 4).

3.3 | Processing durations and cost

qPCR was the quickest method to process the entire set of samples, at 7 h compared with 27 for light microscopy (Table 4). The second quickest method was fluorescence microscopy, which took approximately one-third of the duration of light microscopy, indicating a substantially faster procedure. Fluorescence microscopy was also the least expensive method in terms of equipment and expendables (Table 4), particularly with the low cost of lamp and filters compared with PCR instruments. qfPCR was the most expensive and time-consuming method, with similar operation characteristics for ddPCR (Table 4). Automated fluid imaging took comparable processing time to qfPCR and ddPCR, largely due to the need for manual image processing rather than the instrument operation time.

4 | DISCUSSION

When systematically comparing the efficiency of five enumeration methods (to the benchmark technique of light microscopy, only ddPCR was comparative in accuracy and precision of detecting salmon louse copepodids in plankton samples. It should, however, be noted that the HotSHOT DNA isolation method providing template for qPCR, although often used for qualitative detection purposes, may not be suitable for quantitative studies. It was utilized in the present comparison as it is the method used by the

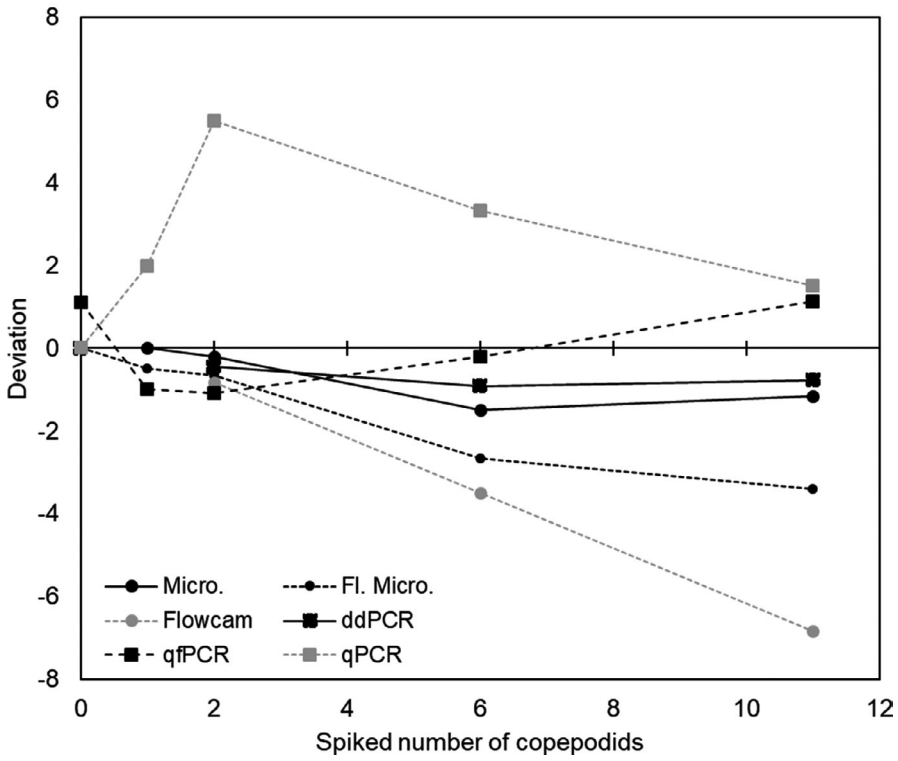

FIGURE 4 Mean deviation from the true value (i.e. mean minus expected counts) across spike levels, for each tested method: light microscopy (micro.), fluorescence microscopy (fl. micro), automated fluid imaging (FlowCam), ddPCR, qfPCR and qPCR. Data are pooled for low- and high-density samples

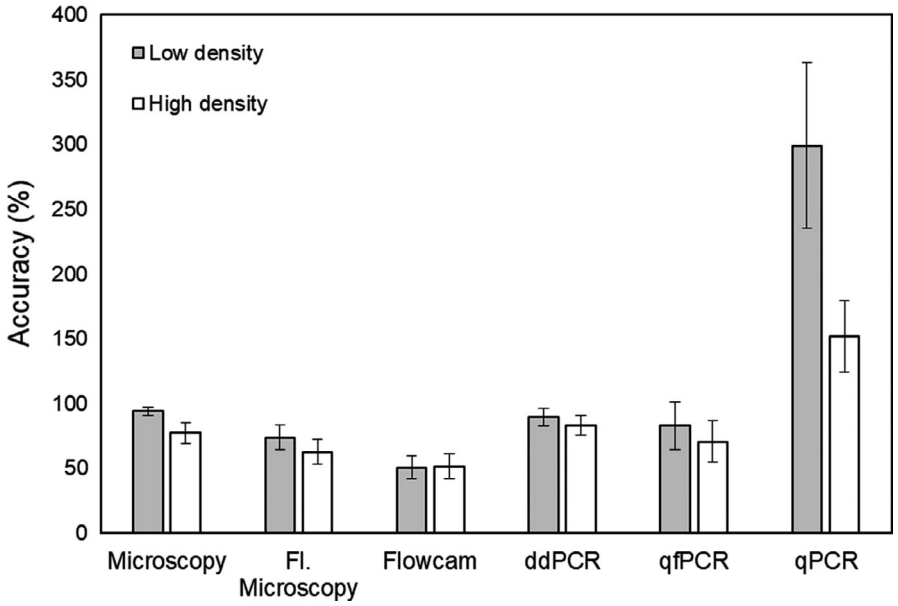

FIGURE 5 Accuracy (% of true value) of test methods with either low- or high-density plankton samples, pooled across spike levels. Methods represented are light microscopy (Micro.), fluorescence microscopy (Fl. micro), automated fluid imaging (FlowCam), ddPCR, qfPCR and qPCR. Error bars represent standard error of the mean

laboratory performing the qPCR analysis and as the goal was to compare protocols in use or under development. The method validation framework functioned intuitively using bias and standard deviation compared with the benchmark, and excluded those methods that did not provide acceptable certainty; for example, ddPCR and FlowCam had similar precision, but only ddPCR recorded a similar observed analytic error due to accuracy. Similarly, the mean accuracy of qfPCR showed a low bias but high variability at a level that did not come within the benchmark threshold of OAE. This demonstrates the usefulness of OAE to compare methods based on both accuracy and precision.

Stock plankton solution and individual samples were not scanned for environmental lice, as the regions were not expected to have any infection pressure at the time of collection. In addition, the volumes of hauls were far lower than what is likely to be able to capture appreciable numbers of individuals; the vertical hauls used here filtered a volume of ~4 m^3, and in regions lacking salmon aquaculture sites, an average of one *L. salmonis* copepodid is found in 30 m^3 (Skarðhamar et al., 2019). Lastly, the only method to scan for environmental lice would be light microscopy, which does not have guaranteed accuracy. As each sample was processed by two enumeration methods, the likelihood of detecting false positives (i.e.

TABLE 3 Mean number of lice found in plankton samples over replicate samples, with number of replicates represented in parentheses. Results are pooled over sample density levels. Dashes indicate that this spike level was not tested for the method

	Number of copepodids spiked into sample				
Method	**0**	**1**	**2**	**6**	**11**
Light microscopy	0 (1)	1 (1)	1.8 (5)	4.5 (6)	9.8 (6)
Fluorescence microscopy	0 (3)	0.5 (2)	1.3 (6)	3.3 (6)	7.6 (5)
Auto. fluid imaging	0 (2)	—	1.2 (6)	2.5 (6)	4.2 (6)
ddPCR	0 (2)	—	1.6 (6)	5.1 (6)	10.2 (6)
qfPCR	1.1 (1)	0 (1)	0.9 (5)	5.8 (6)	12.1 (6)
qPCR	0 (2)	3 (2)	7.5 (6)	9.3 (6)	12.5 (6)

copepodids that were not manually added into the sample) was high; false positives were not detected in these results.

4.1 | Use of visual enumeration methods

The processing time for standard microscopy varies substantially between users, and the time used in the present study (Table 4) was considered by these authors to be very fast compared with previous experiences. Similarly, experience and expertise differ among users, and therefore, accuracy is operator-dependent. Light microscopy remains the most laborious and time-consuming strategy; however, it is the simplest of those tested in terms of the equipment and materials required.

Light microscopy is not only the most simple method, but it also remains the most accurate method for enumeration of salmon lice. However, the additional methods tested in this study provide promising results that encourage further technique development and refinement. Among the visual methods, fluorescence microscopy has the most potential for further development, whereby the lack of accuracy of this method is due to the lack of differential fluorescence in target individuals. Although some specimens were identified through regular morphological detection, the user was instructed to focus on identification using the fluorescence profile as the distinguishing factor. The fluorescence characteristics of *L. salmonis* may be affected by several factors, such as preservation agent, preservation duration or age of individuals. Further investigation into the fluorescence profile of salmon lice could elevate the validity of this technique (Thompson et al., 2020).

On the other hand, the use of automated imaging for enumeration through the FlowCam also has promise, but to refine the method enough to increase its accuracy to an acceptable level is likely to increase the time and effort required to process samples. For instance, increased dilution of samples or increased duration of agitation might bring the processing time to a similar level of light microscopy. The advantage of FlowCam is that the instrument can be left unattended for extended periods, with occasional checks to ensure no clogging of the system and segmenting the data files to ensure a manageable file size. In addition, the classification algorithm could also be improved with more comprehensive libraries to reduce post-processing time, however, is unlikely to reach complete accuracy and precision in detection: it has been estimated that the overall error in FlowCam automated classification is approximately 10% in studies of plankton size structure and composition (Álvarez et al., 2012). Furthermore, in this study all images were checked by the user, and therefore, the unquantified lice that resulted in the low accuracy were likely (a) not imaged, (b) imaged in an angle that was not permissible for visual identification (e.g. from an anterior or posterior direction, or only a portion of the body captured), or (c) imaged in a 'clump' of entangled organisms whereby other individuals would have masked and hidden the target organisms. Figure S1 shows an example of when a copepodid could have easily been hidden by the larger cluster imaged.

TABLE 4 Duration and estimated expenses for each tested method in this study, for the total samples processed for this study (19 for light microscopy and qfPCR, 22 for fluorescence microscopy and qPCR)

Method	**No. of samples processed**	**Time (h)[a]**	**Estimated cost: required equipment**	**Estimated cost: expendables**
Light microscopy	19	27	<€10,000	<€150
Fluorescence microscopy	22	10.5	<€10,000	<€150
Automated fluid imaging (FlowCam)	20	16[b]	>€45,000	<€150
ddPCR	20	15[b]	>€45,000	<€550[c]
qfPCR	19	19[b]	>€45,000	€1200[d]
qPCR	22	7[b]	<€45,000	<€150

[a]Time taken to process all samples.

[b]Time including both processing and image analysis in post processing (FlowCam) or data interpretation (molecular methods).

[c]Includes triplicate isolations of DNA per plankton sample and triplicate ddPCR runs.

[d]The cost does not reflect the cost in a high-throughput system, where the running costs can be expected to be significantly lower.

4.2 | Use of molecular enumeration methods

Molecular methods have a more specific pathway for technique development and refinement. Here, the three tested methods also reflected differences in the operation of the separate laboratories that processed the samples; the overall delivery of each laboratory, technician and protocol can influence performance in addition to the technique itself. Thus, the processing protocols can be adapted, and new protocols can improve accuracy. However, molecular techniques are not likely to replace microscopy completely as morphological identification remains necessary in some cases (McManus & Katz, 2009). In the present study, all three molecular techniques applied are based on the genomic presence of a species-specific part of the COI gene, and thus, stages are undistinguishable. For salmon lice, and potentially other species with multiple planktonic life stages (e.g. *Caligus* spp.), it is important to differentiate between stages when enumerating larvae in a sample; the infective copepodid is the most relevant stage to research and industry, which exhibits different behaviours than nauplii (á Norði et al., 2015; Crosbie et al., 2019). Using alternative molecular markers (e.g. stage-specific mRNA transcripts) may facilitate stage assignment of samples by molecular methods in the future. Aside from the inability to differentiate larval stages, the molecular methods applied were more consistent in estimation, displaying similar variation with increasing number of target animals in the sample, whereas visual methods were more likely to become less efficient with higher quantities of the target animal. One of the main advantages of molecular methods is the large number of samples that can be processed with relatively little increase in time consumption for each additional sample. Moving from a few samples to larger batches enables the possibility of DNA isolation in multi-well plates, reducing both cost and hands-on time. A second advantage of the molecular methods is that samples can be processed with uniformity and variation due to subjectivity of result interpretation could be minimized. The differential characteristics of ddPCR, qfPCR and qPCR as tested in this study influenced the final accuracy recorded. Whereas qPCR is depending on a continuous reading of DNA concentration during a PCR run, the two other methods are end-point methods that are less prone to variation in template concentration, enzyme quality and between-run differences (Hindson et al., 2011). This makes the qPCR method more sensitive to DNA isolation inconsistency and PCR inhibition than the two other methods and may well explain the difference in method performance on high- and low-density samples (Figure 5). Hence, the present study should not be taken to indicate that qPCR is without promise, but rather that extensive refinement of the protocol is required. Interestingly, use of qPCR for quantification seems promising for planktonic copepods in other systems (Jungbluth et al., 2013). It may be speculated that the observed divergence in the present qPCR results may be caused by the different plankton background or methodological challenges, with the DNA isolation protocol being the primary suspect since HotSHOT isolation yields are sensitive to heat treatment duration and results in fragmented DNA (Truett et al., 2000). The different genetic methods were analysed in different laboratories, and isolation of DNA was not standardized across laboratories, with ddPCR and qfPCR using Qiagen's DNeasy kit, whereas qPCR used the HotSHOT method for DNA extraction. Hence, part of the variation among methods could originate from differential treatment of the samples or method of DNA extraction.

4.3 | Method comparison: practicalities and implications

Both visual- and DNA-based methods require training and experience, with morphological identification, use of fluorescence equipment, the use of PCR instruments, laboratory skills and interpretation of outputs. Which method, or combination of methods, to use depends on (a) their validation, (b) the research question and study design and (c) the resources available (see Table 5). For instance, for field studies on the behavioural response of copepodids to environmental conditions, the ability to distinguish between larval stages is essential. However, in studies where total larval abundances is needed (Byrne et al., 2018), molecular quantification would be appropriate and more efficient. A choice between the methods tested in this study would require a trade-off of cost and processing time, where ddPCR was most expensive to acquire equipment and run, but had a comparable processing time to fluorescence microscopy and the FlowCam. Fluorescence microscopy was relatively affordable and had quick processing times, and could be an economic solution (combined with morphological identification) if resources were limited.

The effect of background plankton assemblages and densities on the proficiency of methods tested in this study was not focused on, and little is known about the planktonic assemblages associated with salmon louse larvae. However, there is undoubtedly an effect of the salmon louse density and the abundance and species composition of other zooplankton in the ability to detect a louse in realistic samples—an effect that will vary with the varying plankton assemblages in time and space. For molecular methods, the increased biodiversity and abundance of genetic material may mask the quantification of louse genetic material through several different effects including the presence of PCR inhibitors and similar PCR templates (Sidstedt et al., 2020). Background screening, spiking or standardization between seasonal samples may be necessary. Thus, accuracy and precision reported here should be validated with true plankton samples from the field that would represent the density and plankton assemblage expected from field surveys.

The morphological uniqueness of the salmon louse requires low taxonomic resolution; however, their sparseness in the water masses renders field studies difficult to perform and result in excessive quantities of samples that require processing (Nelson et al., 2018). Many studies concerning salmon lice target their research questions at the larval stages in order to develop prevention or management tools. Understanding the distribution and dispersal of planktonic stages of salmon lice can provide answers to many facets of this problem: this knowledge can confirm behaviours and biology

TABLE 5 Advantages and disadvantages of the operation and efficiency of tested methods

Method	Advantages	Disadvantages
Fluorescence microscopy	Fluorescence microscopy was the simplest method after traditional microscopy, with essentially the same processing procedure as light microscopy with the addition of fluorescent lamps and an emission filter. The target individuals fluoresced a duller yellow colour compared with the non-target organisms, and this was often enough to be able to distinguish them	Not all target specimens fluoresced in the same manner; therefore, not all individuals were identifiable solely through a unique fluorescence profile. Further investigation into the auto-fluorescent profile of copepodids is required to understand under what conditions a consistent and unique fluorescence would be observed
Automated fluid imaging	Use of FlowCam was relatively straightforward and user-friendly. The processing of fluid samples ran independently, thus requiring little hands-on time	There was a consistently high frequency of clumping and aggregation of individual plankton specimens (Figure S1), particularly due to species with antennae and appendages that were more 'sticky'. This resulted in multiple individuals passing the camera together, and the post-processing software considering them a single particle (Figure S1); filtering and sorting of images were therefore not accurate, and more time was required to go through the images to seek potential matches (Table 4). The occurrence of clumping was not improved with increased dilution or time being agitated prior to processing through the FlowCam
ddPCR	ddPCR was the only technique that exhibited adequate accuracy and precision to reliably enumerate copepodids in a plankton sample. The methodology for this technique is relatively straightforward with little risk of contamination	Because of the unknown average of DNA copies per louse in these specific samples, the processing team were given the number of lice in one high- and one low-density sample to provide the baseline quantities of DNA copies per louse, which was then used to estimate quantities in the remaining samples. This could potentially have influenced the accuracy of the methodology, but not the precision. For future use, the method would need to be adapted to cater for unknown mean DNA copies per louse, which may change seasonally or regionally
qfPCR	qfPCR relies on fractions of the samples to be generated *before* isolation of DNA. Hence, a plankton splitter, or other means of creating aliquots, is required. A drawback from this is increased handling of the sample, while a benefit is that the nature of the sample (e.g. density) becomes apparent to the operator	The method requires many DNA isolations, and interpretation requires generation of a table similar to Table S1 customized to the number of fractions generated
qPCR	Standard method used in many laboratories	The instrument is expensive, and generation of a realistic standard curve is required

of larval stages (Brooker et al., 2018), ground truth to depth-related cage prevention technologies (such as submerged or snorkel cages, or louse skirts; Geitung et al., 2019; Sievers et al., 2018; Stien et al., 2016, 2018), validate particle dispersal models that form the basis of zone management (Adams et al. ,2012, 2016; Asplin et al., 2011; Myksvoll et al., 2018; Salama et al., 2013; Sandvik et al., 2016) and substantiate realistic impacts of farm presence on infestation pressure in coastal waters (á Norði et al., 2016; McKibben & Hay, 2002; Nelson et al., 2018; Penston et al., 2011). This study demonstrates the capacity for ddPCR as a solution for louse quantification, but also highlights the potential of visual-based techniques for further advancement towards an acceptable accuracy level.

5 | CONCLUSIONS

This study highlights some of the current available enumeration methods for planktonic salmon lice, indicating the experienced weaknesses and areas for potential development. From these results, ddPCR was the only technique comparable to the benchmark method, although different developmental stages could not be separated. With the increasing demand to characterize the epidemiology of salmon lice, new or further refined methods will undoubtedly arise in the near future, which should be tested against the benchmark method (light microscopy) and validated using the framework proposed in this study. This framework and these tested techniques could be applied to other species that require enumeration in plankton surveys.

ACKNOWLEDGEMENTS

The authors are grateful to Stig Mæhle, Eirikur Danielsen, Elin Jacobsen and the other colleagues for their field and laboratory assistance.

CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

All authors contributed substantially to this article; SB, SD, FO, FF, ÁJ, GáN, BF, MJF and RS-M conceived and designed the study, and conducted data acquisition/analysis along with TV, HB and HKM. All authors contributed to drafting the manuscript and approved of the final submitted version.

ETHICS STATEMENT

This study adhered to the Norwegian Food Safety Authority (Mattilsynet) regulations for ethical use of animals in research, whereby lice were produced using salmon hosts according to application ID 11912.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Samantha Bui https://orcid.org/0000-0002-7885-2989
Sussie Dalvin https://orcid.org/0000-0002-6092-4710
Frode Oppedal https://orcid.org/0000-0001-8625-0331
Gunnvør á Norði https://orcid.org/0000-0003-1550-6007
Bengt Finstad https://orcid.org/0000-0003-3796-0884
Rasmus Skern-Mauritzen https://orcid.org/0000-0002-6983-8907

REFERENCES

á Norði, G., Simonsen, K., Danielsen, E., Eliasen, K., Mols-Mortensen, A., Christiansen, D. H., Steingrund, P., Galbraith, M., & Patursson, Ø. (2015). Abundance and distribution of planktonic *Lepeophtheirus salmonis* and *Caligus elongatus* in a fish farming region in the Faroe Islands. *Aquaculture Environment Interactions*, *7*, 15–27.

á Norði, G., Simonsen, K., & Patursson, Ø. (2016). A method of estimating in situ salmon louse nauplii production at fish farms. *Aquaculture Environment Interactions*, *8*, 397–405.

Adams, T. P., Aleynik, D., & Black, K. D. (2016). Temporal variability in sea lice population connectivity and implications for regional management protocols. *Aquaculture Environment Interactions*, *8*, 585–596.

Adams, T., Black, K., MacIntyre, C., MacIntyre, I., & Dean, R. (2012). Connectivity modelling and network analysis of sea lice infection in Loch Fyne, west coast of Scotland. *Aquaculture Environment Interactions*, *3*, 51–63.

Álvarez, E., López-Urrutia, Á., & Nogueira, E. (2012). Improvement of plankton biovolume estimates derived from image-based automatic sampling devices: Application to FlowCAM. *Journal of Plankton Research*, *34*, 454–469.

Asplin, L., Boxaspen, K. K., & Sandvik, A. D. (2011). Modeling the distribution and abundance of planktonic larval stages of *Lepeophtheirus salmonis* in Norway. In *Salmon lice* (pp. 29–50). Wiley-Blackwell.

Benfield, M. C., Grosjean, P., Culverhouse, P. F., Irigoien, I., Sieracki, M. E., Lopez-Urrutia, A., Dam, H. G., Hu, Q., Davis, C. S., Hanson, A., Pilskaln, C. H., Riseman, E. M., Schultz, H., Utgoff, P. E., & Gorsky, G. (2007). Research on automated plankton identification. *Oceanography*, *20*, 172–187.

Bjørn, P. A., Sivertsgård, R., Finstad, B., Nilsen, R., Serra-Llinares, R. M., & Kristoffersen, R. (2011). Area protection may reduce salmon louse infection risk to wild salmonids. *Aquaculture Environment Interactions*, *1*, 233–244.

Brooker, A. J., Skern-Mauritzen, R., & Bron, J. E. (2018). Production, mortality, and infectivity of planktonic larval sea lice, *Lepeophtheirus salmonis* (Krøyer, 1837): Current knowledge and implications for epidemiological modelling. *ICES Journal of Marine Science*, *75*, 1214–1234.

Byrne, A. A., Pearce, C. M., Cross, S. F., Jones, S. R. M., Robinson, S. M. C., Hutchinson, M. J., Miller, M. R., Haddad, C. A., & Johnson, D. L. (2018). Planktonic and parasitic stages of sea lice (*Lepeophtheirus salmonis* and *Caligus clemensi*) at a commercial Atlantic salmon (*Salmo salar*) farm in British Columbia, Canada. *Aquaculture*, *486*, 130–138.

Costelloe, M., Costelloe, J., O'Donohoe, G., Coghlan, N. J., Oonk, M., & Van Der Heijden, Y. (1998). Planktonic distribution of sea lice larvae, *Lepeophtheirus salmonis*, in Killary Harbour, west coast of Ireland. *Journal of the Marine Biological Association of the United Kingdom*, *78*, 853–874.

Crosbie, T., Wright, D. W., Oppedal, F., Johnsen, I. A., Samsing, F., & Dempster, T. (2019). Effects of step salinity gradients on salmon lice larvae behaviour and dispersal. *Aquaculture Environment Interactions*, *11*, 181–190

Fordyce, M. J. (2017). An improved method for the visualisation of salmon louse *Lepeophtheirus salmonis* copepodids from plankton by fluorescence stereomicroscopy. In *18th International Conference on Diseases of Fish and Shellfish*.

Geitung, L., Oppedal, F., Stien, L. H., Dempster, T., Karlsbakk, E., Nola, V., & Wright, D. W. (2019). Snorkel sea-cage technology decreases salmon lice infestation by 75% in a full-cycle commercial test. *International Journal for Parasitology*, *49*:843–846.

Goffredi, S. K., Jones, W. J., Scholin, C. A., Marin, R. 3rd, & Vrijenhoek, R. C. (2006). Molecular detection of marine invertebrate larvae. *Marine Biotechnology (NY)*, *8*, 149–160.

Hamre, L. A., Eichner, C., Caipang, C. M., Dalvin, S. T., Bron, J. E., Nilsen, F., Boxshall, G., & Skern-Mauritzen, R. (2013). The salmon louse *Lepeophtheirus salmonis* (Copepoda: Caligidae) life cycle has only two Chalimus stages. *PLoS One*, *8*, e73539.

Hamre, L. A., Glover, K. A., & Nilsen, F. (2009). Establishment and characterisation of salmon louse (*Lepeophtheirus salmonis* (Kroyer 1837)) laboratory strains. *Parasitology International*, *58*, 451–460.

Henzler, C. M., Hoaglund, E. A., & Gaines, S. D. (2010). FISH-CS—A rapid method for counting and sorting species of marine zooplankton. *Marine Ecology Progress Series*, *410*, 1–11.

Hindson, B. J., Ness, K. D., Masquelier, D. A., Belgrader, P., Heredia, N. J., Makarewicz, A. J., Bright, I. J., Lucero, M. Y., Hiddessen, A. L., Legler, T. C., Kitano, T. K., Hodel, M. R., Petersen, J. F., Wyatt, P. W., Steenblock, E. R., Shah, P. H., Bousse, L. J., Troup, C. B., Mellen, J. C., … Colston, B. W. (2011). High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Analytical Chemistry*, *83*, 8604–8610

Igboeli, O. O., Burka, J. F., & Fast, M. D. (2013). *Lepeophtheirus salmonis*: a persisting challenge for salmon aquaculture. *Animal Frontiers*, *4*, 22–32.

Jansen, P. A., Kristoffersen, A. B., Viljugrein, H., Jimenez, D., Aldrin, M., & Stien, A. (2012). Sea lice as a density-dependent constraint to salmonid farming. *Proceedings of the Royal Society B: Biological Sciences*, *279*, 2330–2338.

Johnsen, I. A., Asplin, L. C., Sandvik, A. D., & Serra-Llinares, R. M. (2016). Salmon lice dispersion in a northern Norwegian fjord system and the impact of vertical movements. *Aquaculture Environment Interactions*, *8*, 99–116.

Johnson, S. C., & Albright, L. J. (1991). The development stages of Lepeophtheirus salmonis (Krøyer, 1837) (Copepoda: Caligidae). *Canadian Journal of Zoology 69*, 929–950

Jungbluth, M. J., Goetze, E., & Lenz, P. H. (2013). Measuring copepod naupliar abundance in a subtropical bay using quantitative PCR. *Marine Biology*, *160*, 3125–3141.

Komai, T., Gotoh, R. O., Sado, T., & Miya, M. (2019). Development of a new set of PCR primers for eDNA metabarcoding decapod crustaceans. *Metabarcoding and Metagenomics*, *3*, 1–19.

Krkosek, M., Ford, J. S., Morton, A., Lele, S., Myers, R. A., & Lewis, M. A. (2007). Declining wild salmon populations in relation to parasites from farm salmon. *Science*, *318*, 1772–1775.

McBeath, A., Penston, M. J., Snow, M., Cook, P. F., Bricknell, I. R., & Cunningham, C. O. (2006). Development and application of real-time PCR for specific detection of *Lepeophtheirus salmonis* and *Caligus elongatus* larvae in Scottish plankton samples. *Diseases of Aquatic Organisms*, *73*, 141–150.

McKibben, M. A., & Hay, D. W. (2002). *Planktonic distribution of sea lice* Lepeophtheirus salmonis *larvae in inter-tidal plankton samples in Loch Shieldaig, Western Scotland in relation to local salmon farm production cycles, p. ICES CM 2002/T:2006*. International Council for the Exploration of the Sea.

McManus, G. B., & Katz, L. A. (2009). Molecular and morphological methods for identifying plankton: What makes a successful marriage? *Journal of Plankton Research*, *31*, 1119–1129.

Myksvoll, M. S., Sandvik, A. D., Albretsen, J., Asplin, L., Johnsen, I. A., Karlsen, Ø., Kristensen, N. M., Melsom, A., Skardhamar, J., & Ådlandsvik, B. (2018). Evaluation of a national operational salmon lice monitoring system-From physics to fish. *PLoS One*, *13*, e0201338.

Nelson, E. J., Robinson, S. M. C., Feindel, N., Sterling, A., Byrne, A., & Pee Ang, K. (2018). Horizontal and vertical distribution of sea lice larvae (*Lepeophtheirus salmonis*) in and around salmon farms in the Bay of Fundy. *Journal of Fish Diseases*, *41*, 885–899.

Nilsen, M. K. (2016). *Sampling strategies, distribution and concentration of planktonic salmon lice copepods in the outer Hardangerfjord and the Altafjord*. University of Tromsø.

Olaussen, J. O. (2018). Environmental problems and regulation in the aquaculture industry. Insights from Norway. *Marine Policy*, *98*, 158–163.

Penston, M. J., McBeath, A. J. A., & Millar, C. P. (2011). Densities of planktonic *Lepeophtheirus salmonis* before and after an Atlantic salmon farm relocation. *Aquaculture Environment Interactions*, *1*, 225–232.

Penston, M. J., Millar, C. P., Zuur, A., & Davies, I. M. (2008). Spatial and temporal distribution of *Lepeophtheirus salmonis* (Kroyer) larvae in a sea loch containing Atlantic salmon, *Salmo salar* L., farms on the north-west coast of Scotland. *Journal of Fish Diseases*, *31*, 361–371.

R Development Core Team. (2019). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Rocha, G. M., Martinelli, A. B., Utz, L. R. P., & Fernandes, L. F. L. (2019). Using internal transcribed spacers for Calanoida (Crustacea, Copepoda) species identification. *Marine Biodiversity*, *49*, 1565–1571.

Salama, N. K., Collins, C. M., Fraser, J. G., Dunn, J., Pert, C. C., Murray, A. G., & Rabe, B. (2013). Development and assessment of a biophysical dispersal model for sea lice. *Journal of Fish Diseases*, *36*, 323–337.

Samsing, F., Oppedal, F., Dalvin, S., Johnsen, I., Vågseth, T., & Dempster, T. (2016). Salmon lice (*Lepeophtheirus salmonis*) development times, body size, and reproductive outputs follow universal models of temperature dependence. *Canadian Journal of Fisheries and Aquatic Sciences*, *73*, 1841–1851.

Sandvik, A. D., Bjørn, P. A., Ådlandsvik, B., Asplin, L., Skarðhamar, J., Johnsen, I. A., Myksvoll, M., & Skogen, M. D. (2016). Toward a model-based prediction system for salmon lice infestation pressure. *Aquaculture Environment Interactions*, *8*, 527–542.

Schram, T. A. (2004). Practical identification of pelagic sea lice larvae. *Journal of Marine Biological Association UK*, *84*, 103–110.

Serra-Llinares, R. M., Bjørn, P. A., Finstad, B., Nilsen, R., Harbitz, A., & Berg, M. (2014). Salmon lice infection on wild salmonids in marine protected areas: an evaluation of the Norwegian 'National Salmon Fjords'. *Aquaculture Environment Interactions*, *5*, 1–16.

Sidstedt, M., Rådström, P., & Hedman, J. (2020). PCR inhibition in qPCR, dPCR and MPS—Mechanisms and solutions. *Analytical and Bioanalytical Chemistry*, *412*, 2009–2023.

Sieracki, C. K., Sieracki, M. E., & Yentsch, C. S. (1998). An imaging-in-flow system for automated analysis of marine microplankton. *Marine Ecology Progress Series*, *168*, 285–296.

Sievers, M., Korsøen, O., Dempster, T., Fjelldal, P. G., Kristiansen, T., Folkedal, O., & Oppedal, F. (2018). Growth and welfare of submerged Atlantic salmon under continuous lighting. *Aquaculture Environment Interactions*, *10*, 501–510.

Skarðhamar, J., Nilsen Fagerli, M., Reigstad, M., Sandvik, A. D., & Bjørn, P. A. (2019). Sampling planktonic salmon lice in Norwegian fjords. *Aquaculture Environment Interactions*, *11*, 701–715.

Skern-Mauritzen, R., Sissener, N. H., Sandvik, A. D., Meier, S., Sævik, P. N., Skogen, M. D., Vågseth, T., Dalvin, S., Skern-Mauritzen, M., & Bui, S. (2020). Parasite development affect dispersal dynamics; infectivity, activity and energetic status in cohorts of salmon louse copepodids. *Journal of Experimental Marine Biology and Ecology*, *530–531*, 151429.

Stien, L. H., Dempster, T., Bui, S., Glaropoulos, A., Fosseidengen, J. E., Wright, D. W., & Oppedala, F. (2016). 'Snorkel' sea lice barrier technology reduces sea lice loads on harvest-sized Atlantic salmon with minimal welfare impacts. *Aquaculture*, *458*, 29–37.

Stien, L. H., Lind, M. B., Oppedal, F., Wright, D. W., & Seternes, T. (2018). Skirts on salmon production cages reduced salmon lice infestations without affecting fish welfare. *Aquaculture*, *490*, 281–287.

Thompson, C., Bron, J., Bui, S., Dalvin, S., Fordyce, M. J., Furmanek, T., á Norði, G., & Skern-Mauritzen, R. (2020). Illuminating the planktonic stages of salmon lice: a unique fluorescence signal for rapid identification of a rare copepod. *Journal of Fish Diseases*, early view, https://doi.org/10.1111/jfd.13345

Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O. T., Nilsen, F., Horsberg, T. E., & Jackson, D. (2013). Salmon lice – Impact on wild salmonids and salmon aquaculture. *Journal of Fish Diseases*, *36*, 171–194.

Truett, G. E., Heeger, P., Mynatt, R. L., Truett, A. A., Walker, J. A., & Warman, M. L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and Tris (HotSHOT). *BioTechniques*, *29*, 52–54.

Westgard, J. O., Carey, R. N., & Wold, S. (1974). Criteria for judging precision and accuracy in method development and evaluation. *Clinical Chemistry*, *20*(7), 825–833.

SUPPORTING INFORMATION

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

How to cite this article: Bui S, Dalvin S, Vågseth T, et al. Finding the needle in the haystack: Comparison of methods for salmon louse enumeration in plankton samples. *Aquaculture Research*. 2021;00:1–14. https://doi.org/10.1111/are.15202