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Published in:
OSA Continuum

Link to article, DOI:
10.1364/OSAC.389089

Publication date:
2020

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Nielsen, J. H., Pedersen, C., Kørboe, T., Nikolajsen, T., Brydegaard, M., & Rodrigo, P. J. (2020). Dual-band fluorosensor for discriminating non-eating from algae-eating zooplankton in aquatic environments. OSA Continuum, 3(7), 1730-1738. https://doi.org/10.1364/OSAC.389089

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Dual-band fluorosensor for discriminating non-eating from algae-eating zooplankton in aquatic environments

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Abstract: We present a fluorosensor for the detection of laser-induced autofluorescence of zooplankton in marine environments. The sensor uses an inexpensive 410 nm laser diode as excitation source and simultaneously measures two fluorescence bands, 500-550 nm and 675-725 nm, using two identical 16-bit linear array detectors. We show continuous measurements at 200 Hz of zooplankton swimming through a water volume illuminated by the 410 nm laser. The sensor can distinguish salmon lice (Lepeophtheirus salmonis) larvae from an algae-eating reference species (Acartia tonsa) with a sensitivity of up to 99%. The system successfully differentiates the two species using mixed-species cultures at different ratios. This work shows the potential of fluorescent pest monitoring in the salmon farming industry and paves the way for single-ended aquatic lidars.

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1. Introduction

In the past decade, we have seen a growing interest in the use of photonics technologies to monitor and survey fauna in both air and water [1]. For marine and freshwater ecology, progress has particularly been made in optical monitoring of zooplankton – small aquatic fauna with sizes from tens of micrometers to a few millimeters. The attention gained by in situ optical monitoring systems from both the scientific community and the aquaculture industry is due to the advantages of optical systems over conventional manual sampling, which is cumbersome, time-consuming, and unable to provide real-time data. A number of optical systems have been developed based on different optical properties of target species. These systems have been dominated by camera-based approaches such as the video plankton recorder and the optical plankton counter [2] and holographic based techniques [3]. Laser-induced fluorescence (LIF) based systems have been used for in situ monitoring and classification of chlorophyll-rich phytoplankton [4,5], and remote sensing of water quality [6] but have yet to be applied to underwater sensing of zooplankton.

In this work, we present an extension of recent findings from our LIF microspectroscopy study, which show that six zooplankton species possess observable differences in their autofluorescence spectra [7]. In our previous study, one evident feature is the presence or absence of a peak near 680 nm (i.e. chlorophyll a fluorescence [8]) in the zooplankton’s LIF spectrum – indicating whether the animal is algae-eating or non-algae-eating, respectively. Here, we develop a compact dual-band autofluorescence detection system designed for discriminating a non-eating zooplankton species of interest, salmon lice copepodid (Lepeophtheirus salmonis), from similarly
shaped algae-eating zooplankton. Salmon lice are parasitic pests in salmon aquaculture and have significant economic impact on the industry [9]. Current monitoring is almost exclusively conducted manually on adult lice after infection, while optical monitoring on the parasitic larval stages would provide continuous information about their abundance and distribution. Such knowledge could improve treatment planning, mathematical spread models, and infection prevention. Most crustacean zooplankton feed on algae, and so to identify a non-eating crustacean zooplankter narrows the field of possible species considerably. The only other major non-eating group in coastal water are the cypris larvae of barnacles, but they could be distinguished from salmon lice larvae if differences in autofluorescence spatial distribution are exploited [7]. In this preliminary (proof-of-concept) investigation, the presented instrument is tested in a laboratory setting with the animals swimming in filtered seawater inside a water tank positioned at some distance from the optical system – akin to a setup in a recent work on hyperspectral aquatic lidar [10]. The following sections describe the experimental setup and the results that characterize the performance of the current zooplankton fluorosensor. Finally, we discuss the potential challenges that we face in realizing an experimental demonstration in the field.

2. Experimental setup and methods

A dual-band fluorescence detection system was built as sketched in Fig. 1(a). A water volume containing zooplankton was illuminated using a collimated violet 410 nm, 500 mW laser diode, LD, with beam size $3 \times 5$ mm. An achromatic doublet lens (Thorlabs, AC508-100-A-ML), L, with a focal length of 100 mm was used to collect the induced fluorescence at 90° from incidence. The fluorescence was imaged unto two identical 16-bit linear array detectors (Synertronic Designs, LineScan-I-Gen2), LA1 and LA2, using a longpass dichroic mirror (Thorlabs, DMLP550L), DM, with a cut-on wavelength of 550 nm. For each detector, a filter set was used to dictate the relevant fluorescence band and eliminate elastic light from the laser. FS1 contains an OD5 longpass filter with a cut-on wavelength of 500 nm (Thorlabs, FELH0500) and an OD4 bandpass filter of 50 nm bandwidth and center wavelength at 525 nm (Edmund Optics #86-951). FS2 contains an OD6 longpass filter with a cut-on wavelength of 550 nm (Thorlabs, FEL0550) and an OD4 bandpass filter of 50 nm bandwidth and center wavelength at 700 nm (Edmund Optics #84-787).

![Fig. 1.](image-url)

(a) Schematic overview of the setup. A collimated beam from a 410 nm laser diode, LD, illuminates a volume of water with free-swimming zooplankton. An achromatic lens, L, images the induced fluorescence unto two linear array detectors, LA1 and LA2, using a dichroic mirror, DM, with a cut-on wavelength of 550 nm. Two filter sets, FS1 and FS2, are used to filter the relevant wavelength bands of 500-550 nm and 675-725 nm, respectively. (b) Fluorescence spectra of $L. salmonis$ and $A. tonsa$, with the spectral bands of FS1 and FS2 (shaded regions).
The fluorescence bands are chosen based on the fluorescence spectra of zooplankton obtained in our previous work [7]. The typical spectra of L. salmonis and A. tonsa are shown in Fig. 1(b), with the bands as defined by FS1 and FS2, marked in the colored boxes. For simplicity, the bands are in the following referred to as the cyan channel (500-550 nm) and the red channel (675-725 nm) based on the color of the fluorescence light measured in each band. The magnification of 1/10 is chosen so the image of a salmon louse covers a minimum of 5 pixels, and most of the laser beam is imaged within the 200 µm height of the detector pixel (pixel size is 14 µm × 200 µm). Spatially overlapping the field-of-view of the two detectors was done by imaging a fluorescent bead that contained fluorescence in both bands. Linear array detectors were chosen over 2D cameras to allow high framerate to temporally resolve animal transiting the beam, while also limiting the amount of collected data. Employment of linear arrays in this setup also paves the way for future retrieval of oscillatory properties.

The larvae of salmon lice do not eat phytoplankton while A. tonsa are algae-eating. L. salmonis in the parasitic copepodite life stage were supplied by the Industrial and Aquatic Laboratory (ILAB), Bergen, Norway and were kept at 10°C. The A. tonsa in their adult life stage were supplied by DTU Aqua and were kept at 18°C. A species from the Acartia genus was chosen to show the identification capabilities of the system since this genus is abundant in the North Sea [11] and has approximately the same size as the salmon lice copepodites. Further, findings in our previous work [7] showed similar fluorescence spectra from different algae-eating species (including A. tonsa), and we concluded that a single species would be sufficient to investigate the classification capabilities of our proposed dual-band LIF based zooplankton sensor. Adult A. tonsa are 1.2 mm long while copepodite L. salmonis are 0.8 mm long on average [12]. The animals used in this study are alive and swim freely in the water during measurements.

The experiments were performed using a framerate of 200 Hz, and an exposure time of 4.98 ms, which was found to best utilize the dynamic range (16-bit) of the detectors. The linear array detectors were set to output a file every 30 seconds, resulting in data files, each of dimensions M × N, where M = 6000 is the number of acquisitions in time and N = 1000 is the number of pixels. The data files were processed to find events, which is the time and pixel positions where an animal is in the beam. This procedure corresponds to the flowchart shown in Fig. 2.

Step 1 is to remove the background fluorescence of the water. This is done by taking the median along the time axis (for every pixel position of each file) and subtracting the median from the raw signal. The median is used rather than the time average, as it is more robust to high-intensity events. This background subtraction assumes that the events are few and short enough that each pixel (in one file) is dominated by the background fluorescence of filtered seawater. Step 2 is to find events in the files, which is done using a method similar to that described in [13]. We create a binary mask based on the cyan signal to pick out each event. This is done by applying a threshold to the cyan signal, which ensures a minimum signal-to-noise ratio (SNR) (using a similar procedure described in [13]). In our case, a minimum SNR = 3.6 is used, which is found to be high enough to overcome the noise background and filter away the particles that are too small to be animals. We then erode the remaining signal to remove noise peaks and subsequently dilate the signal to contain all the pixels within the event. Finally, the mask is multiplied with the original signal in both channels. Step 3 is to find the signal strength of each event, for each channel. This is done to map each event in a scatter plot. The signal strength of the event is found as the sum along the pixel axis averaged over time. Step 4 is to filter away events that are saturated, too narrow (having < 6 pixels wide masks), or have a mean pixel red signal strength below 1.5 times the standard deviation of the background. This threshold is applied to remove events that have signal strengths in the red channel that do not sufficiently exceed the noise level, since the initial thresholding in step 2 applies only to the cyan channel.
Fig. 2. Flow chart of the procedure to find, extract and filter events. Step 1: Subtract background from the raw signal. Step 2: Find and extract events. Step 3: Calculate the red and cyan signal strength for each event. This is done for all files in a measurement series and all points are plotted together in a scatter plot. Step 4: Filter away events that have a very low signal-to-noise ratio (SNR), are saturated, or are too narrow (with < 6 pixels generated masks).

3. Results and analysis

To eliminate the effect of potential variation in fluorescence between individuals of the same species, measurements were initially carried out on a single individual from each species in filtered seawater. Each measurement was conducted over 3–4 hours and the results show that the two species have significantly different distributions (Fig. 3). The dashed lines indicate the noise limit of the cyan (vertical line) and red (horizontal line) signal, imposed by the thresholding done in step 2 and step 4, respectively.

*Acartia tonsa* shows a stronger red fluorescence as expected from ingested chlorophyll. However, its gut is empty for the majority of the measurements, since *A. tonsa* clears its gut within 30-60 minutes of being in filtered seawater [14]. This suggests that the red fluorescence does not stem exclusively from the ingested chlorophyll in the gut, as there is no large variation in the cyan over red fluorescence ratio over time. Thus, at least part of the red fluorescence signal must stem from algae pigment absorbed in the tissue signal. The distribution from each individual shows a linear relation between the signal strengths of the two channels. In salmon lice, this is expected, as our previous study [7] showed only small variations in the normalized fluorescence spectra of several *L. salmonis* individuals. It is also expected in *A. tonsa* since it was starved for most of this experiment, so there would be no major variation due to gut content. The results in Fig. 3 show that there is a significant variation of signal strength within each species. This variation stems from the different positions of the zooplankton within the laser beam (i.e. different excitation intensities) and how well in focus the animal is. It was observed that the animals from both species tend to react to the laser beam by jumping when they reached the beam center. Thus, our measurements seem to alter the animals’ natural behavior. However, the absence of large variation in the cyan-to-red signal ratio of the same individual over time suggests the applicability of the method. Unlike in our experiments where the animals swim...
across the laser beam several times, excessive exposure of any individual zooplankton is more unlikely in a field measurement or in a submersible fluorosensor with a flow system.

Next, measurements were carried out on monoculture samples of each species, with each sample containing approximately 50 individuals. The system was kept running for about 5 hours to collect a big dataset for generating a classifier. Both species were measured in filtered seawater. Around 500 events were gathered from each species. The results are presented in Fig. 4. A test set is generated by selecting every fifth event, with the remaining 80% of the total events then used as a training set. The training set is shown in a histogram of the ratio of cyan to red fluorescence signal strength in Fig. 4(a). It is evident that the two species have distinct distributions. It is seen that the cyan-to-red ratio, $\gamma$, for $A.\ tonsa$, vary more than in the single individual experiment, likely due to a larger variation in the amount of algae in the gut of the 50 $A.\ tonsa$. Based on the distributions of the training set, we create a simple classification algorithm. The classifier is based on the ratio. The slope of the dividing line between the two groups is the ratio, $\gamma_S$, that is equidistant from the mean cyan-to-red ratio of the two species normalized to their standard deviation. This is given by the equation:

$$\gamma_S = \frac{\mu_{AT}\sigma_{LS} + \mu_{LS}\sigma_{AT}}{\sigma_{AT} + \sigma_{LS}}$$  \hspace{1cm} (1)$$

where $\mu$ and $\sigma$ are the mean and the standard deviation, respectively, of the $\gamma$ of each species indicated by subscripts. AT (for $A.\ tonsa$) and LS (for $L.\ salmonis$). $\gamma_S$ is found to be 16 and is indicated by the black dashed line in Fig. 4. This classifier is then used to predict the groups of the events in the test set, which is shown in the scatter plot in Fig. 4(b). The number of predicted individuals belonging to a species as compared to the actual number is shown in Fig. 4(c), and these are used to calculate the performance of the system in distinguishing $L.\ salmonis$ from $A.\ tonsa$. The sensitivity is calculated as the ratio of the number of salmon lice correctly identified to the actual number of salmon lice. Similarly, specificity is calculated as the ratio of the number of $A.\ tonsa$ correctly identified and the actual number of $A.\ tonsa$. It is found that the system has a sensitivity of 99% and a specificity of 98%. This shows that the system can distinguish these two species with high accuracy. The accuracy can be increased by using harsher filtering on the SNR, with the downside of reducing the number of events.
Fig. 4. Measurements performed for 5 hours on 50 individuals of each species in two separate single-species cultures. The dashed black line indicates the dividing line between the two groups, $\gamma_S = 16$. (a) Histogram of the training set consisting of 80% of the events. (b) Scatter plot of the remaining test set consisting of 20% of the events. (c) Resulting confusion matrix of the test set when using a linear classifier determined from the training set.

Finally, we conducted a measurement with mixed-culture samples of the two species in different ratios. In this case, the actual species of each event is not known. The results are shown in Fig. 5. Each histogram represents a different mix ratio. The predicted species, with the dashed line indicating the dividing line $\gamma_S$, are shown based on the color – red events are predicted as \textit{A. tonsa} and blue events are predicted as \textit{L. salmonis}. From the two single-species measurements (top and bottom histograms), we find that the sensitivity is 94% while the specificity is 97%. That is, the sensitivity is slightly lower than the previous measurement but still high. The predicted distributions of species in the mixed-culture measurements are calculated to see if it follows the known distributions. It is found that for actual concentrations of 83%, 50%, and 17% salmon lice, the predicted percentage of salmon lice are 90%, 34%, and 16%, respectively. The predicted distributions follow the trend that a decreasing percentage of salmon lice shows as a decreasing percentage of predicted salmon lice. It is important to note that it is not expected that the number of actual events of each species perfectly mirrors the distribution of these species. That is because the actual number of events might be affected by certain animals going through the beam several times and each species’ physical distribution around and reaction to the laser beam. The fact that the classifier correctly predicts the trend of the mixed-species distributions shows that it does a good job of grouping the two species without \textit{a priori} knowledge. The results also show that there is no immediate difference in whether the two species are attracted or repelled by the laser beam. Neither does it show any indication that the presence of another species in the
water sample changes the expected fluorescence signal ratio. This is an important result as it indicates the feasibility of obtaining a library of expected fluorescence ratios for different species in single-species samples and using it for classification in a mixed-species sample.

![Fig. 5. Measurements on single-species and mixed-species culture of the two species in different ratios as indicated on the respective histograms. LS is the number of *L. salmonis* and AT is the number of *A. tonsa* in each measurement. The colors indicate which species the classifier has predicted for the events (blue = LS and red = AT).](image)

4. Conclusion and outlook

We have presented a dual-wavelength-band fluorosensor for the detection of zooplankton and demonstrated its ability to detect individual animals of 0.7-1 mm body lengths. We have measured the variation of the fluorescence signal from a single animal of a non-eating species, *L. salmonis*, and an algae-eating species, *A. tonsa*. This showed a clear distinction in the fluorescence band ratios between the species. The sensitivity of the system depends on the gut content of the algae-eating species, but the system is robust even for starved algae-eaters. Based on measurements on single-species cultures of approximately 50 animals, we have made a simple classifier showing a high degree of discrimination, with a sensitivity of 99%. Finally, we showed that the classifier is successful in grouping the two species in cultures of mixed species at different ratios. These results, combined with the findings in our previous work [7], show that the system can be used to distinguish non-eating zooplankton like salmon lice from algae-eating zooplankton, constituting the vast majority of all zooplankton.

The results were conducted in lab conditions, with the animal samples in filtered seawater. This is an important first step towards a submersible fluorosensor for use to detect salmon lice in the salmon farming industry. Despite this measurement conducted at a perpendicular angle, the technology presented is directly compatible with single-ended laser diagnostics and Scheimpflug lidar [15], which can be implemented from the sea surface adjacent to floating salmon farms.

While we have shown a good potential for discriminating salmon lice from algae-eating species, differentiation of salmon lice from other non-algae-eating species will require other signatures than the dual-band fluorescence signal. This could for example be movement patterns or spatial differences in the fluorescence patterns, the latter of which was shown in [7] to have the potential of distinguishing salmon lice copepodites from barnacle cypris larvae. This could be implemented by replacing the linear array detectors of the dual-band fluorosensor with high-speed
monochrome cameras. The most abundant species around the Norwegian salmon farms are algae-eating [11]. This is important since salmon lice abundances, in general, are low compared to the total abundance of zooplankton [16] and high abundances of other non-algae-eating species would decrease the ability of the fluorosensor to detect variation in salmon lice numbers.

A thing to address in further work is the effects of water quality. In the brackish water surrounding the salmon farms we expect there to be an increased background fluorescence, respectively for the cyan band from dissolved organic matter [17] and the red band due to chlorophyll from algae in the water. This will reduce the effective dynamic range of the 16-bit detectors and degrade the SNR in the two channels. A solution to address this could be the application of a liquid sample filtering technique, e.g. a combination of a pump and an oscillating mesh-filter as used in [18].

Funding

Innovationsfonden (5189-00132B).

Acknowledgments

We would like to acknowledge Klas Rydhmer and the rest of the data science team of FaunaPho-tonics ApS for valuable suggestions on our measurements and providing the starting point for the event extraction software.

Disclosures

The authors declare no conflicts of interest.

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