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Milos Stupar, Wejden Gongi, Silke Van den Wyngaert, Alexandra Kraberg, Albert Reñé, Serena Rasconi

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Miloš Stupar[1], Wejdene Gongi[2], Silke Van den Wyngaert[3], Alexandra Kraberg[4], Albert Reñé[5] & Serena Rasconi[6]

[1]University of Belgrade, Faculty of Biology, Belgrade, Serbia

[2]Institute of Materials Science of Mulhouse (IS2M), CNRS Mulhouse, France

[3]Department of Biology, University of Turku, Finland

[4]Alfred Wegener Institute, Bremerhaven, Germany

[5]Institut de Ciències del Mar (ICM-CSIC). Dpt. Biologia Marina i Oceanografia. Pg. Marítim de la Barceloneta, 37-49 08003 Barcelona, Catalonia, Spain

[6]Université Savoie Mont Blanc, INRAE, CARRTEL, Thonon les Bains, France

Abstract

The objective of this methodological paper is to outline a microscopy protocol for detecting and observing fungal zoosporic parasites of phytoplankton in natural environments, with potential applications in algal biotechnology to enhance the success and sustainability of algal cultivation endeavors. The fluorescent dual staining with wheat germ agglutinin (WGA) and Calcofluor White (CFW) showcased during the Training School "Identification and detection of zoosporic parasites" in Neuglobsow (Germany) from 16th till 18th of May 2023 and organized and funded by COST Action ParAqua (European Cooperation in Science and Technology) illustrated its ease of implementation and suitability for diverse natural and artificial systems.

Key words: algal biotechnology, calcofluor white, chytrids, microscopy, wheat germ agglutinin

Introduction

Recently, the microalgae industry has shown an increasing market demand not only for biomass production but also for algal metabolites, such as proteins, lipids, carbohydrates, carotenoids, vitamins, natural pigments as well as a variety of biologically active compounds. Microalgae industry has a wide range of applications for the production of biofuel, nutraceuticals, pharmaceuticals, foods and feeds, biofertilisers, additives, and cosmetics (Balasubramaniam et al., 2021). Thus, to respond to this demand, large-scale production has increased immensely. Indeed, the market potential for large-scale microalgae cultivation products has been estimated in the trillion-dollar range (Kothari et al., 2017). However, sustainable industrial production is still challenged by their stability, which is mainly affected by the presence of contaminants in production system (mainly open ponds, but also in closed systems such as photobioreactors), leading to contamination of the final product, and severe reduction or even crashes in the production (Wang et al., 2016).

Indeed, the broad variety of common and as yet unknown parasite epidemics associated with microalgae will pose a considerable biological and economical challenge to the commercial cultivation of algae at the industrial level (Carney and Lane, 2014). Zoosporic parasites are nowadays recognized as important ecological drivers of phytoplankton community dynamics in both freshwater and marine ecosystems, as they can have a significant role in controlling host population size (Scholz et al., 2016). Due to their strong impact on plankton communities, their presence in microalgae industry facilities must not be neglected and novel methods for fast but reliable detection and monitoring of zoosporic parasites which could be applied in the microalgae industry must be developed and implemented.

Microscopic analysis is one of the most conventional laboratory methods for the observation of morphological features of microorganisms, and as such, it can be implemented as an easy routine method for the detection of the presence of zoosporic parasites in natural and artificial systems. However, traditional light microscopy is regarded as an ineffective detection tool for the detection of zoosporic parasites in samples with a mixed assemblage of microorganisms. Small and inconspicuous thalli, minuscule zoospores (2-5 µm), as well as the various development stages without distinctive morphological features, lead to overlooking and misidentification of zoosporic parasites in traditional microscopy survey. Few routine microscopy techniques for

reliably detecting and counting zoosporic parasites in natural environments have been proposed (Rasconi et al 2009, Sime-Ngando et al 2013, Klawonn et al 2021). The major groups of zoosporic parasites of phytoplankton are chytrids (members of divisions Chytridiomycota in the kingdom Fungi) which have chitinaceous cell walls, and hence are suitable for detection by chitin-binding dyes like Congo Red (CR), Lactophenol-cotton blue (LCB), Trypan Blue (TB), Calcofluor White (CFW), and Fluorochrome conjugated-wheat germ agglutinin (WGA). Due to their ability to bind to chitin, these dyes are regarded as fungal cell wall markers. Ecologically, chytrids can be the infectious agents in all major phytoplankton groups. For example, the green algae *Haematococcus pluvialis*, which is cultivated in photobioreactors due to astaxanthin production (Balasubramaniam et al., 2021), is documented as a host for chytrids *Quaeritorhiza haematococci* (Longcore et al., 2020) and *Paraphysoderma sedebokerensis* (Gutman et al., 2009). Green algae from the genus *Scenedesmus*, commercially grown for the production of photoprotective pigment lutein, as well as for bioethanol production (Balasubramaniam et al., 2021), are susceptible to infection by chytrid *Phlyctidium scenedesmi* (Laezza et al., 2022).

By utilizing microscopic methods, researchers and biotechnologists can identify and track the presence of these parasites, enabling them to implement strategies to mitigate their effects and promote the growth of healthy algae for various industrial and environmental applications. Thus, the aim of this methodological paper is to describe the application of a validated microscopy method for the detection and visualization of fungal zoosporic parasites in natural systems which could be also applied in algal biotechnology, aiding to ensure the success and sustainability of algal cultivation efforts.

Protocol

The described protocol is based on the publication by Klawonn et al. (2021)

1. Sampling

Mixed plankton communities are sampled from the water system directly filling the container from the surface water body of interest, or either using a water sampler (e.g. a Niskin bottle) or an appropriate plankton net to concentrate phytoplankton cells. In algal biotechnology, such as production systems, or very eutrophic systems with massive blooms a plankton net to increase detection rate of host cells (and thus parasites) is not needed. In other water systems, the

appropriate mesh size of the plankton net will depend on the size of the target hosts of interest. If species of interest are expected to be large sized algae (>25µm) a 25 µm mesh size plankton net is appropriate. However if the expected size of the host of interest is smaller than 10-25µm, it is recommended to use a 10µm mesh size plankton net.

2. Sample fixation

The water samples are fixed with neutral or alkaline Lugol's solution (10 µL mL^{-1}) and stored overnight at room temperature. For long-term storage, a temperature of 4°C and dark is recommended. If plankton sampling was not carried out through a plankton net, an additional cell concentration step may be needed in order to obtain sufficient cell density and to increase the parasite detection rate in case the parasite prevalence is very low. In that case, phytoplankton cells are concentrated as follows: samples are homogenized and transferred to a centrifugation tube (50 mL), and stored at room temperature for 24h to allow cells to settle to the bottom. The top water layer (approx 90%) is then siphoned to reach an enrichment of cells 10x.

3. WGA and CFW dual-staining

For analysis, samples (1-2 mL) are transferred to a 2mL microcentrifuge tube. Sodium thiosulfate (20-40 µL, 30 g L^{-1}) is added to the sample to remove Lugol's staining. Sample is shaken manually till it becomes transparent. Both stains (WGA and CFW) are added in a final concentration of 5 µg mL^{-1} and the samples are incubated in the dark for 15 minutes. The order of stain addition does not influence the staining efficiency. The sample is then transferred into an Utermöhl chamber (or other type of microscopy chamber suitable for inverted microscopy) and left undisturbed for 15 minutes in order to let cells sink to the bottom of the chamber. The chambers are kept in the dark to avoid light degradation of the fluorescent dyes. The prepared samples can be stored in the fridge for 1–2 days if microscopy observation is not done immediately.

4. Microscopy analysis

Samples are observed under inverted epifluorescence microscopes equipped with ultraviolet (for CFW) and blue (for WGA-488) excitation filters. For visualization of chytrid infections and sporangia associated with microphytoplankton (20–200 µm), 300 – 400 x magnification is used. On the other hand, visualization of chytrids associated with nanophytoplankton (2–20 µm) is

accomplished with 600 – 900 x magnification. Simultaneous visualization of CFW-fluorescence along with phytoplankton autofluorescence is enabled through UV excitation/long pass emission filters, which can be combined with transmitted light observation (bright-field or DIC), but it could be applied only on samples non-fixed with Lugol's solution. Blue excitation/long pass emission filters can be used for simultaneous visualization of WGA-488 along with phytoplankton autofluorescence for live samples. This described protocol leads to the enumeration of phytoplankton cells (transmitted light) as well as the detection of attached fungal sporangia (CFW/WGA) within the same field of view.

5. Diagnostic criteria for detecting parasite chytrids

The chytrid infection will be confirmed if the following diagnostic criteria are fulfilled:

1) positive CFW/WGA staining of the chytrid structure attached to the host (sporangium);

2) stained sporangia have a distinctly outlined globose to ovoid shape and are physically attached to a phytoplankton cell (via stalks and/or rhizoids);

3) the size of the stained sporangia do not exceed the host cell size;

4) infected phytoplankton cells show signs of chloroplast degradation indicated by low red autofluorescence (applied only for samples non-fixed with Lugol's solution);

5) stained sporangia display no Chl *a* autofluorescence;

6) re-occurrence of sporangia (at different developing stages) on the same phytoplankton taxon.

Results

During the Training School "Identification and detection of zoosporic parasites", which took place in Neuglobsow (Brandenburg, Germany) from 16th till 18th of May 2023 organized by the Action ParAqua and funded by COST (European Cooperation in Science and Technology) samples for demonstration of WGA-CFW fluorescent staining were taken in Lake Stechlin.

During the microscopy analysis, the usage of chitin-binding dyes along with fluorescence showed good results in visualizing chytrid–microalgae associations and sporangia of fungal parasites infecting centric and pennate diatoms as well as filamentous cyanobacteria were observed (Figure 1).

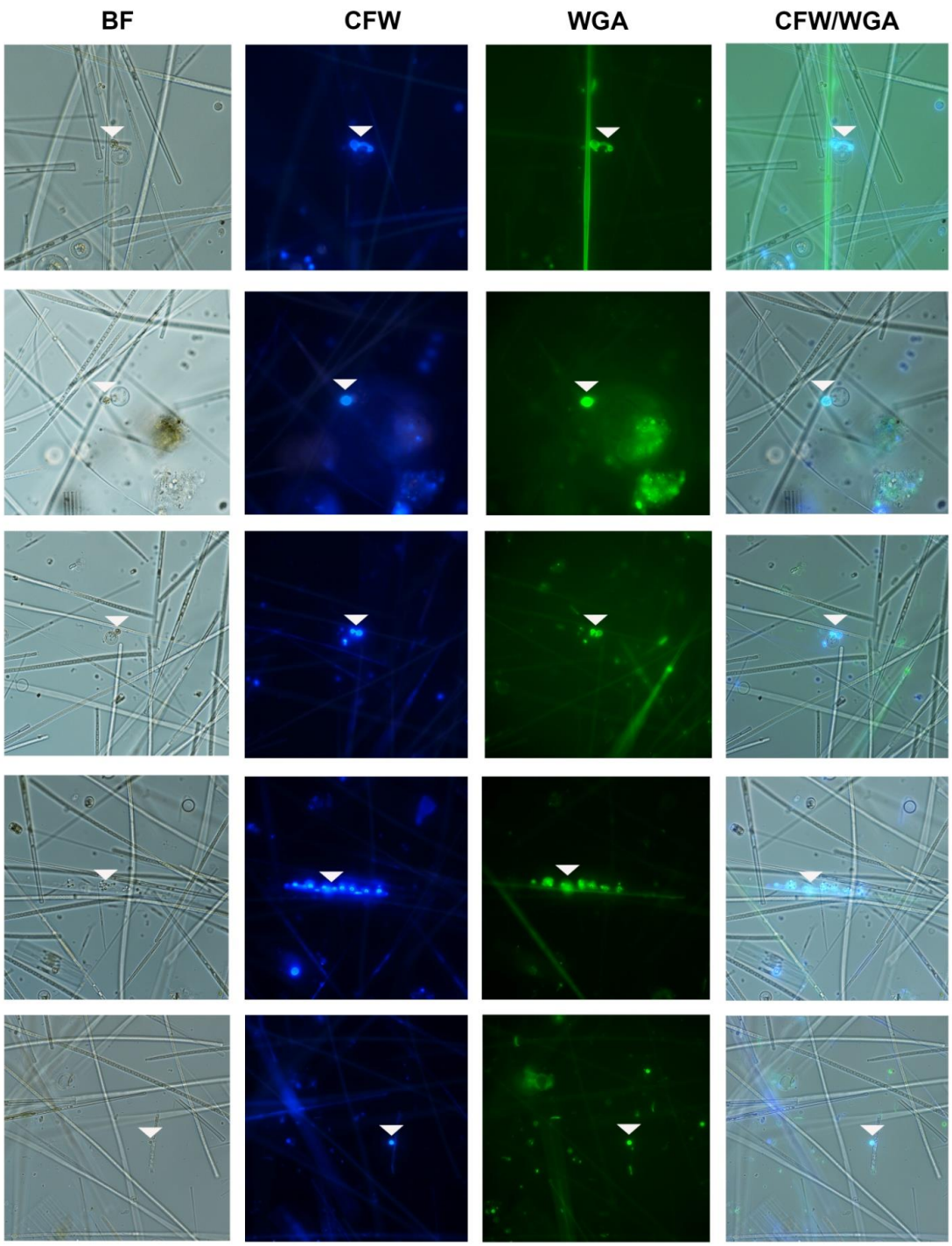

Figure 1. Microscopy images of various diatoms (rows 1-4) and filamentous cyanobacteria (row 5) with associated morphological structures of zoosporic parasites (arrow). Samples from Lake Stechlin, Neuglobsow, Germany): BF- bright field; CFW - Calcofluor White; WGA - wheat germ agglutinin

Discussion

The ParAqua COST Action aims to develop new, efficient, and sustainable methods for the detection and management of parasites in algae aquaculture, which will help to improve the biomass production yield and stability. The project also aims to develop new strategies for the control and prevention of parasitic infections that will not only be effective but also environmentally sustainable and economically feasible. Indeed, during the Training School “Identification and detection of zoosporic parasites” which took place in Neuglobsow (Germany) and was organized by ParAqua, various techniques for detection of zoosporic parasites of microalgae including microscopy methods were introduced and demonstrated to trainees. Participants learned how to implement the microscopy protocol in their own projects, contributing to the success of the training program. The demonstration of the WGA-CFW fluorescent staining method showed that this method is easy to be implemented and could be potentially applied to a multitude of natural and artificial systems. However, application of this method in artificial systems is still to be validated.

In summary, facilitating the transfer of knowledge regarding a straightforward and affordable technique for identifying fungal zoosporic parasites, which can be grasped by those with basic microscopy skills, encourages broad acceptance and application of knowledge. This advancement has the potential to enhance microalgal cultivation practices and empower researchers to address potential challenges more effectively.

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