Check for updates

METHOD ARTICLE

# REVISED Chlorophyll-a determinations in mesocosms under varying nutrient and temperature treatments: in-situ fluorescence sensors versus in-vitro measurements

[version 3; peer review: 6 approved]

Eti Ester Levi[1], Erik Jeppesen[1-5], Jens C. Nejstgaard[6], Thomas Alexander Davidson[1]

[1]Department of Ecoscience & WATEC, Aarhus University, Aarhus, Central Denmark Region, 8000, Denmark
[2]Limnology Laboratory, Department of Biological Sciences and Centre for Ecosystem Research and implementation (EKOSAM), Middle East Technical University, Ankara, 06800, Turkey
[3]Institute of Marine Sciences, Middle East Technical University, Mersin, 33731, Turkey
[4]Sino-Danish Centre for Education and Research, University of the Chinese Academy of Sciences, Beijing, 100190, China
[5]Institute for Ecological Research and Pollution Control of Plateau Lakes, School of Ecology and Environmental Science, Yunnan University, Kunming, Yunnan, 650091, China
[6]Department of Plankton and Microbial Ecology, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Stechlin, 16775, Germany

**v3** **First published:** 12 Apr 2024, **4**:69
https://doi.org/10.12688/openreseurope.17146.1
**Second version:** 22 Oct 2024, **4**:69
https://doi.org/10.12688/openreseurope.17146.2
**Latest published:** 13 Feb 2025, **4**:69
https://doi.org/10.12688/openreseurope.17146.3

## Abstract

Harmful algal blooms (HABs) are a significant threat to freshwater ecosystems, and monitoring for changes in biomass is therefore important. Fluorescence in-situ sensors enable rapid and high frequency real-time data collection and have been widely used to determine chlorophyll-*a* (Chla) concentrations that are used as an indicator of the total algal biomass. However, conversion of fluorescence to equivalent-Chla concentrations is often complicated due to biofouling, phytoplankton composition and the type of equipment used. Here, we validated measurements from 24 Chla and 12 phycocyanin (cyanobacteria indicator) fluorescence in-situ sensors (Cyclops-7F, Turner Designs) against spectrophotometrically (in-vitro) determined Chla and tested a data-cleaning procedure for eliminating data errors and impacts of non-photochemical quenching (i.e. light-induced decrease in fluorescence intensity). The test was done across a range of freshwater plankton communities in 24 mesocosms (i.e. experimental tanks) with a 2x3 (high and low nutrient x ambient, IPCC-A2 and IPCC-A2+50% temperature scenarios) factorial design. For

**Open Peer Review**

**Approval Status** ✓ ✓ ✓ ✓ ✓ ✓

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **version 3** (revision) 13 Feb 2025 | | ✓ view | | | | ✓ view |
| **version 2** (revision) 22 Oct 2024 | | | ✓ view | ✓ view | ✓ view | ✓ view |
| **version 1** 12 Apr 2024 | ✓ view | ✓ view | | | | |

1. **Tammi L. Richardson**, University of South Carolina, Columbia, USA
2. **Michela Rogora**, National Research Council, Water Research Institute (CNR IRSA), Verbania Pallanza, Italy
3. **Catherine Schmechtig**, Sorbonne

most mesocosms (tanks), we found accurate ($r^2 \geq 0.7$) calibration of in-situ Chla fluorescence data using simple linear regression. An exception was tanks with high in-situ phycocyanin fluorescence, for which multiple regressions were employed, which increased the explained variance by >10%. Another exception was the low Chla concentration tanks ($r^2 < 0.5$). Our results also show that the high frequency in-situ fluorescence data recorded the timing of sudden Chla variations, while less frequent in-vitro sampling sometimes missed these or, when recorded, the duration of changes was inaccurately determined. Fluorescence in-situ sensors are particularly useful to detect and quantify sudden phytoplankton biomass variations through high frequency measurements, especially when using appropriate data-cleaning methods and accounting for factors that can impact the fluorescence readings. Nevertheless, corroborating these data with in-vitro Chla assessments would provide additional validation for the early warnings provided by sensor data.

**Plain language summary**

Harmful algal blooms (HABs) may pose a significant threat to freshwater ecosystems and to animal and human health. Therefore, it is important to monitor changes in algal biomass. Traditional methods, while effective, lack the ability to provide rapid, high-frequency, real-time data. In-situ fluorescence sensors, specifically designed to measure chlorophyll-*a* (total phytoplankton indicator) and phycocyanin (Blue-green algae indicator), offer a promising solution. However, challenges such as biofouling, temporal changes in phytoplankton composition, and equipment variations complicate the conversion of fluorescence data into equivalent chlorophyll-*a* concentrations.

Our study aimed to validate measurements from 24 chlorophyll-*a* and 12 phycocyanin fluorescence in-situ sensors (Cyclops-7F, Turner Designs). We compared these measurements against spectrophotometrically determined (in-vitro method) chlorophyll-*a* concentrations. Additionally, we tested a data-cleaning procedure to eliminate errors caused by different sources, such as light. The validation and testing were conducted at Lemming Experimental Mesocosm site (Denmark), in 24 experimental tanks (mesocosms) representing 2 different nutrient levels and 3 temperature scenarios.

This study underlines that high-frequency in-situ fluorescence sensors can be useful, only if the user is aware of the possible interacting factors that can influence fluorescence readings (e.g. turbidity, daylight). Therefore, in-situ fluorescence sensors, when properly calibrated and validated, offer a valuable tool for monitoring harmful algal blooms. The high-frequency data provides insights into sudden variations in phytoplankton biomass, demonstrating the potential for improved real-time understanding of freshwater ecosystems.

Université, Paris, France

4. **Karsten Rinke**, UFZ-Helmholtz Centre for Environmental Research, Department Lake Research, Brueckstr, Germany
5. **Marju Tamm**, Estonian University of Life Sciences, Tartu, Estonia
6. **Kaisa Kraft**, Marine Research Centre, Helsinki, Finland

Any reports and responses or comments on the article can be found at the end of the article.

**Keywords**
phytoplankton bloom detection, phycocyanin fluorescence, in-vivo method, spectrophotometry, high frequency data, time series, mesocosm




This article is included in the Horizon 2020 gateway.

**Corresponding author:** Eti Ester Levi (eel@ecos.au.dk)

**Author roles: Levi EE**: Data Curation, Formal Analysis, Investigation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Jeppesen E**: Conceptualization, Funding Acquisition, Methodology, Project Administration, Resources, Supervision, Writing – Review & Editing; **Nejstgaard JC**: Supervision, Writing – Review & Editing; **Davidson TA**: Conceptualization, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Writing – Review & Editing

**Competing interests:** No competing interests were disclosed.

**Grant information:** This work was supported by the AQUACOSM and AQUACOSM-plus projects funded by the European Union's Horizon 2020 research and innovation programme under grant agreements No. 731065 and 871081 to EEL, EJ, JCN and THD. In addition, support was provided by TÜBITAK outstanding researcher programme 2232 (project 118C250) to EJ and by AnaEE, Denmark, to EEL, EJ and THD. EEL and THD were also supported by the European Union's Horizon 2020 research and innovation programmes under grant agreement No 869296 –The PONDERFUL Project.
*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

**Copyright:** © 2025 Levi EE *et al*. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**How to cite this article:** Levi EE, Jeppesen E, Nejstgaard JC and Davidson TA. **Chlorophyll-a determinations in mesocosms under varying nutrient and temperature treatments: in-situ fluorescence sensors versus in-vitro measurements [version 3; peer review: 6 approved]** Open Research Europe 2025, **4**:69 https://doi.org/10.12688/openreseurope.17146.3

**First published:** 12 Apr 2024, **4**:69 https://doi.org/10.12688/openreseurope.17146.1

**REVISED** **Amendments from Version 2**

An error in the code for the fluorescence temperature correction was identified and corrected. As a result, Figure 3–Figure 12 have been updated, and some numerical values in the text have been adjusted. While this correction improved the fit between in-vitro and in-situ measurements in some tanks, it did not affect the main results.

Other changes in the manuscript include:

1. Abstract
    a. A brief explanation of photochemical quenching is added: "light-induced decrease in fluorescence intensity."
2. Introduction
    a. "Flowcytometry" is removed as an example of phytoplankton sampling methods, as automated flowcytometry did not fit the context. Consequently, "Lombard et al., 2019" was removed from the references.
    b. New references on open data sources (Argo, 2024; Sauzède *et al.*, 2015) and the importance of sensor manufacturers (Roesler *et al.*, 2017) are added.
3. Methods
    a. A reference (Jespersen & Christoffersen, 1987) for spectrophotometric Chla analysis is added.
    b. A brief explanation of fluorescence signal quenching: "reduction in signal intensity due to very high concentrations." Is added.
    c. At the end of the Methods, a new section is added: "Annual and Summer Chl-a Means: In-Vitro vs. Equivalent-Chl-a Comparison."
4. Results
    a. The caption of Figure 4 is updated, to clarify: "x1 corresponds to in-situ Chla fluorescence, and x2 to in-situ Phyco fluorescence."
5. Conclusion
    a. The section on Mean Absolute Error (MAE) is updated: "Equivalent-Chla concentrations, calculated from the fluorescence in-situ sensor calibrations, were mostly in agreement with the in-vitro concentrations. The mean absolute error (MAE) between Equivalent-Chla and in-vitro Chla was 12, while the median absolute error was 2. Among the overestimated variables, 18% had a MAE greater than 12, whereas for the underestimated variables, this proportion was 22%."

**Any further responses from the reviewers can be found at the end of the article**

## Introduction

Anthropogenic activities and global climate change pose a significant threat to the ecological status of freshwater ecosystems, resulting in e.g. eutrophication, which in turn alters biological community structures, including phytoplankton, and causes higher risk of harmful algal blooms (HAB) (Jeppesen *et al.*, 2014). HABs threaten human and animal health when the waterbody is used for drinking water (e.g. Gatz, 2019). Phytoplankton is, moreover, of key importance in the nutrient cycling and energy flow and is a key metric for evaluating the water quality in lakes in the EU Water Framework Directive (WFD) (Poikane *et al.*, 2015).

There are several ways to collect information on phytoplankton in waterbodies. Two of the frequently used sampling/analysis methods are microscopy (e.g. Hillebrand *et al.*, 1999) and pigment analysis with HPLC (Roy *et al.*, 2011). These methods are especially useful when detailed information on phytoplankton species or groups is required. Alternatively, spectrophotometric or fluorometric analyses of chlorophyll-*a* (Chla) are used as proxies for total phytoplankton biomass. All these methods are time consuming to a varying degree. They are also prone to human error, need expertise and require transport to a laboratory for analysis. The most important disadvantage, however, is the fact that the number of samples that can be retrieved and analysed is limited, often resulting in low spatial and temporal resolution, and leaving short-term changes unnoticed (Zamyadi *et al.*, 2012). Moreover, such ex-situ analyses do not provide the real-time information that may be needed for functional societal services such as effective HAB monitoring and warning systems.

An alternative is to use various in-situ fluorescence methods to obtain high frequency (HF) and real-time measurements of Chla and/or other pigments. The majority of the Chla in eukaryotic algae is found in the strongly fluorescent photosystem II (PSII) region, while for cyanobacteria it is mostly located in the non-fluorescent PSI region. Therefore, in-situ Chla fluorescence of cyanobacteria is low, causing underestimation of extracted (in-vitro) Chla concentrations (Campbell *et al.*, 1998; Seppälä, 2009). The main light-harvesting pigments in the PSII region of cyanobacteria are phycobilins, such as phycocyanin (Phyco) or phycoerythrin (Hodges *et al.*, 2018; Seppälä, 2009; Zamyadi *et al.*, 2012). The in-situ measurement method for phycobilins is the same as for Chla, but Chla and phycobilins differ in fluorescence characteristics. Thus, Chla absorbs and emits light in the blue (ca. 440 nm) and red (ca. 680 nm) regions of the spectrum (Egeland *et al.*, 2011), while phycocyanin, the dominant pigment in freshwater cyanobacteria, absorbs and emits light in the red-orange region, 610–630 nm and 600 – 700 nm (maximum emission 647 nm), respectively (Zamyadi *et al.*, 2012). Phycoerythrin pigment, on the other hand, is more common in marine species, and it has an absorption peak in the 490 – 575 nm and an emission peak in the 570 – 580 nm regions (Seppälä, 2009), though some freshwater species, like *Planktothrix rubescens*, have a high phycoerythrin content.

The fluorescence signals are dependent on phytoplankton species composition (Bertone *et al.*, 2019; Lawrenz & Richardson, 2011) but also influenced by cell size (Hodges *et al.*, 2018), ambient light conditions (Carberry *et al.*, 2019; Ralph *et al.*, 2010; Rousso *et al.*, 2021), nutrient concentrations (Kiefer, 1973b), temperature (Lorenzen, 1966; Watras *et al.*, 2017), turbidity (Zamyadi *et al.*, 2016), dissolved organic matter and biofouling (Kuha *et al.*, 2020; Xing *et al.*, 2017), all of which affect the fluorescence yield per Chla (i.e. the ratio of in-vivo Chla fluorescence to extracted Chla) (Kiefer, 1973a;

Lorenzen, 1966). Moreover, Chla and Phyco fluorescence might interfere with each other, and the strength of the interference depends on the concentration of these pigments (Zamyadi *et al.*, 2012). Therefore, concentrations determined by in-vivo methods are considered semi-quantitative and caution should be taken when interpreting results (Seppälä, 2009).

Multiple calibration studies of in-situ Chla and Phyco fluorometer measurements have been conducted (e.g. Gregor & Maršálek, 2004; Zamyadi *et al.*, 2012), and they have emphasised the importance of eliminating or including the interference effects from turbidity (Zamyadi *et al.*, 2012), organic matter (Kuha *et al.*, 2020; Xing *et al.*, 2017) and ambient light (Carberry *et al.*, 2019; Rousso *et al.*, 2021). Moreover, several studies have investigated fluorescence sensor performances by comparing measurements from field samples with single algal species cultures (Bastien *et al.*, 2011; Hodges *et al.*, 2018; Zamyadi *et al.*, 2012). These studies have shown that frequent validation of fluorescence readings is needed and also that the success of calibration varies – both strong (Gregor & Maršálek, 2004) and weak (Hodges *et al.*, 2018) correlations have been observed. By employing multivariate analysis and including DOM fluorescence readings in the Chla calibration, Kuha *et al.* (2020) found a much-improved fit (increase in $r^2$ from ca. 0.5 to 0.8) between spectrophotometric and in-situ Chla fluorescence values in 14 lakes with variable trophic state. They also pointed out that using water colour, instead of DOM, gave better calibration results.

The importance of open data sources for sharing is increasingly emphasised by the scientific community, and such sources are becoming more available (e.g. Argo, 2024; Sauzède *et al.*, 2015). However, this represents an important challenge when using in-situ fluorescence data as there is a great variety of instruments from different manufacturers with different optical and mechanical settings. Several studies have shown that raw fluorescence sensor data can differ among brands (Hodges *et al.*, 2018; Zamyadi *et al.*, 2016), highlighting the importance of sensor calibration to achieve harmonisation of data sets from different sources to allow comparable estimation of phytoplankton biomass across water bodies, as it was also emphasized by Roesler *et al.* (2017), in their global analysis of WET Labs ECO sensors.

The objective of this study was to find ways to efficiently clean, calibrate and thus harmonise in-situ fluorescence data collected by chlorophyll-*a* (Chla) and phycocyanin (Phyco) sensors (Cyclops 7F, Turner Designs). We took advantage of a long running mesocosm (experimental tank) based climate change experiment facility in Denmark (Liboriussen *et al.*, 2005, including 24 fully mixed shallow mesocosms (1 m depth) with contrasting nutrient concentrations crossed with three different temperature scenarios.

## Methods

### Study site

The current study was conducted between October 2018 and August 2020 in outdoor mesocosms (from here onwards tanks) at an experimental facility established in 2003 in Lemming, Denmark (for details see Liboriussen *et al.*, 2005). The facility consists of 24 stainless steel tanks (diameter = 1.9 m, tank depth = 1.5 m, water depth = 1 m) with mixed water columns and a flow-through system (a groundwater inlet and an outlet) with a water residence time of about 2.5 months. The tanks cover six different treatments consisting of three temperature scenarios crossed with two nutrient levels, each with four replicates.

The temperature treatments consist of unheated ambient temperature (AMB), IPCC climate scenario A2 (A2), which is 2–4 °C higher than the AMB, and climate scenario A2+50% (A2+), which is 4–6 °C higher than the AMB. Half of the tanks only receive nutrients via the input of groundwater, representing low nutrient (LN) treatment (Table 1). The other tanks, which are high nutrient treatments (HN), have been loaded with nitrogen (N) and phosphorus (P) since May 2003; $Na_2HPO_4$ and $Ca(NO_3)_2$ solutions have been added every week as sources of phosphorus and nitrogen, constituting during our study period a constant dose of 2.7 mg P $m^{-2}$ $day^{-1}$ (Liboriussen *et al.*, 2005) and 108 mg N $m^{-2}$ $day^{-1}$. Between

**Table 1. Summary data from low (LN) and high (HN) nutrient tanks for October 2018–August 2020 period.**

| Variables | | Low nutrient (LN) tanks | | | | High nutrient (HN) tanks | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *mean* | *median* | *min* | *max* | *mean* | *median* | *min* | *max* |
| Water temp | (°C) | 12.9 | 11.5 | -0.1 | 31.0 | 13.1 | 11.7 | -0.1 | 30.7 |
| pH | | 8.0 | 7.8 | 6.5 | 10.3 | 8.7 | 8.8 | 5.5 | 12.2 |
| Dissolved oxygen | (mg $L^{-1}$) | 11.6 | 11.6 | 0.1 | 20.9 | 12.6 | 12.3 | 0.0 | 30.0 |
| Conductivity | (µS $m^{-1}$) | 373 | 380 | 240 | 525 | 400 | 392 | 263 | 920 |
| In-vitro Chla | (µg $L^{-1}$) | 6.6 | 2.7 | 0.3 | 118 | 165 | 45 | 0.6 | 2959 |
| TP | (µg $L^{-1}$) | 30 | 13 | 1.0 | 906 | 264 | 217 | 2.0 | 1219 |
| TN | (µg $L^{-1}$) | 422 | 351 | 1.0 | 2411 | 2085 | 1293 | 1.0 | 9418 |

June 2018 and June 2019, N addition (apart from the input with groundwater) to the high nutrient (HN) tanks was suspended and subsequently N loading was resumed.

The tank letters A, D, F and G indicate high nutrient (HN) treatments, while B, C, E and H are low nutrient (LN) treatments. The numbers 1, 2 and 3 indicate the different temperature treatments – as ambient (AMB), A2 and A2+, respectively.

### Sampling for chlorophyll-*a*: in-vitro determination

Water samples from three locations were collected from the entire water column of each tank with a 1 m long polypropylene tube sampler (d: 10 cm), which was equipped with a stopper at the bottom. Two litre sub-sample was then transferred to dark bottles for spectrophotometric Chla analysis. Depending on the concentration, between 300 and 900 mL of water was filtered through Whatman GF/C filters (diameter: 47 mm), whereafter the filters were extracted for Chla using 10 mL 96% ethanol for 6–20 hours at room temperature. Afterwards, in-vitro Chla concentrations were determined spectrophotometrically (Shimadzu UV-1800) based on absorbance measured at 665 and 750 nm (DS 2201, 1986; Jespersen & Christoffersen, 1987). The whole process in the laboratory was conducted in a darkened environment.

The frequency of sample collection from the HN tanks was every second week in autumn, every month in winter and every week in spring and summer. Sampling in the LN tanks was undertaken at a slightly lower frequency, i.e. every second week for the first three months and then every month for the rest of the study.

### Chla and Phyco fluorescence sensors: in-situ determination

i. Sensors and their setup

Stainless steel Cyclops-7F fluorescence sensors from Turner Designs were used for in-situ determination of chlorophyll-*a* (Chla) and phycocyanin (Phyco) pigments. These in-situ sensors have three gain setting, defining their sensitivity. We used two of these gains: X1, which has a lower sensitivity with a measurement range of 0–5000 mV (equivalent to 0–500 µg Chla $L^{-1}$), and X10, which has a higher sensitivity with a measurement range of 0–500 mV (0–50 µg Chla $L^{-1}$).

PVC shade caps (length: 12.7 cm, diameter: 4.3 cm) were used on each sensor to provide a fixed distance for sample measurement, in order to reduce the effect from ambient light and to minimise the number of small animals (e.g. water snails, leeches) crawling on to the sensor tips.

The sensors were connected to a Campbell Scientific (Logan, UT, USA) CR6 datalogger through a Campbell Scientific AM16/32B multiplexer, which was used to increase the number of sensors that can be connected to the datalogger (AppendixE Fig. E1).

ii. Sensor quality checks

Sensor quality checks were performed by measuring a concentration range of Rhodamine B standard solution (linearity check-1) and water samples representing specific Chla concentrations (linearity check-2), as detailed below. Moreover, blank sample measurements using deionised water were also made (AppendixB Fig. B1).

All the measurements were conducted at the tank site with the original in-situ sensor setup (i.e. Cyclops-7F sensors connected to CR6 datalogger). Each Chla and Phyco in-situ sensor fitted with a shade cap was inserted into a glass beaker filled with relevant solution, and data were recorded in the dark.

For linearity check-1, Rhodamine B (Sigma-Aldrich R6626) stock solution was prepared according to YSI Environmental (2005) by dissolving 0.05 g Rhodamine B in distilled water. This stock solution was diluted to prepare seven different concentrations of standard solutions, representing Chla concentrations between 10 and 480 µg $L^{-1}$ at ca. 21.5 °C (YSI Environmental, 2005).

Linearity check-2 was conducted for a subset of the in-situ Chla sensors. Deionised water served as the blank (0 µg $L^{-1}$). A water sample from an LN tank was used for Chla concentrations of 5 µg $L^{-1}$ (1:2 diluted) and 10 µg $L^{-1}$ (undiluted). Rhodamine B (Turner Designs) for Chla concentrations of 20 µg $L^{-1}$ (1:2 diluted) and 40 µg $L^{-1}$ (undiluted). Finally, a water sample from an HN tank was used for Chla concentrations of 75 µg $L^{-1}$ (1:2 diluted) and 150 µg $L^{-1}$ (undiluted).

iii. Dissolved organic carbon effect check

On July 2020, approximately 20 L water sample, representing the entire water column, were collected from all tanks using a polypropylene tube sampler (d: 10 cm, L: 1 m), with a bottom stopper. For each tank, a 60 mL subsample was filtered through GF/F grade Whatman glass microfiber filters that were combusted for 2h at 550°C. Dissolved organic carbon (DOC) was measured from the filtered water samples with a TOC-L analyser (Shimadzu Corporation, Japan) using standard method from DS/EN 1484 (1997).

Five tanks, covering low to high DOC concentrations, were chosen for further analysis. Between 400 and 600 mL of water was filtered with combusted GF/F to remove phytoplankton and to keep DOC sources. Filtered water was then divided into three subsamples and stored in dark brown bottles for at least an hour. Subsequently, Rhodamine B standards (Sigma-Aldrich R6626) were added so that the concentrations of each subsample were 62.5, 125 and 500 µg Rhod $L^{-1}$. These Rhodamine B concentrations are approximate algal Chla equivalents of 10 µg $L^{-1}$, 20 µg $L^{-1}$ and 80 µg $L^{-1}$, respectively (YSI Environmental, 2005).

The Chla and Phyco in-situ fluorescence of the filtered water samples and the Rhodamine B-added water samples were measured using only one Chla and one Phyco sensor, each connected to a multimeter in a laboratory. These measurements were done in dark bottles in a room kept as dark as possible at room temperature (21.2 – 21.4 °C). The data from the Rhodamine B-added samples were then compared with the values of standard Rhodamine B solutions.

### Implementation of the fluorescence in-situ sensors

In October 2018, Chla in-situ sensors were placed in all 24 tanks, while Phyco in-situ sensors were deployed only in the HN treatments (12 tanks). The instruments were placed so that the in-situ sensors were located in the centre of the tanks and at middle depth (50 cm below the water surface/above the bottom in these fully mixed tanks (AppendixE Fig. E1)). The gain settings of the in-situ sensors were adjusted so that the sensors in the HN tanks had lower sensitivity (X1 gain) and higher X10 gain than in the LN tanks. The X10 gains were changed to X1 when necessary, ideally before running into signal-quenching (i.e. reduction in signal intensity due to very high concentrations).

As the sensors were not equipped with automatic cleaning brushes, they were instead generally cleaned every second week. As an additional control, the graphs from each sensor were examined every second or third day with LoggerNet software version 4.5 (Campbell Scientific, Logan, UT, USA) and in the case of an unexpected abrupt change, the relevant sensor was cleaned again.

### In-situ sensor data collection and cleaning

Data from the fluorescence in-situ sensors were recorded every minute during the whole period, stored as ASCII files and retrieved with LoggerNet software version 4.5 (Campbell Scientific). A data-cleaning protocol was developed and consisted of several steps (AppendixA).

Photosynthetically active radiation (PAR) was measured with S-LIA-M003 Photosynthetic Light (PAR) Smart Sensor (Onset Computers, Bourne, MA, USA) and downloaded from the Hobolink website (www.hobolink.com, Accessed in 10.01.2022). PAR values equal to one are used to select night-time in-situ fluorescence sensor data.

The water temperature of the tanks was measured every half an hour with a PR electronics temperature sensor (see Liboriussen *et al.*, 2005 for details). Because fluorescence is temperature dependent (decreases with increasing temperature), the output from the in-situ Chla sensors was corrected according to following equation by applying 20 °C as reference temperature (www.turnerdesigns.com/technical-support, Accessed in 07.03.2023);

$$\%\ \text{Change in Chla in-situ} = \text{Temperature (°C)} * 1.4\%$$

Fluorescence signals from the in-situ Phyco sensors were temperature corrected based on the phycocyanin-extract equation by Watras *et al.* (2017). These authors used equivalent type of sensors, Cyclops-7 instead of the 7F (faster readout version used here), from the same manufacturer, and their study was based on a 5–30 °C temperature range, which overlaps with the temperature range in our study (Table 1). The following equation was used:

$$F_r = F_m / (1 + \rho(T_m - T_r))$$

where F: fluorescence, T: temperature, r: reference, m: measured and ρ: temperature coefficient (Watras *et al.*, 2017).

Instead of establishing specific coefficients for each HN tank, the temperature coefficient was chosen based on the commercial phycocyanin extract (Watras *et al.*, 2017) because each tank has its own unique cyanobacteria community, which possibly changes through the year. However, it is important to note that the optimal way of correcting the sensor signals would be by developing specific coefficients for each study environment, as recommended by Watras *et al.* (2017).

### In-situ Chla sensor calibration

Fluorescence in-situ Chla sensor readings were calibrated against in-vitro Chla measurements. Linear (i.e. in-vitro Chla ~ in-situ Chla) or multiple linear regression (i.e. in-vitro Chla ~ in-situ Chla + in-situ Phyco) analysis was used to establish equations for each in-situ Chla sensor, enabling us to predict Chla concentrations as μg $L^{-1}$ (from here onwards "Equivalent-Chla") based on the in-situ fluorescence data.

The reason for using multiple regression was that Phyco is the main fluorescing pigment in cyanobacteria, and its contribution to Chla fluorescence is generally low, potentially resulting in underestimation of the spectrophotometrically measured (in-vitro) Chla in water bodies (Campbell *et al.*, 1998; Seppälä *et al.*, 2007). Therefore, the in-situ Chla fluorescence sensors in the tanks with high cyanobacteria presence were calibrated based on multiple regression. This method was not used for all the HN tanks because a high Chla fluorescence signal can also interfere with Phyco fluorescence (Zamyadi *et al.*, 2012), causing multicollinearity (correlation coefficient > 0.6) between the fluorescence signals of Chla and Phyco.

The Cyclops-7F (Turner Designs) in-situ sensor measurement limit is 500 μg Chla $L^{-1}$; therefore, sampling dates exceeding this limit (in total 19 days) were excluded from the calibrations of eight of the HN tanks.

Potential outliers were identified and removed based on combined information from linear regression diagnostic plots and robust linear regression weights with method of moments (MM) estimation; the information was obtained by comparing the models with and without removing possible outliers. MM estimation is a robust regression method, which is resistant to outlier influence due to its high breakdown property, i.e. the proportion of outliers that can be handled without affecting the model is high (Susanti *et al.*, 2014). We used this method as a check to prevent removal of legitimate data points as outliers.

Residuals were calculated as the difference between in-vitro Chla concentrations and Equivalent-Chla values. Moreover, relative error was calculated as absolute residuals divided by in-vitro Chla concentrations.

Data handling (cleaning and interpolation) and regression analysis were conducted in R version 4.2.2 (R Core Team, 2022) with the rB3 (Muraoka & McBride, 2018) and stats (R Core Team, 2022) packages, respectively. All the datasets used for calibration is added as underlying data (Levi *et al.*, 2024a) and analysis code is added as extended data (Levi, 2024), as well as the supplementary material (Levi *et al.*, 2024b).

### Annual and summer Chla means: in-vitro vs equivalent-Chla comparison

The summer or annual mean of Chla is frequently used in the literature, for instance as a biological quality element in Water Framework Directive (Søndergaard *et al.*, 2016). Therefore, in the current study, annual and summer means of both in-vitro and equivalent-Chla were compared to assess their agreement.

To perform this comparison, daily in-vitro Chla concentrations for year-2019 were obtained by linearly interpolating the biweekly or monthly in-vitro Chla data. The interpolated values were then used to calculate the mean annual and summer concentrations for each tank (from here onwards interpolated-$Chla_{in\text{-}vitro}$). Similarly, annual and summer means of the Equivalent-Chla concentrations were also calculated. Subsequently, Student's t-test was used to compare the means between the two methods for each tank.

## Results

### Study site between October 2018 and August 2020

During the study period, average nutrient concentrations were ca. 30 and 264 µg $L^{-1}$ for total phosphorus (TP) and 422 and 2085 µg $L^{-1}$ of total nitrogen (TN) in the LN and HN treatments, respectively (Table 1). The average in-vitro Chla concentrations of the treatments also differed substantially, being 6.6 µg $L^{-1}$ in the LN and 165 µg $L^{-1}$ in the HN treatments. The maximum in-vitro Chla concentration in the HN tanks was as high as 2959 µg $L^{-1}$.

### Fluorescence in-situ sensor quality check

i. Linearity check

The **linearity check-1** conducted across a range of Rhodamine B (Sigma-Aldrich R6626) concentrations showed, as expected, a good fit (AppendixB, Fig. B2).

The results of linearity check-2 indicated that the in-situ fluorescence measurements of Rhodamine B dye approximated our expectations with ca. 350-400 mV and 150-170 mV for non-diluted and diluted solutions, respectively (Figure 1). The water sample with low Chla concentration (10 µg $L^{-1}$) showed an average of ca. 50 mV and the sample with 150 µg Chla $L^{-1}$ an average of 300 mV. Since the in-situ sensor range for X1 gain (0-5000 mV) is equal to 0-500 µg Chla $L^{-1}$, the expected values for low and high Chla samples were around 100 mV and 1500 mV, indicating that the in-situ sensor readings were approximately 2 to 5 times lower than expected (Figure 1). Moreover, the in-situ Phyco fluorescence signal from the low concentration sample was ca. 1 mV, while relatively high values of ca. 35 mV were found for the high concentration sample.

ii. Dissolved organic carbon effect check

The impact of organic matter on Chla fluorescence readings is well documented (e.g. Kuha *et al.*, 2020). In our enclosures, however, Zhou *et al.* (2019) showed that the concentration of organic matter was relatively low and mainly derived from algae. Therefore, we did not expect to see substantial interference from DOC.

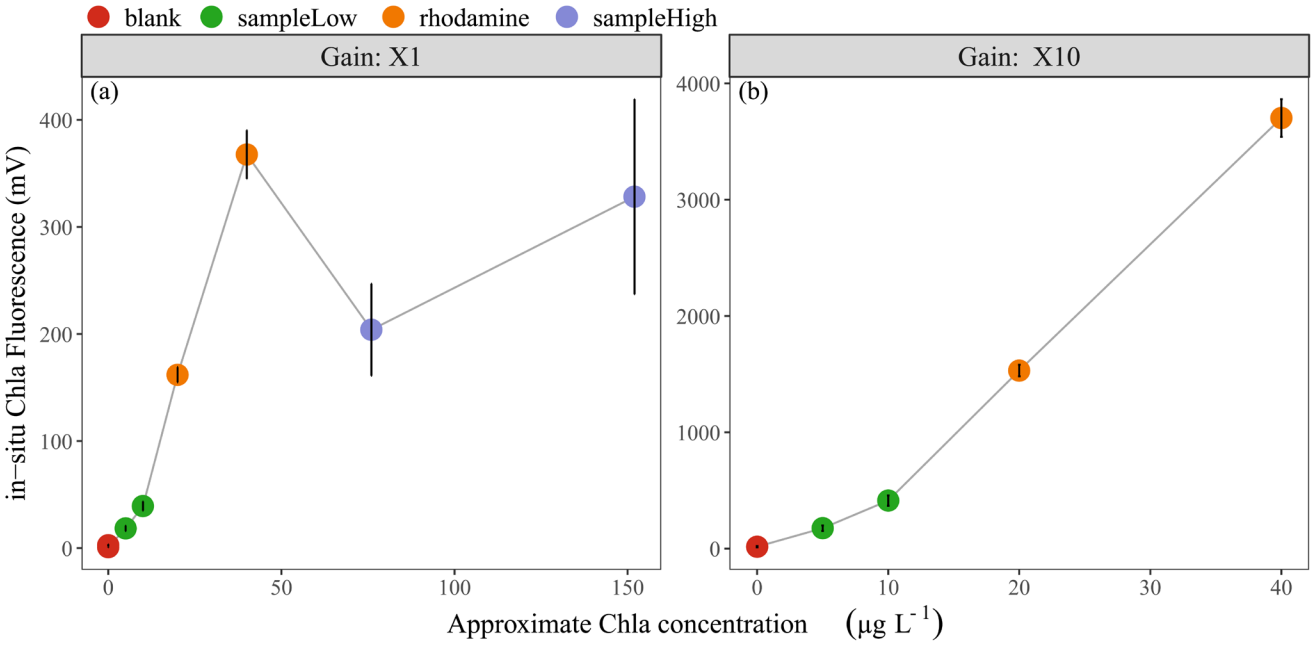


**Figure 1. Linearity check-2**; in-vitro Chla concentrations vs fluorescence in-situ sensor (Cyclops-7F, Turner Designs) readings, showing the mean and standard deviation of (**a**) 11 sensors with X1 gain and (**b**) four sensors with X10 gain. In-vitro Chla concentrations for undiluted samples are; blank: 0 µg $L^{-1}$, sampleLow: 10 µg $L^{-1}$, rhodamine: 40 µg $L^{-1}$, sampleHigh: 150 µg $L^{-1}$. Note the scale differences on the axes.

Dissolved organic carbon (DOC) in the samples collected on 27 July 2020 ranged between 2.6 and 25.5 mg $L^{-1}$, with a mean of 3.7 mg $L^{-1}$ and 8.8 mg $L^{-1}$ for the LN and HN treatments, respectively (Figure 2). Comparison of in-situ fluorescence readings between Rhodamine B standard solutions and Rhodamine B added water samples showed that the in-situ Chla fluorescence increased with increasing DOC, and at the highest DOC the difference was ca. 40 mV. However, when DOC was <5 mg $L^{-1}$, the impact was relatively low, with a ca. 3 mV increase at 2.6 mg DOC $L^{-1}$ (Figure 2). In our case, in-situ Phyco fluorescence readings were not affected by DOC (Figure 2).

We collected DOC samples from all the tanks an additional four times between the end of August and the start of September 2020, and the concentration ranged between ca. 2–5 mg $L^{-1}$ and 4–9 mg $L^{-1}$ in the LN and HN tanks, respectively. These values correspond well with those recorded in summer 2016 by Zhou *et al.* (2019) (ca. 2–5 mg and 5–8 mg $L^{-1}$ for the LN and HN tanks, respectively), indicating rather stable DOC concentrations with occasional exceptions in the HN tanks. Therefore, we did not consider the DOC effect further.

### In-situ fluorescence data cleaning and problems

Throughout the study period, some inconsistencies were observed in the fluorescence in-situ sensor data, some of which reflected biofouling on the shade caps and electricity cuts, resulting in an unexpectedly high and gradually increasing variance (e.g. PhycoG3, Nov/2018, AppendixD page 6) and negative or missing values, respectively. Biofouling on the optics of the sensors, which fully covered the LED, further caused a gradual decrease in in-situ fluorescence values, following a sudden increase after sensor cleaning (e.g. PhycoG1, Date: 14-09-2019, AppendixD page 2). Filamentous algae cover around the shade caps of the in-situ sensors (e.g. ChlB3, Aug/2019, AppendixD page 9) also led to erroneous signal-quenching. In addition, we identified problems with the sensor pins or with the multiplexer causing structural data changes (jumps or drops) (ChlC1 and ChlE2, AppendixD pages 7 and 8). Inconsistent sudden and steady jumps or drops were also found, for which no reasons could be provided. Moreover, pond snails, snail eggs, leeches and damselfly larvae were observed on the shade caps, possibly occasionally creating questionable signal values.

Fluorescence in-situ sensor data collection over a year at high resolution (every minute, 533101 data points for each sensor) allowed data cleaning in several steps (see AppendixA). Even though the cleaning procedure worked well, it did not allow us to remove all faulty readings, such as the ones due to structural changes caused by electronic problems or the gradual decrease caused by biofouling. With this method, on

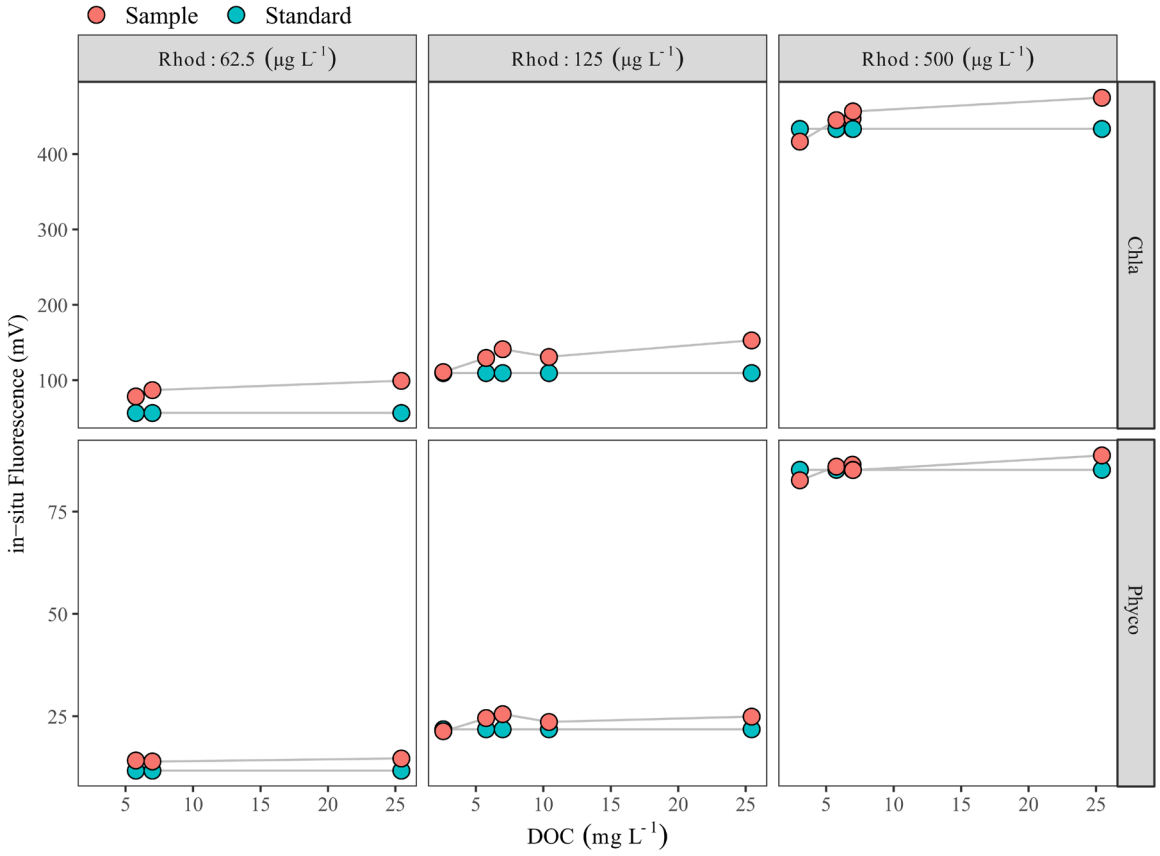


**Figure 2. Dissolved organic carbon (DOC) effect check.** DOC vs Rhodamine B-added filtered water samples (red circles) Rhodamine B standards (green circles) measured with Cyclops-7F sensors (Turner Designs). Each red circle represents a different water sample with a different DOC concentration. Note the scale difference on the y-axis.

average only 15 days of data out of ca. 500–670 days with measurements had to be removed (see AppendixA, for details).

### Fluorescence in-situ Chla sensor calibration for each tank

The night-time medians of fluorescence in-situ readings from each sensor were calibrated against in-vitro Chla measurements from the same tanks and days, resulting in ca. 22 – 30 calibration points for the HN tanks and ca. 18 – 25 points for the LN tanks.

Following removal of outliers, there were generally good linear relationships ($r^2 \geq 0.7$) between the in-vitro and in-situ measurements in the LN tanks (Figure 3 upper panel, AppendixB, Fig. B3). An exception was the sensor from tank C3, which had a lower coefficient of determination ($r^2$ ca. 0.45). This tank had relatively low in-vitro Chla concentrations throughout the study period (<10 µg $L^{-1}$). Similarly, two other tanks (B1 and C1) with relatively low in-vitro Chla concentrations also showed low explained variance prior to the removal of outliers (data not shown).

The sensors in the HN tanks (Figure 3 lower panel, AppendixB Fig. B4) generally had a high $r^2$ (> 0.6). However, high cyanobacteria concentrations in some tanks led to weaker relationships (AppendixB Fig. B5), but inclusion of Phyco night-time median readings in multiple regressions resulted in a better model fit and a 10–60% increase in explained variability (AppendixB, Fig. B5 vs B6), resulting in $r^2 > 0.5$ (Figure 4, AppendixB Fig. B6). The calibration plots of the D1 and G1 sensors showed a high in-vitro Chla concentration either as a result of high in-situ Phyco fluorescence or high in-situ Chla fluorescence (Figure 4).

### Comparing Equivalent-Chla and in-vitro Chla

Time series plots comparing Equivalent-Chla and in-vitro Chla showed good agreement for the LN tanks, especially for B2 and H3, where the Chla concentration range was wide (0–125 µg $L^{-1}$) (Figure 5). However, some mismatch occurred, especially in the C3 and, to some extent, in B1 and C1 where in-vitro Chla concentrations were low (0.5 – 5 µg $L^{-1}$), being in the lowest 1–10% of the sensor range at X10 (0–50 µg $L^{-1}$).

The time series plots of the HN tanks showed good correspondence as well (Figure 6). In general, the sensors captured the spring/summer/autumn peaks and the decreases in the colder months. There were, some inconsistencies, however, not least in the D2 tank. At the start of the sampling period, the agreement was better for D2 where the predicted values followed the trend of in-vitro Chla, but after February-2019 the change in in-vitro Chla was not captured well by the sensors. A similar pattern was observed for G1, especially after mid-August 2019 when there was a recurring biofouling problem on the optical part of the sensor; this was obvious from the gradual decrease in in-situ fluorescence followed by a sudden increase after sensor cleaning (e.g. ChlG1 and PhycoG1, Date: 14-09-2019, AppendixD page 2). Moreover, in both of these cases in-situ Phyco fluorescence showed a higher explained variance with in-vitro Chla, with $r^2 = 0.42$ for D2 and, $r^2 = 0.55$ for G1 (data not shown) compared to in-situ Chla fluorescence.

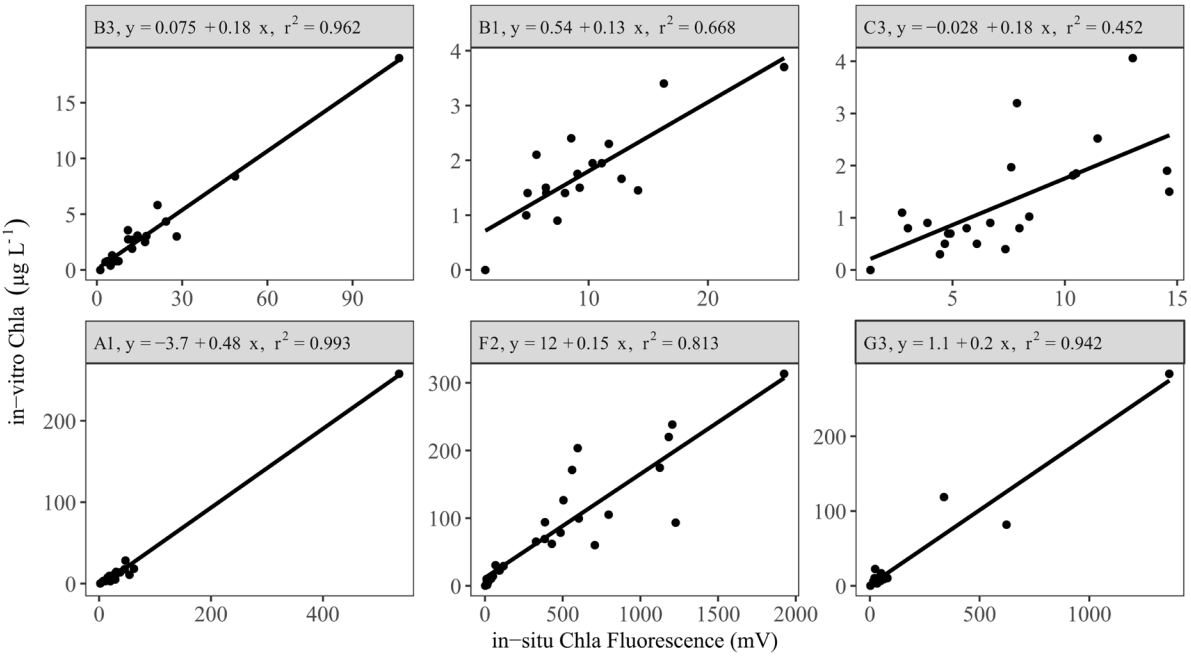


**Figure 3. Linear regression results for a selection of low (upper panel) and high (lower panel) nutrient tanks, showing calibration equations and the coefficient of determination ($r^2$) for each tank (see AppendixB, Fig. B3 and B4 for all the tanks).**

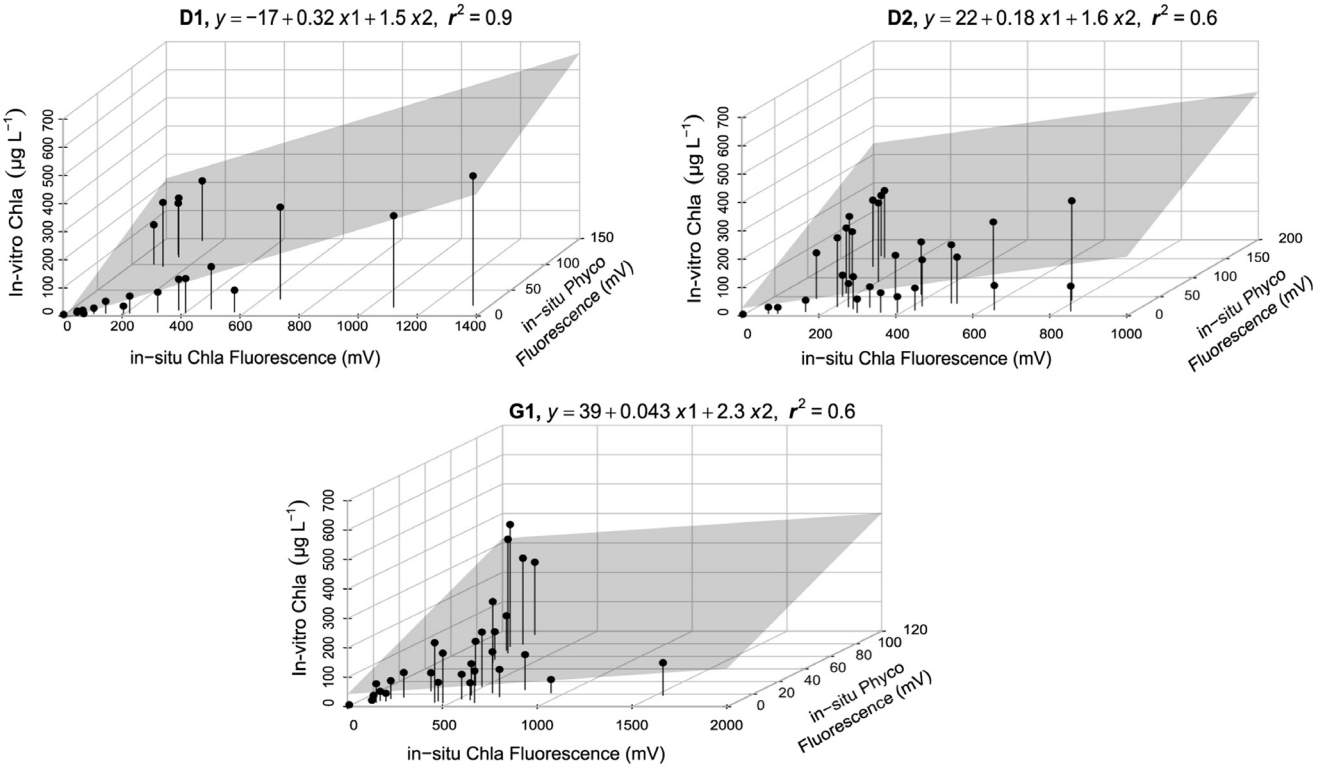


**Figure 4. Multiple linear regression results for a selection of high nutrient (HN) tanks, showing calibration equations and the coefficient of determination ($r^2$) for each tank.** x1 corresponds to in-situ Chla fluorescence and x2 to in-situ Phyco fluorescence. The area highlighted in grey indicates the regression plane (see AppendixB, Fig. B5 for all the tanks).

## Accuracy of the Equivalent-Chla concentration

The comparison of Equivalent-Chla and in-vitro Chla showed a relatively good 1:1 relation (Figure 7), although some deviation was observed. Especially in spring, there was a tendency to overestimation of Chla by Equivalent-Chla for both the HN and LN treatments and underestimation in the summer, particularly for the HN treatments (Figure 7), particularly for AMB and A2 (AMB + 2–4˚C) temperature treatments. Moreover, underestimation in the summer, particularly for the HN–A2 treatment (Figure 7) was also observed.

Relative error (RE) was used to compare the performance of the calibration among treatments. Even though RE was highest in the HN tanks, the comparison did not show any significant differences among the treatments; the warmer treatments, especially A2+, tended to have a slightly higher RE (Figure 8a), Overall, RE was higher at low in-vitro Chla concentrations (Figure 8b).

## Comparing daily Equivalent-Chla with in-vitro Chla

The daily Chla concentrations for each tank were predicted for the whole study period (October 2018–August 2020) by using the calibration equations developed in this study between in-vitro Chla and in-situ fluorescence sensor measurements (Figure 9 and Figure 10, AppendixC). The results illustrate that major events could go unnoticed by the in-vitro Chla measurements. For instance, in tank-H3 (having a high coefficient of determination ($r^2$ = 0.98) between the two measurements), only one of the peaks observed with the in-situ sensors between June and August 2019 was recorded with regular in-vitro sampling (Figure 9). The tanks with moderate explained variance ($r^2$: 0.4 – 0.6) provided similar results, e.g. the small peak at the end of May-2019 in tank C3 was missed by in-vitro sampling.

Observations from high nutrient (HN) tanks were similar (Figure 10, AppendixC); here Chla peaks were also missed by the in-vitro sampling. Moreover, even if the Chla peaks were caught with the regular in-vitro sampling method, e.g. tank-F3, the true duration of these high-Chla periods was not well captured.

Our results further emphasised the significance of employing multiple regression by including Phyco in-situ fluorescence measurements as a predictor variable in the calibration. For example, around October 2018 in tank-D1, the period with high in-vitro Chla was missed by the in-situ Chla fluorescence sensors but caught by the in-situ Phyco sensor (AppendixD page 1), demonstrating that the high in-vitro Chla reflected an increase in cyanobacteria concentrations. Additionally, as expected, Phyco in-situ fluorescence sensors were also useful

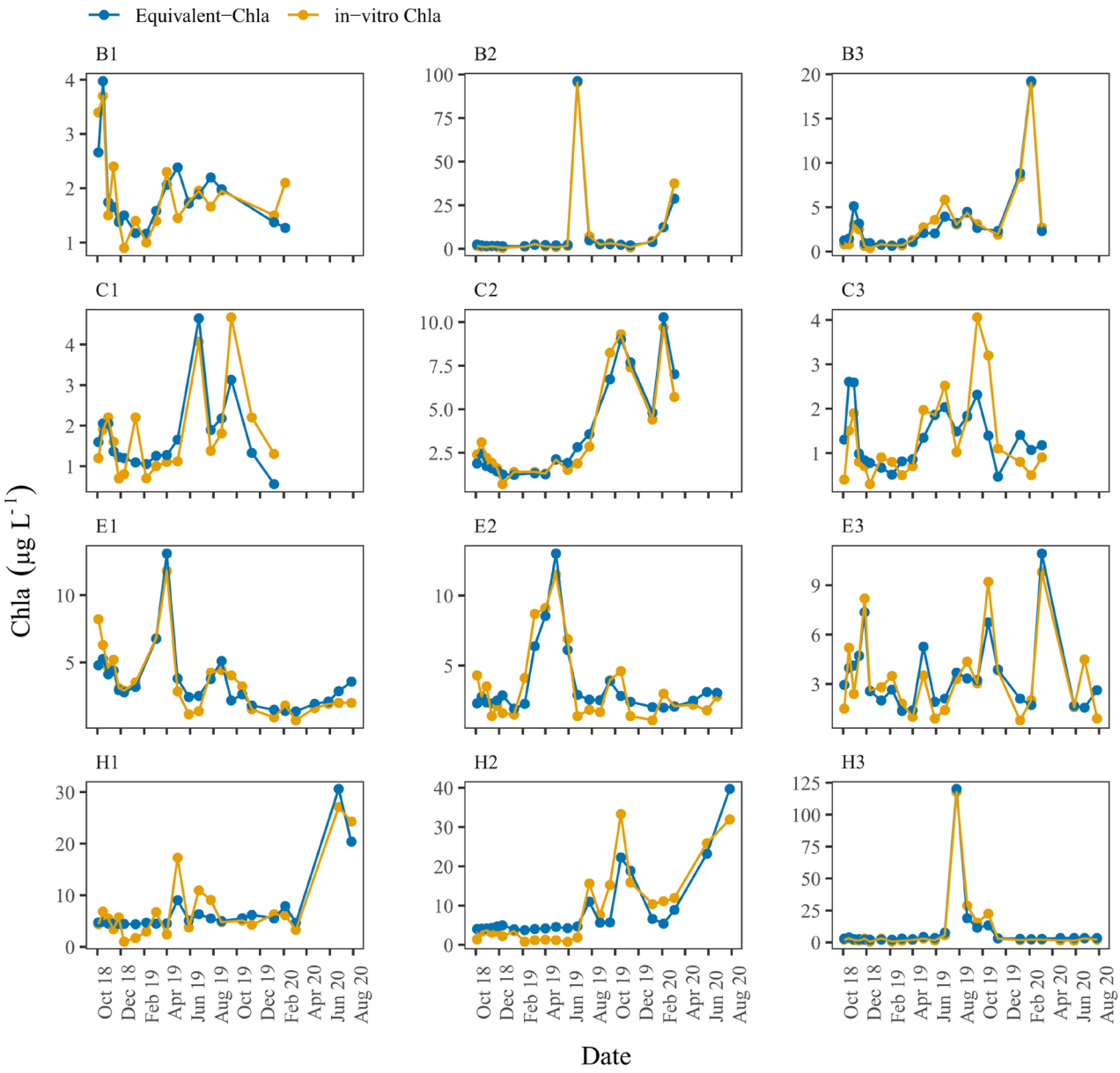


**Figure 5. Biweekly/monthly time series plots for low nutrient (LN) tanks between October 2018 and August 2020.** Note the scale differences on the y-axis.

in monitoring the changes of relative cyanobacteria concentrations, notwithstanding the lack of calibration. These measurements provide information on the timing of main cyanobacteria changes, while also emphasising the possible impact of in-situ Chla fluorescence on in-situ Phyco values. This can be seen e.g. in tank-D3 where the three in-situ Chla peaks likely caused an increase in in-situ Phyco fluorescence (AppendixD page5).

## Comparing annual and summer means: in-vitro vs Equivalent-Chla

The comparison between the annual mean values with 95% confidence interval (CI) of the two methods revealed no significant differences for many of the low nutrient (LN) tanks. For instance, tank-E1 had annual means (±CI) of 4.2 (±0.4) and 3.9 (±0.3) for Equivalent-Chla and interpolated-$Chla_{in\text{-}vitro}$, respectively (Figure 11). Nevertheless, significant differences were observed especially in the summer means of most of the LN tanks, and the interpolation of in-vitro Chla concentrations either caused over- or under-estimation of the mean values. For example, in tank-B2, both annual and summer means of interpolated-$Chla_{in\text{-}vitro}$ were higher than the mean of Equivalent-Chla. Due to linear interpolation, the duration of the interpolated-$Chla_{in\text{-}vitro}$ peak was wider in comparison to the observed peak width of Equivalent-Chla (AppendixC page4). This was also the case for tank-H1. On the other hand,

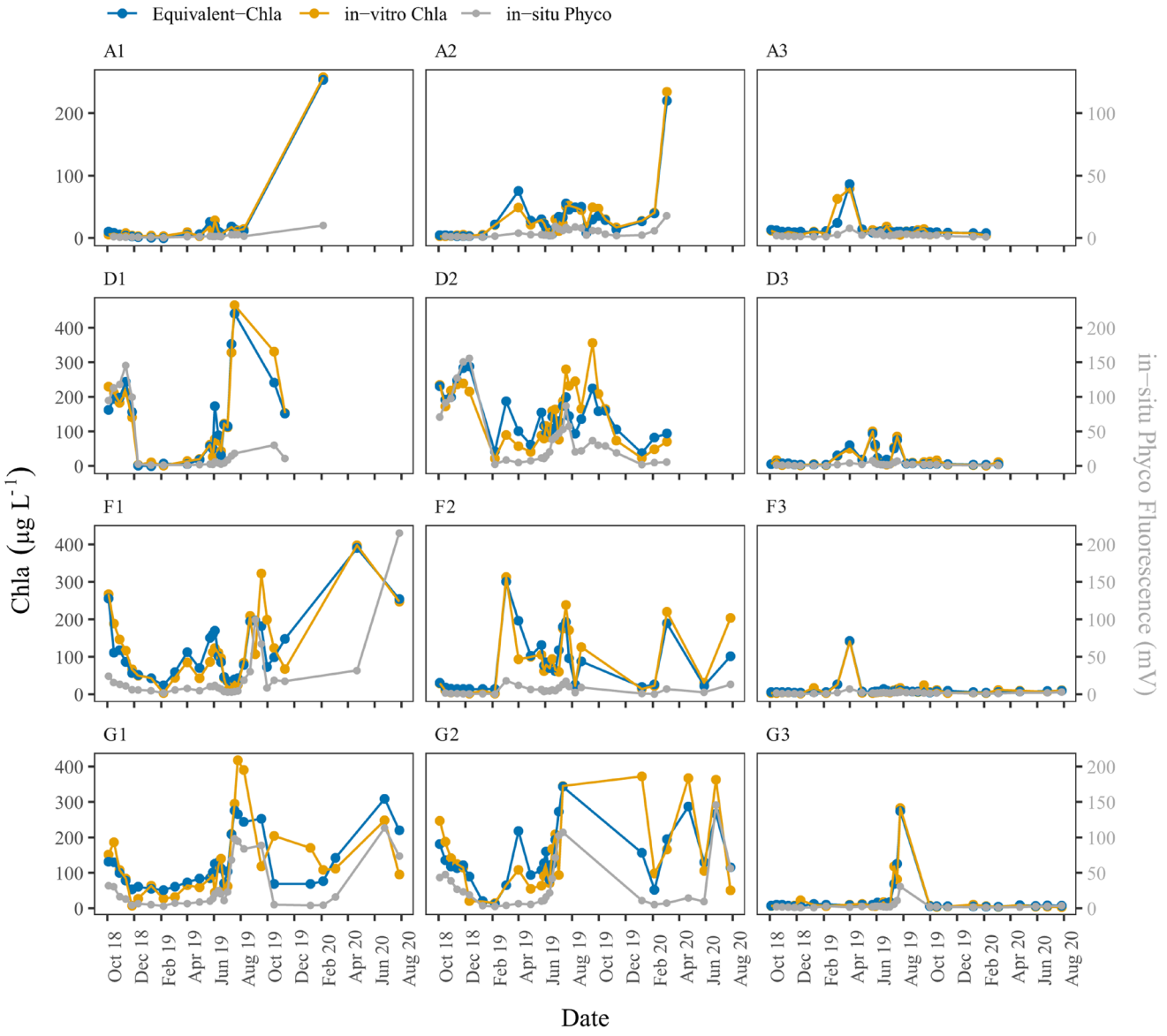


**Figure 6. Biweekly/monthly time series plots for high nutrient (HN) tanks between October 2018 and August 2020.** Note the scale and unit differences on the primary and secondary y-axis.

mean Chla was under-estimated with interpolated-Chla$_{in\text{-}vitro}$ in tank-C1 because the regular sampling completely missed the Chla peak in June-2019 (Figure 11 and AppendixC page 3).

The high nutrient (HN) tanks showed a somewhat different pattern (Figure 12), partly as a consequence of the Chla range limit of the sensors, i.e. concentrations higher than 500 µg Chla L$^{-1}$ were not measured, but also due to more frequent sampling. Out of eight tanks with high in-vitro Chla concentrations, the mean values from both methods were similar for tanks A1, F1 and partly for D1, while for the other five tanks (G1, D2-F2-G2, G3) mean interpolated-Chla$_{in\text{-}vitro}$ was significantly higher, both for the summer and annual means (Figure 12). The tanks (A2, A3-D3-F3) with lower in-vitro Chla concentrations (≤500 µg L$^{-1}$) mostly had similar annual and summer mean values for both methods, except for A3 summer. This similarity is likely related to the higher regular in-vitro sampling frequency in the HN compared to the LN tanks, enabling us to capture some of the short-term Chla peaks with regular sampling (e.g. Figure 10 and Figure 12 tank-D3).

## Discussion

We tested chlorophyll-*a* fluorescence in-situ sensors over the course of 22 months covering a diverse set of environmental conditions in 24 tanks (mesocosms) simulating fully mixed shallow lakes with contrasting nutrient concentrations and

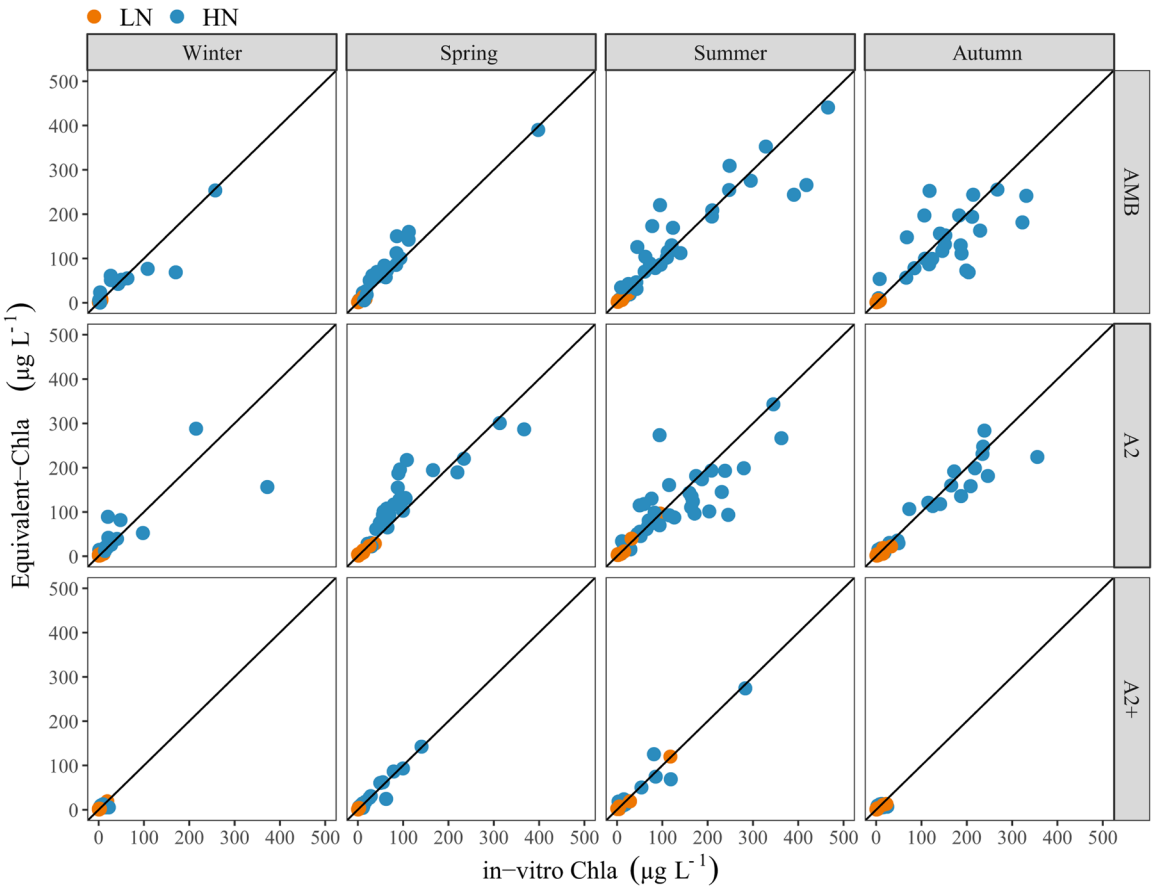


**Figure 7. Comparison of Equivalent-Chla and in-vitro Chla concentrations, grouped according to season and temperature treatments.** Solid line indicates the 1:1 relation. Nutrient treatments are low (LN) and high (HN). Temperature treatments are ambient (AMB), IPCC-A2 (A2) and IPCC-A2+50% (A2+).

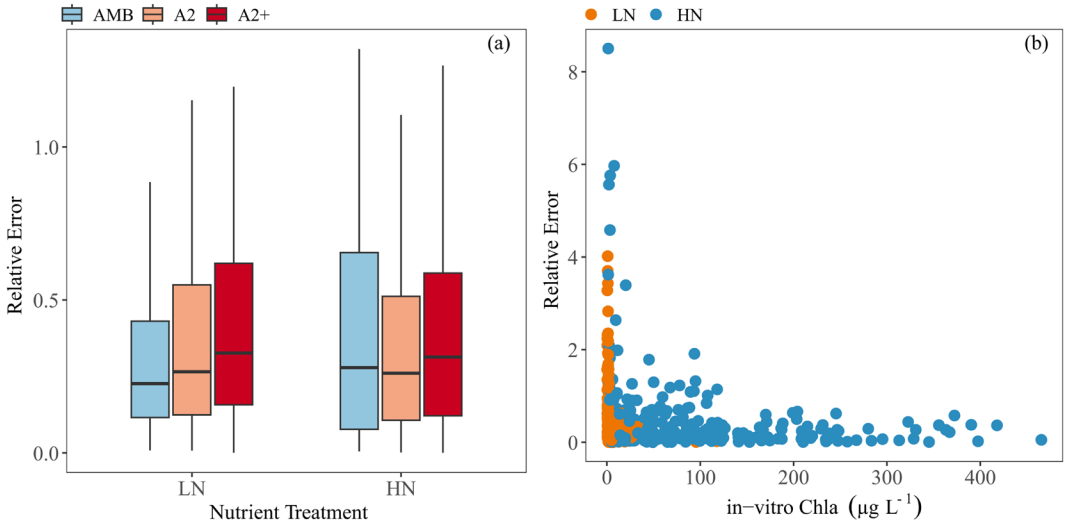


**Figure 8.** Relative errors (RE) against (**a**) each nutrient and temperature treatment (**b**) and in-vitro Chla concentrations. Temperature treatments are Ambient (AMB), A2 (+2-4 °C) and A2+ (+ 4-6 °C). Nutrient treatments are low (LN) and high (HN). Outliers were removed from the boxplot for visual purposes. The boxplots show: minimum and maximum values (range bars), interquartile range (boxes: 25 – 75%), and median value (line within the box). Note the scale differences on the y-axis.

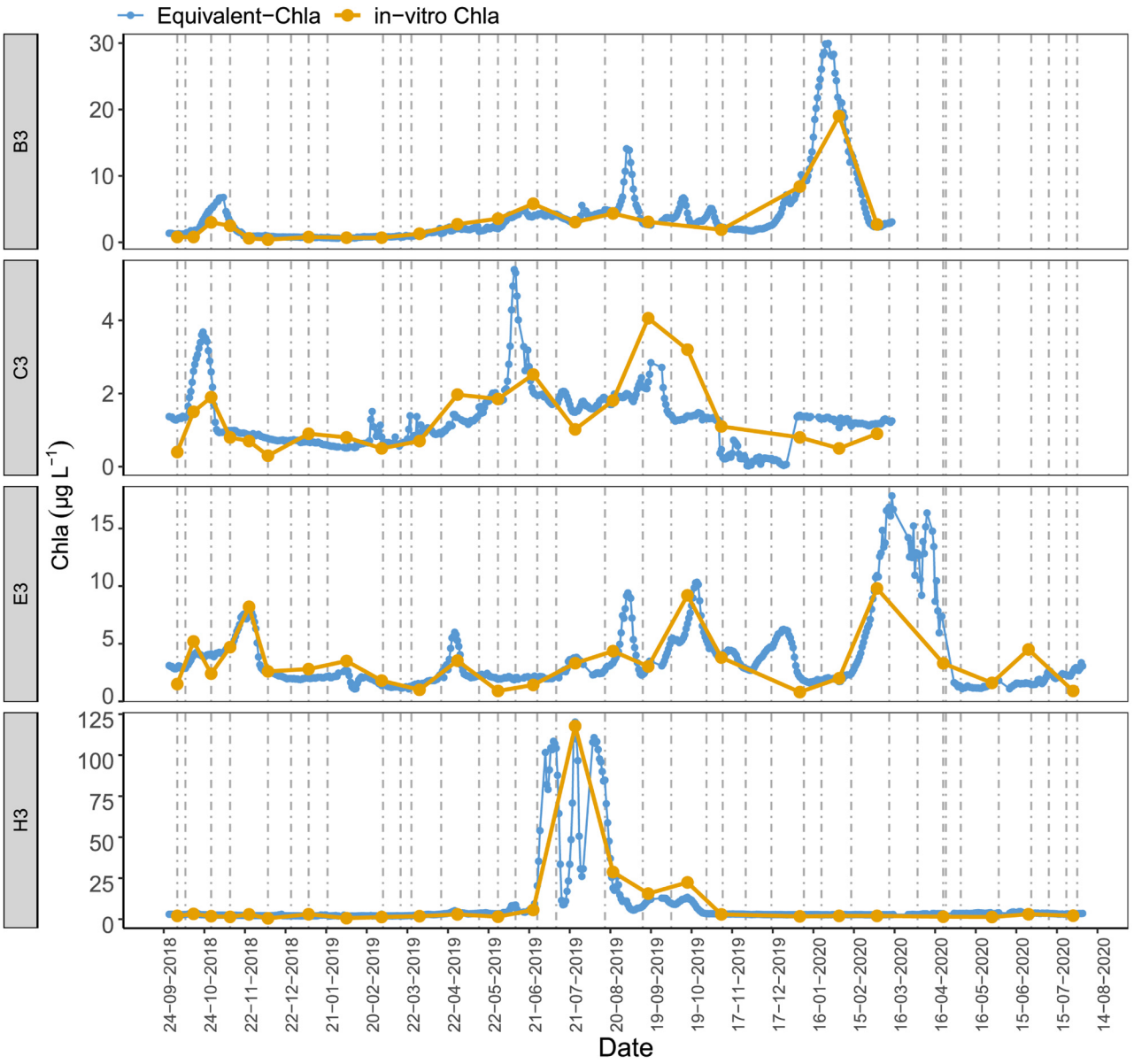


**Figure 9. Daily Equivalent-Chla vs in-vitro Chla for low nutrient (LN), A2+ climate scenario treatments.** Vertical lines are sensor cleaning days. See AppendixC for figures of the remaining tanks.

temperatures. The tanks had relatively low and stable concentrations of dissolved organic matter, and turbidity was dominated by phytoplankton. After cleaning of sensor data, the Chla fluorescence in-situ sensors gave reliable concentration estimates; however, the concordance between our Equivalent-Chla concentrations and in-vitro measured values was not perfect since we also observed high relative errors, especially at low Chla concentrations. It is important to recognise that in-situ sensors of Chla fluorescence do not measure Chla concentrations directly.

## Data cleaning: pros and cons

Data-cleaning and interpolation were conducted for all sensors following a comprehensive 11-step procedure (AppendixA). The first steps followed suggestions from the literature, for example removing daytime data, defining a minimum data limit and removing repeated values (e.g. Jaccard *et al.*, 2021), aiming to exclude data affected by non-photochemical quenching, negative values and erroneous signal-quenching. We also used a rate of change (ROC) approach to remove extreme values and to reduce high variation, for example during shade cap-biofouling periods. Jaccard *et al.* (2021) suggested another approach for cleaning Chla in-situ fluorescence data collected from moving platforms in the ocean. They mathematically defined the periods showing a gradual increase of measured values, followed by a sudden drop after sensor cleaning. They were able to identify fouling periods and assigned a possibility scale as higher or lower

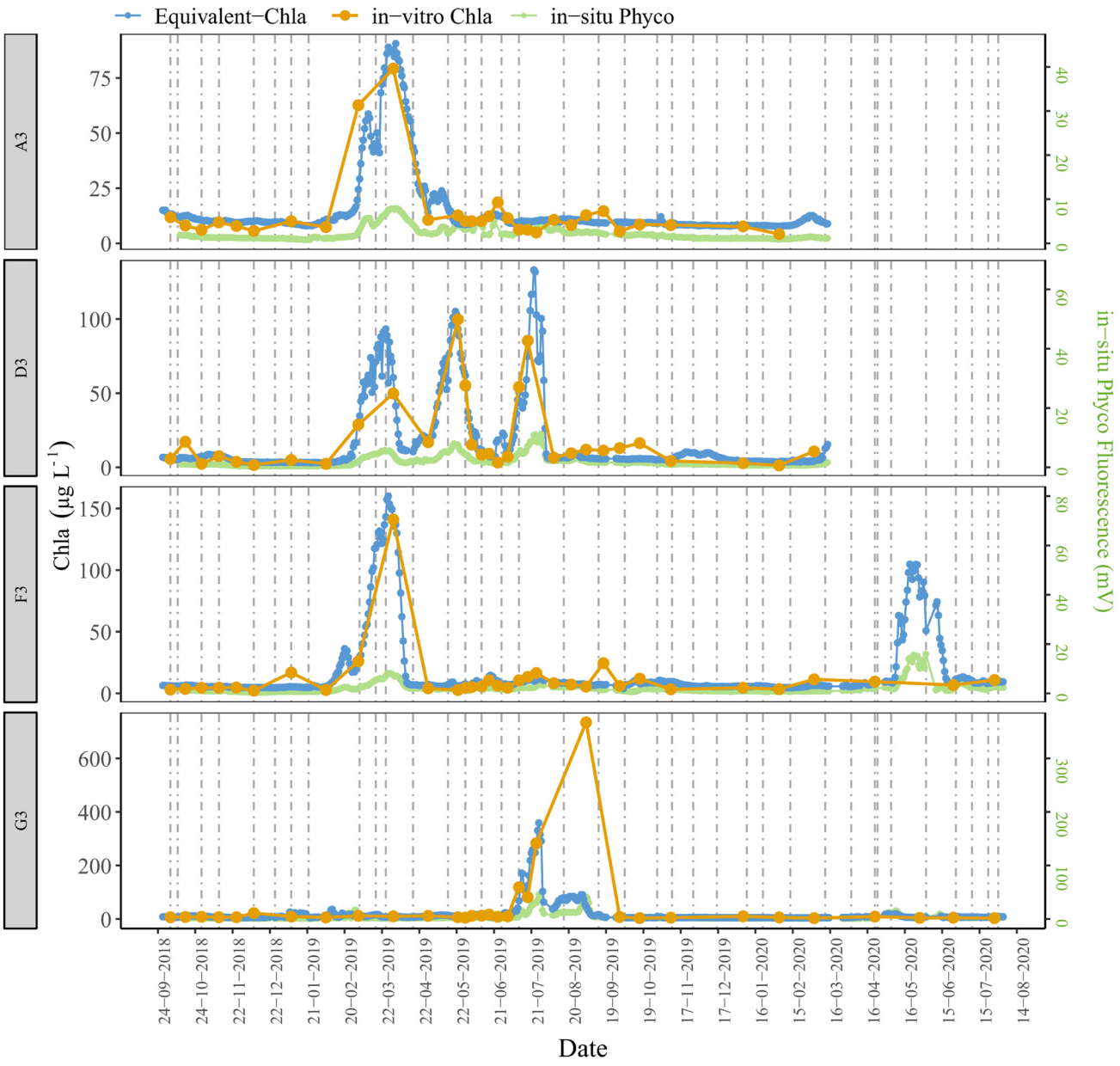


**Figure 10. Daily Equivalent-Chla vs in-vitro Chla for high nutrient (HN), A2+ climate scenario treatments.** Vertical lines are sensor cleaning days. See AppendixC for figures of the remaining tanks.

probability of biofouling (Jaccard *et al.*, 2021). Although our rate of change approach removed the high variance caused by shade cap biofouling, it was, at least to some extent, not sufficient to identify the in-situ fluorescence decrease caused by the biofouling on the optical parts of the sensors that prevented the transmission of the light beam. Even though biofouling is a well-known issue in water environments, we acknowledge that the challenge we encountered was partly due to the relatively low cleaning frequency of the sensors (mainly 2-week intervals). This issue was mostly prominent in our HN tanks, emphasising that biofouling is a relatively minor issue in oligo- mesotrophic systems.

Following the rate of change approach, data noise and possible remaining anomalies, such as questionable spikes or drops, were filtered out if they were 1.2 times higher or lower than the standard deviations from the rolling 48-hour mean. The latter data cleaning process is widely used for HF time series observations (Sharma *et al.*, 2010; Teh *et al.*, 2020), but, when used alone, it did not suffice to clean our data when

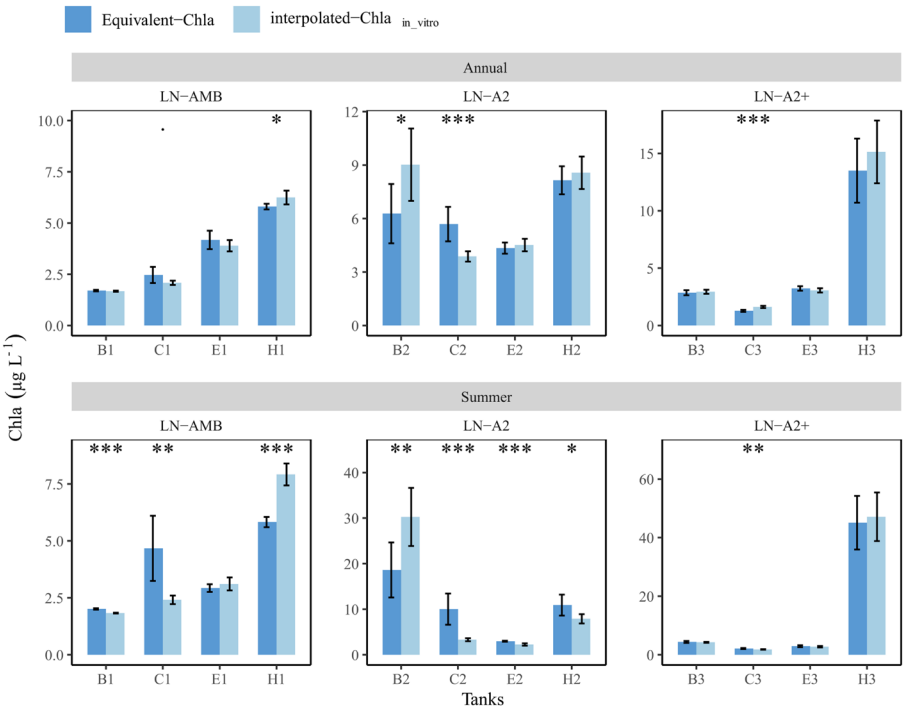


**Figure 11. Annual and summer means of Chla concentrations in low nutrient (LN) tanks, calculated based on interpolated-Chla$_{in\text{-}vitro}$ and Equivalent-Chla values.** Temperature treatments are Ambient (AMB), A2 (+2-4 °C) and A2+ (+ 4-6 °C). Error bars show 95% confidence intervals. The significance (*p*-value from t-test) is shown as stars where *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$.

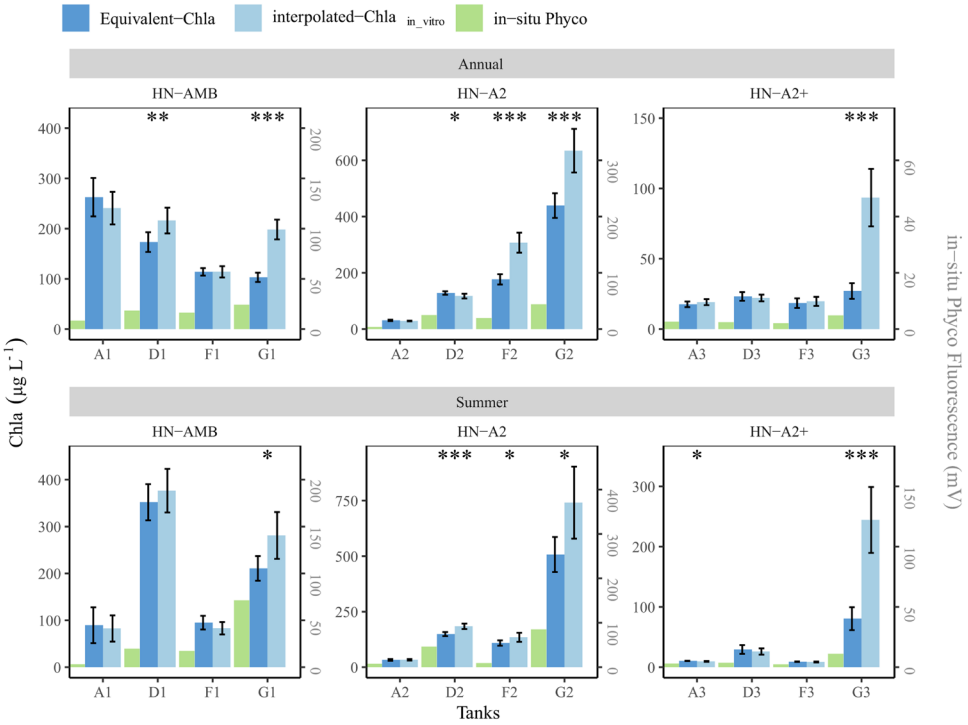


**Figure 12. Annual and summer means of Chla concentrations in high nutrient (HN) tanks, calculated based on interpolated-Chla$_{in\text{-}vitro}$ and Equivalent-Chla.** Temperature treatments are Ambient (AMB), A2 (+2-4 °C) and A2+ (+ 4-6 °C). Error bars show 95% confidence intervals. The significance (*p*-value from t-test) is shown as stars where *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$.

there was a false high variation. This data cleaning procedure resulted in an average data loss of ca. 8% of all night-time 5-minute data collected during the two years.

### Basic inspection of in-situ Chla and Phyco sensors

The first sensor check (linearity check-1) showed, as expected, strongly linear Rhodamine B standard curves. Although the subsequent linearity check-2 indicated more complex conditions in the natural water samples, the sensors were confirmed to be "linear with a proportional increase" within each diluted/undiluted group (Rhodamine dye, low-Chla water sample, high-Chla water sample). A comparison between these groups showed that the increase in in-situ fluorescence values was not proportional. However, optimal fluorescence measurements, e.g. with Rhodamine dye, are not to be expected for water samples due to variation in environmental factors as well as in phytoplankton species composition and size, all being factors that affect the fluorescence signals (Petersen & Möller, 2017). The comparison of low and high Chla concentration water samples highlighted inconsistencies as the high-Chla in-situ signals were ca. 2.5 times lower than expected compared with the low-Chla in-situ signals when comparing to the respective in-vitro measurements. This was probably due to the relatively high Phyco in-situ fluorescence (ca. 35 mV) in the high-Chla sample, leading to reduced Chla in-situ fluorescence. This is consistent with previous investigations testing the precision of Cyclops-7 fluorescence in-situ sensors, showing a decrease in sensor response with addition of cyanobacterial Chla to a diatom Chla media (Carroll *et al.*, 2005). Thus, Seppälä *et al.* (2007) found that Chla concentrations could not be estimated by employing only Chla fluorescence measurements during the periods with cyanobacteria blooms in the Baltic Sea. We found similar results in five of twelve HN tanks; using multiple regression, we observed an average 38% increase in coefficient of determination (AppendixB Fig. B5 vs B6). By contrast, Kara *et al.* (2012) did not measure in-situ Phyco fluorescence and observed a very weak relation between in-situ and in-vitro Chla measurements for summer samples ($r^2$ of 0.06, n = 17) from cyanobacteria-dominated Lake Mendota (USA).

### In-situ Chla sensor calibration and evaluating Equivalent-Chla

Overall, we found good correspondence between in-situ Chla fluorescence and in-vitro Chla concentration measurements, especially after employing temperature correction for both in-situ Chla and Phyco fluorescence readings. Linear and multiple regression analyses showed that the coefficient of determination ($r^2$) was >0.5 for 23 of the 24 sensors. Several other studies conducted in lakes (Kuha *et al.*, 2020), rivers and reservoirs (Gregor & Maršálek, 2004), fish farms (Brager *et al.*, 2015) or in the marine environment (Aiken *et al.*, 2011; Maung-Saw-Htoo-Thaw *et al.*, 2017) have identified similar relationships for in-situ fluorescence and in-vitro measurements, the latter based on HPLC measurements, microscopy or spectrophotometry. It is important to note that the results of these other studies were not obtained using the same brand of sensors and that fluorescence readings can vary, especially among sensors from different manufacturers (e.g. Hodges *et al.*, 2018 for Phyco sensors).

Our results further indicate an impact of phytoplankton composition. Especially in spring, there was a tendency of overestimation of Chla with Equivalent-Chla for both the HN and LN treatments and of underestimation in summer, particularly for warmer HN–A2 treatment, possibly reflecting the seasonal variation in phytoplankton communities in these tanks (a shift from a diatom dominated community in spring to cyanobacteria in summer (Dashkova *et al.*, 2022; Strandberg *et al.*, 2022)). This was supported by the lack of significant differences in relative error between treatments, indicating that the major treatment differences were not the main cause of the error. Lawrenz and Richardson (2011) studied the effect of species composition on Chla fluorescence and showed that a fluorometer calibrated on diatom culture either over- or underestimated the Chla concentration from cultures of dinoflagellates and chlorophytes, respectively. Similarly, Bertone *et al.* (2019) found variations in Phyco in-situ signals between different cyanobacteria cultures of *Sphaerospermopsis* sp., *Aphanocapsa* sp. and *Raphidiopsis raciborskii* compared to total biovolume and to spectrophotometric Phyco measurement.

Shortcomings related to the technical features of the sensors and spectrophotometric methods should also be considered. For example, due to the sensor measurement limit, it was not possible to calibrate the sensors for values higher than 500 µg Chla $L^{-1}$, resulting in lower Equivalent-Chla concentrations for some of the tanks. The point sampling method also has uncertainties regarding handling, transportation and analysis of the water samples, and together with the lower sensitivity of this method compared to HF fluorescence in-situ sensors (Lakowicz, 2006; Millie *et al.*, 2010), this may explain the lower explained variance in the tanks with low in-vitro Chla concentrations ($\leq$ 5.0µg $L^{-1}$). Even though DOC data suggested that the tanks had relatively low and stable concentrations over the two years, we also acknowledge that by not including DOC in the calibration, discrepancies between in-situ fluorescence and in-vitro measurements may have occurred since impact of dissolved organic matter (DOM) on phytoplankton fluorescence has been found in various studies of different sensor brands (e.g. Kuha *et al.*, 2020; Proctor & Roesler, 2010; Twiss, 2011).

### Time series comparison of Equivalent-Chla and interpolated-Chla

High frequency (HF) Equivalent-Chla concentration data allowed us to observe sudden and often large Chla changes (peaks) that were, in part, missed by the point sampling (in-vitro method). These unobserved Chla peaks resulted, in our case, in an underestimation of the mean concentrations of interpolated-$Chla_{in\text{-}vitro}$ and were one of the reasons for the significant differences in mean values (annual and summer) between the two methods. Furthermore, HF measurements provided the exact timing of the main changes. Even when the peaks were recorded with the point sampling interpolation of the in-vitro data, the real start and end points of the concentration increase remained undetected; thus, the HF data

widened the time period of higher Chla concentrations with implications for the mean values. Overestimation was also found at low Chla concentrations, which might reflect a lower sensitivity of absorbance compared to fluorescence spectroscopy (Lakowicz, 2006), exhibiting more precise measurement at lower concentrations of in-situ fluorescence methods. Unfortunately, besides the benefits of HF measurements, the upper Chla concentration limit of Cyclops-7F fluorescence in-situ sensors (Turner Designs) prevented correct calculation of the means in many of the HN tanks with >500 µg Chla $L^{-1}$. Fortunately, such high values are not common in lakes and would thus not be a problem in most cases.

The time series data also highlighted the importance of including in-situ Phyco fluorescence measurements in the calibration as the periods with relatively high in-vitro Chla concentrations and simultaneously low in-situ Chla fluorescence measurements could only be explained by high cyanobacteria concentrations. While multiple regression enhanced the explained variability, it fell short for providing highly reliable calibration in specific cases, such as tank D2. This limitation may be attributed to the stronger correlation of in-situ Phyco fluorescence with in-vitro Chla and the higher relative change between in-situ Phyco and Chla fluorescence, which exhibited greater variation during the study period compared to other tanks.

## Conclusion

The experimental setup, with two nutrient concentrations and three temperature scenarios (in total six treatments), gave us an advantage in testing the fluorescence in-situ sensors in a diverse set of environments during a ca. two-year period.

In-vitro (spectrophotometric) and in-situ (fluorescence) measurements demonstrated an overall good correlation with occasional exceptions, especially related to sensitivity differences of two methods (fluorescence being more accurate), possibly causing a mismatch regarding low Chla concentrations, and to biofouling on the sensors. Moreover, in-situ Phyco fluorescence (cyanobacteria indicator) was a non-negligible factor that needs to be corrected for when calibrating in-situ Chla fluorescence sensors to compensate for an underestimated cyanobacteria increase by in-situ Chla.

Equivalent-Chla concentrations, calculated from the fluorescence in-situ sensor calibrations, were mostly in agreement with the in-vitro concentrations. The mean absolute error (MAE) between Equivalent-Chla and in-vitro Chla was 12, while the median absolute error was 2. Among the overestimated variables, 18% had a MAE greater than 12, whereas for the underestimated variables, this proportion was 22%. Seasonal comparison of Equivalent-Chla and in-vitro concentrations underlined the possible impact by a shift in dominant species during the study period and pointed to overestimated concentrations in spring and underestimation in summer by in-situ sensors, which may be related to a shift from diatom to cyanobacteria communities. To validate phytoplankton seasonality as a source of error in estimating equivalent-Chla concentrations, further studies investigating and comparing the phytoplankton community and its size in the tanks are needed.

High frequency (HF) in-situ fluorescence data, i.e. Equivalent-Chla concentrations, allowed us to observe sudden changes that would otherwise be overlooked in regular sampling and to identify the exact start and end time point of these concentration variations. Furthermore, the investigation and comparison of Equivalent-Chla and interpolated-$Chla_{in\text{-}vitro}$ concentrations emphasised the difference between the annual and summer means determined by these two methods. The significant differences in the means were mostly caused by the unobserved Chla peaks or from the misidentified time periods of the sudden changes when using regular low-frequency sampling for in-vitro measurements. The upper Chla concentration limit of the fluorescence sensors was another reason for the disagreement between means as it sets a lower limit to the calibration.

Overall, this study pointed out the importance of frequent Chla recordings despite the known challenges of in-situ fluorescence measurements. We are confident that using a careful approach, fluorescence sensors are a useful tool to monitor sudden and/or short-term changes in aquatic ecosystems. In accordance with previous studies, this study strongly emphasises the need for site-specific calibration as phytoplankton composition and possible interference factors, like DOM, are site-specific.

## Data availability

### Underlying data

- Data available from: Zenodo (RRID:SCR_004129); Lemming Mesocosm (Denmark): in-situ fluorescence chlorophyll-*a* calibration, underlying data, https://doi.org/10.5281/zenodo.10520248 (Levi *et al.*, 2024a).

The datasets listed below are used in;

a. Testing the variability of in-situ fluorescence sensor measurements in Blank (i.e. distilled water)
   - *Data used*: LemCP_BlankFull

b. Testing the linearity (Blank vs Rhodamine B std vs Water samples from the tanks) of in-situ fluorescence sensors
   - *Data used*: LemCP_BlankFull, LemCP_RhodamineFull, LemCP_HighLowChlaSample

c. Testing the impact of DOC concentration on in-situ fluorescence sensor readings
   - *Data used*: LemCP_RhodamineStd, LemCP_RhodamineAddition, LemCP_DOC

d. Linearly interpolating (daily) in-vitro chlorophyll-*a* analysis results from August 2018 until August 2020
   - *Data used*: LemCP_Spectro_AugSep18, LemCP_Spectro_OctOnw,

e. Taking the night-time median of the cleaned in-situ fluorescence data

- *Data used*: LemCP_PAR1Med5_QAQCdata, LemCP_DateNewGroup

f. Implementing temperature correction to cleaned in-situ fluorescence data

- *Data used*: Lem_TempOxyPh (and night-time median in-situ fluorescence data)

g. Calibrating in-situ chlorophyll-*a* sensor data against in-vitro chlorophyll-*a* measurements

- *Data used*: LemCP_Spectro_OctOnw, (and night-time median in-situ fluorescence data)

h. Marking sensor cleaning dates against biofouling

- *Data used*: LemCP_SensorCleaning

i. Plotting raw data (i.e. no quality check)

- *Data used*: LemCP_PAR1Med5_RB3data

Metadata file contains detailed information on all the underlying datasets;

- LemCP_metaData_v1: The metadata comprises 3 sheets, with detailed information on
  - main funding/projects,
  - organization where the study was conducted,
  - datasets used for the current study.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

## Extended data

*a. Analysis code*

- Analysis code available from: Zenodo (RRID:SCR_004129), Lemming Mesocosm (Denmark): in-situ fluorescence chlorophyll-*a* calibration analysis code, https://doi.org/10.5281/zenodo.14208033 (Levi, 2024).

  Analysis codes used are listed below:

  - LemCP_InSitu_DataClean: These are the rB3 codes that were used to clean the in-situ fluorescence data.
  - LemCP_DataWork_Graphs: This is the main document to prepare the data, calibrate the in-situ fluorescence sensors and to plot all the figures.
  - LemCP_OutliersTest: This is an example (chlorophyll-*a* data collected from tank G2 and A1) of how outliers were defined.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

*b. Appendix*

- Appendix tables and figures are available from: Zenodo (RRID:SCR_004129), Lemming Mesocosm (Denmark): in-situ fluorescence chlorophyll-*a* calibration, extended data, https://doi.org/10.5281/zenodo.10523065 (Levi *et al*., 2024b).

  List of appendix included as extended data are as below:

  - AppendA_TableA1: In-situ chlorophyll-*a* and phycocyanin fluorescence data cleaning procedure
  - AppendB_FigB1_Chl_BlankVariation: Blank measurement check; mean and standard deviation of in-situ chlorophyll-*a* and phycocyanin fluorescence sensor (Cyclops-7F, Turner Designs) readings in deionised water (blank sample) for X1 gain and for X10 gain. Note the scale differences on the y-axes.
  - AppendB_FigB2_RhodLinearity: Linearity check-1 for Cyclops-7F in-situ sensors (Turner Designs); Rhodamine B vs in-situ chlorophyll-*a* fluorescence (left pane) and in-situ phycocyanin fluorescence (right pane). Note the scale differences on the y-axes.
  - AppendB_FigB3_LinearReg_LN: Linear regression analysis results for the low nutrient (LN) tanks showing calibration equations and the coefficient of determination ($r^2$) for each tank. Note the scale differences on both axes. (Fluorescence in-situ sensors are Cyclops-7F, Turner Designs).
  - AppendB_FigB4_LinearReg_HN: Linear regression analysis results for 7 of the high nutrient (HN) tanks showing calibration equations and the coefficient of determination ($r^2$) for each tank. Note the scale differences on both axes. (Fluorescence in-situ sensors are Cyclops-7F, Turner Designs).
  - AppendB_FigB5_LinearRegTest_HN-noPhyc: Linear regression analysis results for 5 of high nutrient (HN) tanks showing calibration equations and the coefficient of determination ($r^2$) for each tank. Note the scale differences. (Fluorescence in-situ sensors are Cyclops-7F, Turner Designs).
  - AppendB_FigB6_MultReg_HN: Multiple regression analysis results for 5 of the high nutrient (HN) tanks showing calibration equations and the coefficient of determination ($r^2$) for each tank. The area highlighted in grey indicates the regression plane. Note the scale differences. (Fluorescence in-situ sensors are Cyclops-7F, Turner Designs).
  - AppendC_FigC1_4_Daily_Timeseries: Daily Equivalent-Chla vs in-vitro Chla for the tanks with high (HN) and low (LN) nutrient treatments with

ambient (AMB) and IPCC-A2 climate scenario treatments. Vertical lines are sensor cleaning days.

- AppendD_FigD1_9_RawClean_plots: Plots showing raw data for each tank (i.e. logged every minute and 5 min median taken) against quality-controlled data.

- AppendE_FigE1_SensorSetup: Schematic overview of the tank and sensor setup

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

## Acknowledgements

The authors are grateful to Anne Mette Poulsen for editing the manuscript. We also want to thank Lene Vigh, Kathrine Tabermann Uhrenholt, Marlene Venø Skjærbæk, Peter Bedsted Andersen and Viktor Levi for all the chemical analyses and technical assistance.

## References

Aiken CM, Petersen W, Schroeder F, *et al.*: **Ship-of-opportunity monitoring of the Chilean fjords using the pocket FerryBox.** *J Atmos Ocean Technol.* 2011; **28**(10): 1338–135.
**Publisher Full Text**

Argo: **Argo float data and metadata from Global Data Assembly Centre (Argo GDAC).** SEANOE, 2024.
**Publisher Full Text**

Bastien C, Cardin R, Veilleux É, *et al.*: **Performance evaluation of phycocyanin probes for the monitoring of cyanobacteria.** *J Environ Monit.* 2011; **13**(1): 110–118.
**PubMed Abstract** | **Publisher Full Text**

Bertone E, Chuang A, Burford MA, *et al.*: ***In-situ* fluorescence monitoring of cyanobacteria: laboratory-based quantification of species-specific measurement accuracy.** *Harmful Algae.* 2019; **87**: 101625.
**PubMed Abstract** | **Publisher Full Text**

Brager LM, Cranford PJ, Grant J, *et al.*: **Spatial distribution of suspended particulate wastes at open-water Atlantic salmon and sablefish aquaculture farms in Canada.** *Aquac Environ Interact.* 2015; **6**: 135–149.
**Publisher Full Text**

Campbell D, Hurry V, Clarke AK, *et al.*: **Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation.** *Microbiol Mol Biol Rev.* 1998; **62**(3): 667–683.
**PubMed Abstract** | **Publisher Full Text** | **Free Full Text**

Carberry L, Roesler CS, Drapeau S: **Correcting *in situ* chlorophyll fluorescence time-series observations for nonphotochemical quenching and tidal variability reveals nonconservative phytoplankton variability in coastal waters.** *Limnol Oceanogr Methods.* 2019; **17**(8): 462–473.
**PubMed Abstract** | **Publisher Full Text** | **Free Full Text**

Carroll M, Chigounis D, Gilbert S, *et al.*: **Performance verification statement for the TURNER Designs CYCLOPS-7 fluorometer.** *Alliance Coast Technol.* 2005; 37.
**Publisher Full Text**

Dashkova V, Malashenkov DV, Baishulakova A, *et al.*: **Changes in phytoplankton community composition and phytoplankton cell size in response to Nitrogen availability depend on temperature.** *Microorganisms.* 2022; **10**(7): 1322.
**PubMed Abstract** | **Publisher Full Text** | **Free Full Text**

**DS/EN 1484. Water analysis - guidelines for the determination of Total Organic Carbon (TOC) and Dissolved Organic Carbon (DOC)**. The Danish Standardisation Council, Copenhagen, 1997.
**Reference Source**

**DS-2201. Vandundersøgelse: klorofyl a spektrofotometrisk måling i ethanolekstrakt**. Dansk Standardiseringsråd, København. [Water Investigation: Chlorophyll a Spectrophotometrical Measurement in Ethanol Extract]. The Danish Standardisation Council, Copenhagen. 1986.
**Reference Source**

Egeland G, Garrido J, Clementson L, *et al.*: **Part VII: data sheets aiding identification of phytoplankton carotenoids and chlorophylls**. In: S. Roy, C. A. Llewellyn, E. S. Egeland and G. Johnsen (Eds.), *Phytoplankton pigments: characterization, chemotaxonomy and applications in oceanography*. Cambridge University Press, UK, 2011; 665–822.
**Reference Source**

Gatz L: **Freshwater harmful algal blooms: causes, challenges and policy considerations**. Congressional Research Service: Washington, DC USA, 2019; R44871.
**Reference Source**

Gregor J, Maršálek B: **Freshwater phytoplankton quantification by chlorophyll *a*: a comparative study of *in vitro*, *in vivo* and *in situ* methods.** *Water Res.* 2004; **38**(3): 517–522.
**PubMed Abstract** | **Publisher Full Text**

Hillebrand H, Dürselen CD, Kirschtel D, *et al.*: **Biovolume calculation for pelagic and benthic microalgae.** *J Phycol.* 1999; **35**(2): 403–424.
**Publisher Full Text**

Hodges CM, Wood SA, Puddick J, *et al.*: **Sensor manufacturer, temperature, and cyanobacteria morphology affect phycocyanin fluorescence measurements**. *Environ Sci Pollut Res Int.* 2018; **25**(2): 1079–1088.
**PubMed Abstract** | **Publisher Full Text**

Jaccard P, Hjemann DO, Ruohola J, *et al.*: **Quality control of biogeochemical measurements copernicus *in situ* TAC**. CMEMS-INS-BGC-QC, 2021.
**Publisher Full Text**

Jeppesen E, Meerhoff M, Davidson TA, *et al.*: **Climate change impacts on lakes: an integrated ecological perspective based on a multi-faceted approach, with special focus on shallow lakes**. *J Limnol.* 2014; **73**(S1): 88–111.
**Publisher Full Text**

Jespersen AM, Christoffersen K: **Measurements of chlorophyll a from phytoplankton using ethanol as extraction solvent.** *Arch Hydrobiol.* 1987; **109**: 445–454.
**Reference Source**

Kara EL, Hanson P, Hamilton D, *et al.*: **Time-scale dependence in numerical simulations: assessment of physical, chemical, and biological predictions in a stratified lake at temporal scales of hours to months**. *Environ Model Softw.* 2012; **35**: 104–121.
**Publisher Full Text**

Kiefer DA: **Fluorescence properties of natural phytoplankton populations**. *Mar Biol.* 1973a; **22**: 263–269.
**Publisher Full Text**

Kiefer DA: **Chlorophyll *a* fluorescence in marine centric diatoms: responses of chloroplasts to light and nutrient stress**. *Mar Biol.* 1973b; **23**: 39–46.
**Publisher Full Text**

Kuha J, Järvinen M, Salmi P, *et al.*: **Calibration of *in situ* chlorophyll fluorometers for organic matter**. *Hydrobiologia.* 2020; **847**: 4377–4387.
**Publisher Full Text**

Lakowicz JR: **Fluorescence sensing**. In: J. R. Lakowicz (Ed.), *Principles of Fluorescence Spectroscopy*. 3rd edition; Springer US: Boston, MA; 2006; 623–673.
**Publisher Full Text**

Lawrenz E, Richardson TL: **How does the species used for calibration affect chlorophyll *a* measurements by *in situ* fluorometry**. *Estuaries Coasts.* 2011; **34**: 872–883.
**Publisher Full Text**

Levi EE: **Lemming Mesocosm (Denmark): *in-situ* fluorescence chlorophyll-a calibration, analysis code (Version 2)**. [Code]. *Zenodo*. 2024.
**https://zenodo.org/records/14208033**

Levi EE, Jeppesen E, Nejstgaard JC, *et al.*: **Lemming Mesocosm (Denmark): *in-situ* fluorescence chlorophyll-a calibration, underlying data.** (Version 1) [Data set]. *Zenodo*. 2024a.
**http://www.doi.org/10.5281/zenodo.10520248**

Levi EE, Jeppesen E, Nejstgaard JC, *et al.*: **Lemming Mesocosm (Denmark): *in-situ* fluorescence chlorophyll-a calibration, extended data (Version 3)**. *Zenodo*. 2024b.
**https://zenodo.org/records/14207979**

Liboriussen L, Landkildehus F, Meerhoff M, *et al.*: **Global warming: design of a flow-through shallow lake mesocosm climate experiment**. *Limnol Oceanogr Methods.* 2005; **3**(1): 1–9.
**Publisher Full Text**

Lorenzen CJ: **A method for the continuous measurement of *in vivo* chlorophyll concentration.** *Deep-Sea Res Oceanogr Abstr.* 1966; **13**(2): 223–227.
**Publisher Full Text**

Maung-Saw-Htoo-Thaw, Ohara S, Matsuoka K, *et al.*: **Seasonal dynamics influencing coastal primary production and phytoplankton communities**

**along the southern Myanmar coast.** *J Oceanogr.* 2017; **73**: 345–364.
**Publisher Full Text**

Millie DF, Pigg RL, Fahnenstiel GL, *et al.*: **Algal chlorophylls: a synopsis of analytical methodologies**. In: *American Water Works Association, Manual M57, Algae.* American Water Works Association, Denver, Colorado, 2010; 93–122.
**Reference Source**

Muraoka K, McBride C: **rB3: data handling tools for high-frequency lake monitoring buoy observations**. R package version 0.0.0.5000, 2018.
**Reference Source**

Petersen W, Möller KO: **Report on the status of sensors used for measuring nutrients, biology-related optical properties, variables of the marine carbonate system, and for coastal profiling, within the JERICO network and, more generally, in the European context.** 2017.
**Publisher Full Text**

Poikane S, Birk S, Böhmer J, *et al.*: **A hitchhiker's guide to European lake ecological assessment and intercalibration.** *Ecol Indic.* 2015; **52**: 533–544.
**Publisher Full Text**

Proctor CW, Roesler CS: **New insights on obtaining phytoplankton concentration and composition from *in situ* multispectral chlorophyll fluorescence.** *Limnol Oceanogr: Methods.* 2010; **8**(12): 695–708.
**Publisher Full Text**

R Core Team: **R: a language and environment for statistical computing**. R Foundation for Statistical Computing, Vienna, Austria. R Project for Statistical Computing (RRID:SCR_001905), 2022.
**Reference Source**

Ralph PJ, Wilhelm C, Lavaud J, *et al.*: **Fluorescence as a tool to understand changes in photosynthetic electron flow regulation.** *Chlorophyll a Fluorescence in Aquatic Sciences: Methods and Applications.* 2010; **4**: 75–89.
**Publisher Full Text**

Roesler C, Uitz J, Claustre H, *et al.*: **Recommendations for obtaining unbiased chlorophyll estimates from *in situ* chlorophyll fluorometers: a global analysis of WET Labs ECO sensors.** *Limnol Oceanogr Methods.* 2017; **15**(6): 572–585.
**Publisher Full Text**

Rousso BZ, Bertone E, Stewart RA, *et al.*: **Light-induced fluorescence quenching leads to errors in sensor measurements of phytoplankton chlorophyll and phycocyanin.** *Water Res.* 2021; **198**: 117133.
**PubMed Abstract** | **Publisher Full Text**

Roy S, Llewellyn C, Egeland E, *et al.*: **Phytoplankton pigments characterization chemotaxonomy and applications in oceanography**. Cambridge: Cambridge University Press, 2011.
**Reference Source**

Sauzède R, Lavigne H, Claustre H, *et al.*: **Vertical distribution of chlorophyll *a* concentration and phytoplankton community composition from *in situ* fluorescence profiles: a first database for the global ocean.** *Earth Syst Sci Data.* 2015; **7**(2): 261–273.
**Publisher Full Text**

Seppälä J: **Fluorescence properties of baltic sea phytoplankton**. Monographs of the Boreal Environment Research. Helsinki, Finland, 2009.
**Reference Source**

Seppälä J, Ylöstalo P, Kaitala S, *et al.*: **Ship-of-opportunity based phycocyanin fluorescence monitoring of the filamentous cyanobacteria bloom dynamics in the Baltic Sea.** *Estuar Coast Shelf Sci.* 2007; **73**(3–4): 489–500.
**Publisher Full Text**

Sharma A, Golubchik L, Govindan R: **Sensor faults: detection methods and prevalence in real-world datasets.** *ACM Trans Sens Netw.* 2010; **6**(3): 23.
**Publisher Full Text**

Strandberg U, Hiltunen M, Syväranta J, *et al.*: **Combined effects of eutrophication and warming on polyunsaturated fatty acids in complex phytoplankton communities: a mesocosm experiment.** *Sci Total Environ.* 2022; **843**: 157001.
**PubMed Abstract** | **Publisher Full Text**

Søndergaard M, Larsen SE, Johansson LS, *et al.*: **Ecological classification of lakes: uncertainty and the influence of year-to-year variability.** *Ecol Indic* 2016; **61**(Part 2): 248–257.
**Publisher Full Text**

Susanti Y, Pratiwi H, Liana T: **M estimation, S estimation, and MM estimation in robust regression.** *Int J Pure Appl Math.* 2014; **91**(3): 349–360.
**Publisher Full Text**

Teh HY, Kempa-Liehr AW, Wang KIK: **Sensor data quality: a systematic review.** *J Big Data.* 2020; **7**: 11.
**Publisher Full Text**

Twiss MR: **Variations in chromophoric dissolved organic matter and its influence on the use of pigment-specific fluorimeters in the Great Lakes.** *J Great Lakes Res.* 2011; **37**(1): 124–131.
**Publisher Full Text**

Watras CJ, Morrison KA, Rubsam JL, *et al.*: **A temperature compensation method for chlorophyll and phycocyanin fluorescence sensors in freshwater.** *Limnol Oceanogr: Methods.* 2017; **15**(7): 642–652.
**Publisher Full Text**

Xing X, Claustre H, Boss E, *et al.*: **Correction of profiles of *in-situ* chlorophyll fluorometry for the contribution of fluorescence originating from non-algal matter.** *Limnol Oceanogr: Methods.* 2017; **15**(1): 80–93.
**Publisher Full Text**

YSI Environmental: **YSI Technical Note: "Alternate Calibration Methods for the YSI 6025 Chlorophyll Sensor"**. 2005.
**Reference Source**

Zamyadi A, Choo F, Newcombe G, *et al.*: **A review of monitoring technologies for real-time management of cyanobacteria: recent advances and future direction.** *TrAC Trends Anal Chem.* 2016; **85**(Part A): 83–96.
**Publisher Full Text**

Zamyadi A, McQuaid N, Dorner S, *et al.*: **Cyanobacterial detection using *in vivo* fluorescence probes: managing interferences for improved decision-making.** *J Am Water Works Assoc.* 2012; **104**(8): E466–E479.
**Publisher Full Text**

Zhou Y, Zhou L, Zhang Y, *et al.*: **Autochthonous dissolved organic matter potentially fuels methane ebullition from experimental lakes.** *Water Res.* 2019; **166**: 115048.
**PubMed Abstract** | **Publisher Full Text**

# Open Peer Review

## Current Peer Review Status: ✔ ✔ ✔ ✔ ✔ ✔

**Version 3**

Reviewer Report 18 February 2025

https://doi.org/10.21956/openreseurope.21208.r51200

© **2025 Kraft K.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

✔ **Kaisa Kraft**
Marine Research Centre, Helsinki, Finland

I have no further comments for the manuscript

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Phytoplankton ecology, imaging flow cytometry, phytoplankton pigments, in situ continuous observations

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 18 February 2025

https://doi.org/10.21956/openreseurope.21208.r51202

© **2025 Rogora M.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

✔ **Michela Rogora**
National Research Council, Water Research Institute (CNR IRSA), Verbania Pallanza, Italy

I have no further comments on the article

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Limnology; eutrophication; algal nutrients; chemical loads; lake restoration; high frequency monitoring of lakes; impact of climate change on lakes

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

**Version 2**

Reviewer Report 11 January 2025

https://doi.org/10.21956/openreseurope.20322.r48663

© **2025 Kraft K.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Kaisa Kraft**
Marine Research Centre, Helsinki, Finland

The article presents a study comparing continuous fluorometric chlorophyll a measurement with sample-based laboratory analyzed chl a measurements. The measurement have been done in mesocosms with differing nutrient and temperature conditions and therefore differing community compositions. The main aim of the study is to present the advantage of using in situ and continuous fluorometric chl a measurements, also critically addressing the problems related to the method. The study is of high interest to many as in situ chl a fluorescence is a commonly used proxy for algal concentrations. One issue that many face when using the fluorometric data is the proper calibration of the instrument and cleaning of the data, to which the study seeks to answer. The methods were explained thoroughly and discussed well, different aspects were nicely covered.

I have only two specific comments:

-In the introduction, the second chapter: Rationale for using in situ fluorometer for chl a detection is that all the other methods (microscopy, HPLC and various forms of flow cytometry) are time consuming, prone to human error, need expertise and require transport to lab for analysis with low spatial and temporal resolution. I am sorry but that is actually not the case. In situ imaging and pulse shape flowcytometry offers a high-resolution in situ alternative with much more detailed information on the community. Of course, they require expertise, but as seen from this study also calibration, maintenance, and post processing of the data from fluorometers also requires expertise. However, fluorometers are much cheaper and easier to use still. But my point was that one cannot say that all listed negative aspects go for all of the listed methods, at least the authors should exclude the automated in situ sensors to make the paragraph be correct and make sense.

-On page 11, the first chapter of section “Comparing annual and summer means: in-vitro vs equivalent-chla”: This first chapter describes what was done and should belong to the methods section and not the results. Begins with “Daily in-vitro Chla concentrations of year-2019 were linearly interpolated… up till …t-test was used to compare the means between the two methods

for each tank.

**Is the rationale for developing the new method (or application) clearly explained?**
Yes

**Is the description of the method technically sound?**
Yes

**Are sufficient details provided to allow replication of the method development and its use by others?**
Yes

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**
Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Phytoplankton ecology, imaging flow cytometry, phytoplankton pigments, in situ continuous observations

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 10 Feb 2025

**Eti Levi**

*We appreciate the reviewer's time and comments. Below, we have addressed each point separately.*
I have only two specific comments:

**-In the introduction, the second chapter: Rationale for using in situ fluorometer for chl a detection is that all the other methods (microscopy, HPLC and various forms of flow cytometry) are time consuming, prone to human error, need expertise and require transport to lab for analysis with low spatial and temporal resolution. I am sorry but that is actually not the case. In situ imaging and pulse shape flowcytometry offers a high-resolution in situ alternative with much more detailed information on the community. Of course, they require expertise, but as seen from this study also calibration, maintenance, and post processing of the data from fluorometers also requires expertise. However, fluorometers are much cheaper and easier to use still. But my point was that one cannot say that all listed negative aspects go for all of the listed methods, at least the authors should exclude the automated in situ sensors to make the paragraph be correct and make sense.**

- *Response: We thank the reviewer for pointing this out. We updated the information by removing the flowcytometry part from the paragraph. The revised sentences now read: "There are several ways to collect information on phytoplankton in waterbodies. Two of the frequently used sampling/analysis methods are microscopy (e.g. Hillebrand et al., 1999) and pigment analysis with HPLC ( Roy et al., 2011). These methods are especially useful when detailed information on phytoplankton species or groups is required."*

**-On page 11, the first chapter of section "Comparing annual and summer means: in-vitro vs equivalent-chla": This first chapter describes what was done and should belong to the methods section and not the results. Begins with "Daily in-vitro Chla concentrations of year-2019 were linearly interpolated... up till ...t-test was used to compare the means between the two methods for each tank.**
*Response: We agree with the reviewer and moved the suggested part to the end of the methods section under "Annual and summer Chla means: in-vitro vs Equivalent-Chla Comparison"*

***Competing Interests:*** No competing interests were disclosed.

Reviewer Report 27 December 2024

https://doi.org/10.21956/openreseurope.20322.r48263

© **2024 Tamm M.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Marju Tamm**
Estonian University of Life Sciences, Tartu, Estonia

This study examines how fluorescence in-situ sensors can be used to gather real-time, high-frequency data for estimating chlorophyll-a (Chla) concentrations. While these sensors are highly useful, converting their fluorescence readings to accurate Chla concentrations is challenging due to factors like biofouling, the composition of phytoplankton, and variations in sensor types.

To test the reliability of these sensors, researchers compared their readings with traditional spectrophotometric Chla measurements. The study involved 24 freshwater mesocosms, where various combinations of nutrient levels and temperature scenarios were tested.

Findings were interesting and highly valuable. Most sensors worked well after proper calibration, but there were some exceptions. For example, readings in tanks with high phycocyanin fluorescence or very low Chla concentrations were less reliable. One advantage of using these sensors was their ability to capture sudden shifts in Chla levels, which traditional in-vitro methods often missed due to infrequent sampling. The study also emphasized the importance of cleaning the data to correct errors, such as those caused by environmental interference.

Overall, the research demonstrates that fluorescence in-situ sensors are valuable tools for

monitoring rapid changes in algal biomass. When paired with occasional in-vitro tests, these sensors offer a much more comprehensive picture of what's happening in freshwater ecosystems.

The researchers clearly state why the method is needed - there is sufficient discussion about the limitations of the existing methods. The methodological part is clearly explained by the authors and provides sufficient details for replication of the study. All tools, procedures and variables are well-described. The methods used are suitable to address the research objectives.

Authors have done a very nice job describing all the data collection and analysis methods in sufficient detail. Data cleaning process and statistical analyses are clearly explained and reproducible.

Although the results of the study do not revolutionise the field, they are still very valuable for the future studies as most of the hydrobiological studies need a good cost-effective determination of algal biomass. The conclusions of this study reflect the data and are clearly supported by it. Potential biases and uncertainties are well-discussed and suggestions for future applications are pointed out clearly.

**Is the rationale for developing the new method (or application) clearly explained?**
Yes

**Is the description of the method technically sound?**
Yes

**Are sufficient details provided to allow replication of the method development and its use by others?**
Yes

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**
Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Phytoplankton pigments.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 17 December 2024

https://doi.org/10.21956/openreseurope.20322.r47759

**© 2024 Rinke K.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Karsten Rinke**
UFZ-Helmholtz Centre for Environmental Research, Department Lake Research, Brueckstr, Germany

Given the comprehensive revision and overall mature state of the MS I have only two minor comments:

1.)I wondered about inter-sensor variability in the recorded time series. Do all Cyclops sensors behave the same if proper calibrated or is there notable inter-sensor variability? I am not sure whether the data allow to look for that and it is definitely not an issue that deserves another plot. But a statement would be interesting, if doable with the given data

2.) Fig4: it is not clear, which x-axis in the plot corresponds to x1 and x2 in the equation

**Is the rationale for developing the new method (or application) clearly explained?**
Yes

**Is the description of the method technically sound?**
Yes

**Are sufficient details provided to allow replication of the method development and its use by others?**
Yes

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**
Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** aquatic ecology

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 10 Feb 2025
**Eti Levi**

We sincerely appreciate the reviewer's time and feedback. Below, we have provided a response to each point.

1.) I wondered about inter-sensor variability in the recorded time series. Do all Cyclops sensors behave the same if proper calibrated or is there notable inter-sensor variability? I am not sure whether the data allow to look for that and it is definitely not an issue that deserves another plot. But a statement would be interesting, if doable with the given data

- *Response: We appreciate the reviewer's question regarding inter-sensor variability. While we did not focus on this aspect in our manuscript, we conducted measurements of a single water sample (10 µg/L Chl-a, determined spectrophotometrically) with 15 of the Chla in-vivo fluorescence sensors. These measurements indicated some variability among the sensors (please see the figure below). The differences may be related to inter-sensor variation, but also to some extent to field conditions as the measurements were not conducted in a controlled environment.*

*Given these factors, we do not feel confident enough to make definitive comments on inter-sensor variability within the manuscript. However, we acknowledge that variability exists and recommend further controlled studies for a more robust answer.*

Reference Image:
https://s3-eu-west-1.amazonaws.com/openreseurope/linked/246441.Reference_Image.pdf

2.) Fig4: it is not clear, which x-axis in the plot corresponds to x1 and x2 in the equation

- *Response: We thank the reviewer for pointing this out. We added the information to the caption as; "x1 corresponds to in-situ Chla fluorescence and x2 to in-situ Phyco fluorescence."*

***Competing Interests:*** No competing interests were disclosed.

Reviewer Report 13 December 2024

https://doi.org/10.21956/openreseurope.20322.r46334

**© 2024 Schmechtig C.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Catherine Schmechtig**
OSU Ecce Terra, uar3455, Sorbonne Université, Paris, France

The article reports a study that was conducted in mesocosms equipped with fluorometers. The article explains the experimental protocol, the instruments used, all the contaminations they have considered and how they addressed them. The main conclusion of the article is that using high frequency sensors could bring important information regarding short time events that are not

caught by in vitro analysis, assuming that issues like calibration, biofouling, phytoplankton communities, sensor dependant features are considered and corrected (or at least known). The article also highlights the fact that considering cyanobacteria in the study also brought information.
General remarks :

- I would certainly advocate to add other references to all the different analysis protocols to illustrate that they are shared by a large community.
- I think there should be a paragraph explaining what is the quenching. The term is used several times but without any explanation.

1. “and various forms of flowcytometry” => Flow cytometry in two words
2. In the paragraph, “The importance of open data sources”
    1. I would encourage to add references of open data sources and advocates for example for
        1. Argo http://doi.org/10.17882/42182
        2. Sauzede et al. 2015 https://doi.org/10.5194/essd-7-261-2015
    2. I would also encourage to add the reference Roesler et al., 2017 https://doi.org/10.1002/lom3.10185, to complete the references to address the point that the estimation of Chlorophyll a from fluorescence is strongly sensor dependant
3. In the paragraph, “Sampling for chlorophyll-a: in-vitro determination”,
    1. I would add a short sentence to explain why the sampling frequency changes across seasons
    2. Any other references for the analysis protocol ?
4. In the paragraph, “Chla and Phyco fluorescence sensors: in-situ determination”,
    1. I would add a short sentence to explain Phyco ? previously the short term Chla is explained, but Phyco is not fully explained (is it the short version of phycocyanin ?)
5. In the paragraph, “In-situ sensor data collection and cleaning”,
    1. I assume this sentence is not correct :” PAR values equal to one are used to select night-time in-situ fluorescence sensor data.” Isn’t it PAR values below 1 ? or we set a threshold at 1 ?
6. In the paragraph, “Accuracy of the Equivalent-Chla concentration”
    1. The sentence “though RE was high at low in-vitro Chla particularly in the HN–A2+ treatments (Figure 8b)” is a bit confusing as on the figure 8b, while we can see differences between HN/LN, we cannot see any differences in the plot between AMB, A2 and A2+

**References**

1. Sauzède R, Lavigne H, Claustre H, Uitz J, et al.: Vertical distribution of chlorophyll a concentration and phytoplankton community composition from in situ fluorescence profiles: a first database for the global ocean. *Earth System Science Data*. 2015; **7** (2): 261-273 Publisher Full Text
2. Roesler C, Uitz J, Claustre H, Boss E, et al.: Recommendations for obtaining unbiased chlorophyll estimates from in situ chlorophyll fluorometers: A global analysis of WET Labs ECO sensors. *Limnology and Oceanography: Methods*. 2017; **15** (6): 572-585 Publisher Full Text
3. Mignot A, Ferrari R, Claustre H: Author Correction: Floats with bio-optical sensors reveal what processes trigger the North Atlantic bloom.*Nat Commun*. 2018; **9** (1): 1834 PubMed Abstract | Publisher Full Text

**Is the rationale for developing the new method (or application) clearly explained?**
Yes

**Is the description of the method technically sound?**
Yes

**Are sufficient details provided to allow replication of the method development and its use by others?**
Yes

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**
Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** I am a biogeochemical data manager, so I am quite used to work with fluorometer data, but I am not a biologist so I may miss some expertise regarding phytoplancton communities

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 10 Feb 2025

**Eti Levi**

We appreciate the reviewer's time and comments. Below, we have addressed each point separately. General remarks :

- I would certainly advocate to add other references to all the different analysis protocols to illustrate that they are shared by a large community.
  - *Response: Please see below for a reference on Chla analysis.*
- I think there should be a paragraph explaining what is the quenching. The term is used several times but without any explanation.
  - *Response: We thank the referee for pointing this out. Instead of adding a full paragraph we added some information about quenching.*
    - *In the abstract we included: "*non-photochemical quenching (i.e. light-induced decrease in fluorescence intensity)"
    - *Under the "Implementation of the fluorescence in-situ sensors" section, we added: "signal-quenching (i.e. reduction in signal intensity due to very high concentrations)"*

1. "and various forms of flowcytometry" => Flow cytometry in two words

- *Response: Considering another reviewer's comment this part is removed from the paragraph.*

1. In the paragraph, "The importance of open data sources"
    1. I would encourage to add references of open data sources and advocates for example for
        1. Argo http://doi.org/10.17882/42182
        2. Sauzede et al. 2015 https://doi.org/10.5194/essd-7-261-2015
    2. I would also encourage to add the reference Roesler et al., 2017 https://doi.org/10.1002/lom3.10185, to complete the references to address the point that the estimation of Chlorophyll a from fluorescence is strongly sensor dependant

- *Response: We thank the referee for the suggestions. The references are added.*

1. In the paragraph, "Sampling for chlorophyll-a: in-vitro determination",
    1. I would add a short sentence to explain why the sampling frequency changes across seasons'

- *Response : Thank you for pointing this out. The seasonal and treatment-based variation in the sampling frequency was due to a separate, short-term experiment conducted during the same period, which required more detailed monitoring at certain times of the year. Since these details do not directly contribute to the current study's objectives or findings, we opted not to include them in the manuscript.*
    1. Any other references for the analysis protocol ?

- *Response: We added the reference for Chla extraction by "Jespersen and Christoffersen, 1987)*
    - *Jespersen AM, Christoffersen K: Measurements of chlorophyll a from phytoplankton using ethanol as extraction solvent. Archiv für Hydrobiologie. 1987;109:445–454.*

1. In the paragraph, "Chla and Phyco fluorescence sensors: in-situ determination",
    1. I would add a short sentence to explain Phyco ? previously the short term Chla is explained, but Phyco is not fully explained (is it the short version of phycocyanin ?)

- *Response: We added the short of Phycocyanin in the introduction and also in the suggested paragraph*

1. In the paragraph, "In-situ sensor data collection and cleaning",
    1. I assume this sentence is not correct :" PAR values equal to one are used to select night-time in-situ fluorescence sensor data." Isn't it PAR values below 1 ? or we set a threshold at 1 ?

- *Response: We thank the reviewer for the careful check. In our case PAR == 1 is the lowest value we have during night-time. Therefore, PAR values that were equal to 1 were used.*

1. In the paragraph, "Accuracy of the Equivalent-Chla concentration"
    1. The sentence "though RE was high at low in-vitro Chla particularly in the HN–A2+ treatments (Figure 8b)" is a bit confusing as on the figure 8b, while we can see differences between HN/LN, we cannot see any differences in the plot between AMB, A2 and A2+

*Response: We agree with the reviewer and changed the sentence as: "Overall, RE was higher at low in-vitro Chla concentrations (Figure 8b)*"

***Competing Interests:*** No competing interests were disclosed.

**Version 1**

Reviewer Report 18 June 2024

https://doi.org/10.21956/openreseurope.18532.r41121

© **2024 Rogora M.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Michela Rogora**
National Research Council, Water Research Institute (CNR IRSA), Verbania Pallanza, Italy

The manuscript presents an analysis of fluorescence data from in-situ sensors collected trough an experimental design based on mesocosms with varying nutrient and temperature treatments. Chl-a data gathered from the sensors were tested against in-vitro Chl-a measurements, with the aim to critically assessed sensor data and their applicability in detection and monitoring of algal blooms. The topic covered by the study is of broad interest and results are valuable in assessing the usefulness of in-situ high frequency monitoring in the evaluation of water quality and of potential critical issues. Through the results of their study, the authors present a thorough evaluation of both advantages and limits of high-frequency fluorimetric data, also providing suggestion for a proper management of these data (cleaning and data quality check procedures). The manuscript is logically structured, results are extensively and clearly presented and the conclusions sound. I would only suggest a more in-depth discussion on the role of water temperature in the study findings.

Specific comments:

In the abstract and introduction there is focus on harmful algal blooms (HABs) as an important threat to freshwater. Phycocyanin, a pigment indicator of cyanobacteria, is also considered in the study; however, even if providing useful information, phycocyanin sensor readings can only suggest the presence (and a biomass estimate) of cyanobacteria. I would stress the importance of in-vitro assessment to confirm the early warning that can be provided by sensor data.

If I understood correctly, biofouling caused a decrease of the sensor signal, followed by a sudden increase when the optical part of the sensor was cleaned (pag. 9 and 10). We use the same sensors (Cyclops 7, Turner Design, for both Chl and Phycocyanin) for high-frequency monitoring in Lake Maggiore, Italy, and usually get the opposite pattern: fluorescence increases until the upper sensor limit due to biofouling by algae; after the sensor cleaning, the signal immediately goes down. I would suggest to better explain why biofouling caused the signal decrease.

Temperature scenarios are employed in the treatments together with nutrient levels. However, there are much less results and discussion about temperature effect with respect to high and low nutrient treatments. Is it possible to conclude that temperature has a weaker effect on the performance of in-situ sensors?

A shift from diatom to cyanobacteria communities is indicated as the possible reason for the overestimation in spring and underestimation in summer of Chl-a concentration by in-situ sensors (pag. 18). This supports the importance of including phycocyanin sensors in high-frequency monitoring programs, as stated in the manuscript. What about phycoerythrin measurements? I think they could be also potentially useful, especially in oligotrophic environments where the contribution of picocyanobacteria to total phytoplankton biomass may be important.

**Is the rationale for developing the new method (or application) clearly explained?**

Yes

**Is the description of the method technically sound?**

Yes

**Are sufficient details provided to allow replication of the method development and its use by others?**

Yes

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**

Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Limnology; eutrophication; algal nutrients; chemical loads; lake restoration; high frequency monitoring of lakes; impact of climate change on lakes

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 18 Oct 2024

**Eti Levi**

We thank the reviewer for her time and valuable feedback. Below, we have provided responses to each point individually.

1. *In the abstract and introduction there is focus on harmful algal blooms (HABs) as an important threat to freshwater. Phycocyanin, a pigment indicator of cyanobacteria, is also considered in the study; however, even if providing useful information, phycocyanin sensor*

*readings can only suggest the presence (and a biomass estimate) of cyanobacteria. I would stress the importance of in-vitro assessment to confirm the early warning that can be provided by sensor data.*

- **Response:** We agree with the reviewer and added a sentence at the end of the abstract, which reads; "Nevertheless, corroborating these data with in-vitro Chla and Cyanobacteria assessments would provide additional validation for the early warnings provided by sensor data."

2. *If I understood correctly, biofouling caused a decrease of the sensor signal, followed by a sudden increase when the optical part of the sensor was cleaned (pag. 9 and 10). We use the same sensors (Cyclops 7, Turner Design, for both Chl and Phycocyanin) for high-frequency monitoring in Lake Maggiore, Italy, and usually get the opposite pattern: fluorescence increases until the upper sensor limit due to biofouling by algae; after the sensor cleaning, the signal immediately goes down. I would suggest to better explain why biofouling caused the signal decrease.*
   - **Response**: That is true that we also observed increased signals with a partial biofouling on the optical part of the sensors. However, we also had fouling fully covering the LED on the optical part which caused a reduction in the received fluorescence signals.
     - This information is added to the paper as: "Biofouling on the optics of the sensors, which fully covered the LED, caused a gradual decrease in in-situ fluorescence values following a sudden increase after sensor cleaning."
3. *Temperature scenarios are employed in the treatments together with nutrient levels. However, there are much less results and discussion about temperature effect with respect to high and low nutrient treatments. Is it possible to conclude that temperature has a weaker effect on the performance of in-situ sensors?*
   - **Response**: The main reason for the in-depth explanation of high vs. low nutrient tanks was the presence or absence of phycocyanin sensors. Figure 8a indicates no significant difference in relative error between nutrient or temperature treatments, while Figure 8b shows that higher relative error is linked to lower in-vitro Chlorophyll-a concentrations. We have also updated Figure 7 to provide some more detail on temperature treatments. Please see the answer for the other reviewer for more details.
4. *A shift from diatom to cyanobacteria communities is indicated as the possible reason for the overestimation in spring and underestimation in summer of Chl-a concentration by in-situ sensors (pag. 18). This supports the importance of including phycocyanin sensors in high-frequency monitoring programs, as stated in the manuscript. What about phycoerythrin measurements? I think they could be also potentially useful, especially in oligotrophic environments where the contribution of picocyanobacteria to total phytoplankton biomass may be important.*
   - **Response**: Even though in freshwater systems phycoerythrin containing cyanobacteria are typically not dominant, we agree with the reviewer that in environments where picocyanobacteria significantly contribute to the phytoplankton community, measuring phycoerythrin could be important. In such cases, an alternative method may be required for in-vitro Chla analysis, as the method used in the current study does not account for picophytoplankton smaller than 1.2 µm.

***Competing Interests:*** No competing interests were disclosed.

Reviewer Report 29 May 2024

https://doi.org/10.21956/openreseurope.18532.r40259

**© 2024 Richardson T.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Tammi L. Richardson**
University of South Carolina, Columbia, South Carolina, USA

This is an interesting, well-executed comparison of in-situ vs. in-vitro determinations of chl-*a*. The study leverages an on-going mesocosm experiment with varying nutrient concentrations and temperature. Particular attention was paid to effects of cyanobacteria on chl-*a* estimates, and on data 'cleaning' procedures. The multiple regression calibration appears to be successful in explaining additional variance. Overall, I have no major concerns with the data presented or its interpretation.

Specific Comments

1. Title: The title is long and somewhat overwhelming. Perhaps it could be shortened to something like: "Chlorophyll-a determinations by Cyclops-7F sensors vs. in-vitro measurement under varying nutrient and temperature conditions".
2. Introduction, second paragraph. I suggest using the word "concentration" or "biomass" to replace the word "biovolume", as the units are usually expressed in mass not volume.
3. Methods – I suggest adding the duration of the study to the first paragraph of the methods section, i.e., …deployed in October 2018 and recovered in August 2020, and removing it from the paragraph on sampling frequency (as it is a bit confusing there).
4. Methods, 4$^{th}$ paragraph: suggest replacing "points" with "depths".
5. A photograph or sketch of a Cyclops sensor with a shade cap in place would be helpful to understanding the configuration of the Cyclops in each tank.
6. The description of linearity check-2 is a bit confusing. Were chl-a standards used for this calibration? I think it's the words "used to represent" that are problematic. From the LN tank, were the chl-a concentrations truly 5 and 10 ug/l? (There is similar wording on page 6, second paragraph). Please explain and revise as necessary.
7. Why were Phyco sensors put only in HN tanks?
8. Page 7, please define MM in "MM estimation".
9. Page 7, DOC effects – Some of the text on DOC could be reduced since there was no substantial DOC interference. This could also be summarized by saying that most DOC in the tank water wasn't "chromophoric" or "colored" (i.e., CDOM), so not strongly fluorescent.
10. Much of the manuscript focuses on differences among nutrient treatments, but very little

on the different temperatures. I know that temperature was used as part of the calibration, but were there any differences among ambient, IPCC, and IPCC+50%? (Apologies if I missed this).

11. In the conclusions, I suggest giving a range of % underestimation to % overestimation for absolute concentrations of chl-*a* by the Cyclops sensors.

**Is the rationale for developing the new method (or application) clearly explained?**
Yes

**Is the description of the method technically sound?**
Yes

**Are sufficient details provided to allow replication of the method development and its use by others?**
Partly

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**
Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** phytoplankton biology including bulk and spectral fluorometric determinations of phytoplankton biomass and community composition, photosynthesis, primary productivity.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 18 Oct 2024

**Eti Levi**

We appreciate the reviewer's time and comments. Below, we have addressed each point separately.

1. *Title: The title is long and somewhat overwhelming. Perhaps it could be shortened to something like: "Chlorophyll-a determinations by Cyclops-7F sensors vs. in-vitro measurements under varying nutrient and temperature conditions".*
    - **Response**: We agree with the reviewer and updated the title as suggested with a small change. Now it reads: "Chlorophyll-a determinations in mesocosms under varying nutrient and temperature treatments: in-situ fluorescence sensors versus in-vitro measurements"
2. *Introduction, second paragraph. I suggest using the word "concentration" or "biomass" to*

*replace the word "biovolume", as the units are usually expressed in mass not volume.*
    - **Response**: We thank the reviewer for spotting this. The word "biovolume" is replaced as "biomass".
3. *Methods – I suggest adding the duration of the study to the first paragraph of the methods section, i.e., …deployed in October 2018 and recovered in August 2020, and removing it from the paragraph on sampling frequency (as it is a bit confusing there).*
    - **Response**:
        - The first sentence in the methods section is changed as; "The current study was conducted between October 2018 and August 2020 in outdoor mesocosms (from here onwards tanks) at an experimental facility established in 2003 in Lemming, Denmark."
        - The study period information is removed from "sampling frequency" paragraph.
4. *Methods, 4$^{th}$ paragraph: suggest replacing "points" with "depths".*
    - **Response**: Thank you for the suggestion. Water samples were taken from 3 different "locations" in the tanks, covering the whole water column. In this sentence "points" represent different locations in the tanks. We therefore changed the word "points" as "locations".
5. *A photograph or sketch of a Cyclops sensor with a shade cap in place would be helpful to understanding the configuration of the Cyclops in each tank.*
    - **Response**: We added the below photograph to the supplementary materials as AppendixE. 17146 Figure 1 URL
1. *The description of linearity check-2 is a bit confusing. Were chl-a standards used for this calibration? I think it's the words "used to represent" that are problematic. From the LN tank, were the chl-a concentrations truly 5 and 10 ug/l? (There is similar wording on page 6, second paragraph). Please explain and revise as necessary.*
    - **Response:** the word "represent" is removed.
        - The paragraph on page 5 is revised as: "Linearity check-2 was conducted for a subset of the in-situ chlorophyll-a (Chla) sensors. Deionised water served as the blank (0 µg/L). A water sample from an LN tank was used for Chla concentrations of 5 µg/L (1:2 diluted) and 10 µg/L (undiluted). Rhodamine B (Turner Designs) was used for Chla concentrations of 20 µg/L (1:2 diluted) and 40 µg/L (undiluted). Finally, a water sample from an HN tank was used for Chla concentrations of 75 µg/L (1:2 diluted) and 150 µg/L (undiluted)."
        - The part on page 6 is revised as: "These Rhodamine B concentrations are approximate algal Chla equivalents of 10 µg $L^{-1}$, 20 µg $L^{-1}$, and 80 µg $L^{-1}$, respectively"
2. *Why were Phyco sensors put only in HN tanks?*
    - **Response:** Unfortunately, our budget was not enough to have 12 more phycocyanin sensors. Moreover, based on our long-term observations of these mesocosms, we did not expect to have high Cyanobacteria concentrations in low nutrient tanks.
3. *Page 7, please define MM in "MM estimation".*
    - **Response:** The definition of MM is added to the text as "method of moments (MM) estimation".
4. *Page 7, DOC effects – Some of the text on DOC could be reduced since there was no*

*substantial DOC interference. This could also be summarized by saying that most DOC in the tank water wasn't "chromophoric" or "colored" (i.e., CDOM), so not strongly fluorescent.*

- **Response:** We thank the reviewer for pointing this out. We decided to remove the detailed information about "terrestrial humic-like component" and only focused on the DOC concentrations.

5. *Much of the manuscript focuses on differences among nutrient treatments, but very little on the different temperatures. I know that temperature was used as part of the calibration, but were there any differences among ambient, IPCC, and IPCC+50%? (Apologies if I missed this).*
   - **Response**: The main reason for providing a more in-depth explanation of high vs. low nutrient tanks was presence and absence of the phycocyanin sensors in high vs low nutrient tanks, respectively. Overall, Figure 8a shows that the relative error did not differ significantly between the nutrient or temperature treatments. Our results further showed higher relative error to be more related to lower in-vitro Chlorophyll-a concentrations (Figure 8b). Nevertheless, we acknowledge the lack of more in-depth explanation on temperature treatments and have updated Figure7 to address this:

17146 Figure 1 URL

**Figure 7. Comparison of Equivalent-Chla and in-vitro Chla concentrations, grouped according to season and temperature treatments.** Solid line indicates the 1:1 relation. Nutrient treatments are low (LN) and high (HN). Temperature treatments are ambient (AMB), IPCC-A2 (A2) and IPCC-A2+50% (A2+). The paragraph under "Accuracy of the Equivalent-Chla concentration" section is updated as follows: "The comparison of Equivalent-Chla and in-vitro Chla showed a relatively good 1:1 relation (Figure 7), although some deviation was observed. Especially in spring, there was a tendency to overestimation of Chla by Equivalent-Chla for both the HN and LN treatments, particularly for AMB and A2 (AMB + 2–4 $^{O}$C) temperature treatments. Moreover, underestimation in the summer, particularly for the HN–A2 treatment (Figure 7) was also observed.

1. *In the conclusions, I suggest giving a range of % underestimation to % overestimation for absolute concentrations of chl-a by the Cyclops sensors.*
   - **Response:** We added information to the second paragraph of the conclusion section. The sentence reads;

"Overall, among the observations with an absolute error larger than the mean absolute error (MAE) of 13, approximately 21% of the data were overestimated, and 25% were underestimated."

***Competing Interests:*** No competing interests were disclosed.