RBR

Scaling chlorophyll-fluorescence on the RBRtridente

Summary

Factory characterisation of chlorophyll-fluorescence instruments at RBR relies on Secondary Reference Instruments (SRIs) characterised in a chlorophyll-*a* pigment solution, which are then used to evaluate the response of production instruments in fluorescein dye. These reference materials are identified by their Chemical Abstracts Service (CAS) Registry Numbers, enabling third parties to reproducibly prepare reference solutions.

The factory characterisation establishes the relationship between sensor fluorescence response and chlorophyll-*a* pigment concentration measured in vitro. A scaling factor can then be applied to relate the in vitro fluorescence calibration to chlorophyll-*a* concentrations derived from in vivo phytoplankton fluorescence. Because fluorescence yield varies with phytoplankton species composition, physiological state, and environmental conditions, fluorescence measurements cannot be universally converted to chlorophyll-*a* concentration without local scaling. The most accurate approach is therefore to derive a scaling factor from water samples collected at the deployment site, and RBR strongly recommends doing so whenever possible. When local sampling is not practical, users can apply a nominal scaling factor — provided by default at the factory so that every instrument ships on a consistent reporting scale — or adopt one of the reference factors summarised in Table 3.

Introduction

Characterisation of chlorophyll-*a* (chl-*a*) fluorescence instruments at RBR begins with a high-purity chl-*a* pigment as a primary standard. Secondary Reference Instruments (SRIs) are characterised in a chl-*a* pigment solution, where the chl-*a* concentration is quantified using the Jeffrey and Humphrey (J&H) (1975) spectrophotometric method. Once an SRI has been characterised in the reference solution, it is used to determine the chlorophyll-fluorescence equivalent concentration (in vitro scale) of a fluorescein dye solution against which production instruments are characterised (Dever et al., 2024).

In this document, in vitro fluorescence refers to fluorescence from extracted chlorophyll-*a* pigment dissolved in acetone, whereas in vivo fluorescence refers to fluorescence emitted by chlorophyll-*a* contained within intact phytoplankton cells.

Traditionally, chlorophyll fluorescence sensors are characterised in monocultures of microalgae. However, it has been widely demonstrated in the literature that the relationship between fluorescence and chlorophyll-*a* pigment concentration varies with species, growth conditions, light conditions, geographical location, etc. (Johnson et al., 2017; Roesler et al., 2017; Bittig et al., 2019). Because of this, RBR characterises chlorophyll fluorescence sensors using extracted chlorophyll-*a* pigment and applies a scaling factor to relate fluorescence measured in vitro to fluorescence measured in vivo. As this factor is environmentally and biologically variable, a nominal scaling factor ($C_2 = 0.100$) is used. This factor provides a consistent reporting scale but does not represent a universal relationship between fluorescence and chlorophyll-*a* concentration in natural waters. This factor should be replaced with either a published or locally derived value appropriate to the sampled population.

This document outlines the characterisation protocol for chlorophyll fluorescence sensors manufactured by RBR.

Secondary reference instrument characterisation in chl-*a* pigment

RBR's SRIs are characterised in ≥95% pure chl-*a* pigment, validated by high-performance liquid chromatography (HPLC). Pigment, sourced from Sigma-Aldrich (CAS: 479-61-8; ≥95% purity), is suspended in ~30 mL stock solution consisting of 90% acetone and 10% water (v/v). This chl-*a* stock solution is further diluted in 90% acetone to produce an approximately 150 mg/L working solution, which has its precise chl-*a* concentration determined using the Jeffrey and Humphrey (J&H) (1975) spectrophotometric method:

$$[Chl\text{-}a]_{J\&H}\left(\frac{\mu g}{L}\right) = (11.85 \times (E_{664} - E_{750}) - 1.54 \times (E_{647} - E_{750}) - 0.08 \times (E_{630} - E_{750})) \quad (1)$$

An SRI is placed in a stirred beaker (diameter 20 cm, height 30 cm) containing 90% acetone and 10% water. The beaker is sufficiently large that its walls do not encroach on the sensor's measurement volume. The appropriate volume of the chl-*a* working solution is added to the beaker containing the SRI to achieve a chlorophyll-*a* concentration of 500 µg/L in the pigment solution (in vitro conditions), then mixed. The concentration is confirmed using the J&H spectrophotometric method.

Table 1. The relationship between the instrument response in voltage ratio (V_r) and the J&H-quantified concentration of chl-*a* (µg/L) is characterised by a linear regression (Equation 2) for the SRI.

Vr*	J&H quantified chl-a (µg/L)
-0.0000004	0
0.0002573	534

*Vr values reported to a fixed precision of 1×10^{-7}.

$$[Chl\text{-}a]_{J\&H} = C0_{SRI} + Vr_{SRI} \times C1_{SRI} \quad (2)$$

Production instrument characterisation in fluorescein dye

RBR’s production instruments are characterised at two points corresponding to 0 and ~50 µg/L equivalent chl-a concentration (in vivo scale). This two-point approach is justified because the instrument response has been shown to be linear (R2>0.99) across the measurement range, as demonstrated by dilution series in fluorescein dye and by monoculture titration at low concentrations (0–2 µg/L, in vivo scale) on a representative set of units (RBR, 2025). The first point at zero is taken with the instrument’s optics blocked, ensuring no light reaches the detector. The second fluorescence measurement point is a 500 µg/L aqueous fluorescein dye solution prepared from fluorescein disodium salt powder (sourced from Sigma-Aldrich; CAS: 518-47-8). SRIs are used to express the fluorescence signal of the fluorescein solution as an equivalent chlorophyll-fluorescence concentration of ~500 µg/L (in vitro scale), corresponding to an equivalent chl-a concentration of ~50 µg/L (in vivo scale). It is at this step that $C_2 = 0.100$ is applied, converting the SRI's in vitro chlorophyll-fluorescence scale (~500 µg/L) to the in vivo chl-*a* concentration scale (~50 µg/L) against which production instruments are characterised. Production instruments, therefore, output directly on the in vivo scale. The scalar and offset calibration coefficients, C_1 and C_0 (Equation 3), are obtained from a linear regression of SRI-measured chlorophyll fluorescence against the instrument response measured by production instruments.

Table 2. A standard chlorophyll-fluorescence sensor response (V_r) curve in a fluorescein dye solution with chlorophyll-fluorescence concentration (in vitro scale), and equivalent chl-a concentration (in vivo scale), using the default C_2 = 0.100.

Vr*	chl-*a* (µg/L) (in vitro scale)	chl-*a* (µg/L) (in vivo scale)
0.0000002	0.0	0.00
0.0002624	511.9	51.19

*Vr values reported to a fixed precision of 1×10^{-7}.

$$[Chl\text{-}a]_{SRI} = (C0 + Vr \times C1) \quad (3)$$

Relationship between chl-*a* pigment in vitro and chl-*a* in vivo

Since monocultures are challenging to maintain so that their concentration of chl-*a* and accessory pigments remains constant, using them as a primary standard is not ideal due to a lack of reliable repeatability. For that reason, RBR characterises the chlorophyll-fluorescence sensors against a robust standard, ensuring reproducibility of the factory reference scale.

The chlorophyll fluorescence measured in vivo must be scaled appropriately before being reported as chlorophyll concentration. When a sensor characterised in chl-a pigment is compared against in situ chl-a concentrations determined by HPLC, the sensor's readings will not match, because in vivo fluorescence from intact phytoplankton differs from the in vitro fluorescence of extracted pigment or fluorescein. This is expected, as in situ organisms have accessory pigments that transfer absorbed energy to chlorophyll-*a* via resonance energy transfer, producing a higher fluorescence signal than pure chl-*a* in acetone. To account for the difference between the in vitro pigment measurements used in the calibration laboratory and in vivo fluorescence measured in monocultures or natural waters, an in situ scaling factor (C_2 in Equation 4) is incorporated via the sensor response equation.

$$[Chl\text{-}a] = C2 \times [Chl\text{-}a]_{SRI} = C2 \times (C0 + Vr \times C1) \quad (4)$$

The appropriate value for C_2 depends on the population being measured (species, growing conditions, geographical location, etc.). It is strongly recommended that C_2 be determined independently using water samples (Roesler et al., 2017; Bittig et al., 2019). However, as the collection of water samples is not always possible, RBR has implemented a default factor $C_2 = 0.100$ as part of the characterisation. It is important to note that this factor is solely intended to provide a general scaling from chlorophyll fluorescence (in vitro) to chlorophyll-*a* concentration (in vivo). Additionally, RBR has conducted laboratory work to provide common factors that may benefit the community (RBR, 2025). The report includes a scaling factor for the traditionally used monoculture of *Thalassiosira weissflogii*, a detailed protocol for growing the reference culture, and a factor for other common chlorophyll fluorescence sensors. A summary of measured scaling factors for aligning with specific references is provided in Table 3.

Table 3. Scaling factors derived to align RBR's chlorophyll-fluorescence sensors with the listed sources.

Source of alignment	**Scaling factor (C_2)**
Sea-Bird SeaOWL in fluorescein	0.103
Sea-Bird SeaOWL in *T. weissflogii**	0.108
Sea-Bird WET Labs ECO in *T. weissflogii**	0.0740
T. weissflogii (Welschmeyer, 1994)*	0.258

*Growth conditions and treatment of the *Thalassiosira weissflogii* culture are detailed in RBR report 0018411revA. Factor derivation in a *T. weissflogii* culture under different growth conditions may yield a different factor.

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