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RESEARCH

Using picosecond fluorescence lifetime analysis to determine photosynthesis in the world's oceans

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Abstract
Phytoplankton in the ocean account for less than 1% of the global photosynthetic biomass, but contribute about 45% of the photosynthetically fixed carbon on Earth. This amazing production/biomass ratio implies a very high photosynthetic efficiency. But, how efficiently is the absorbed light used in marine photosynthesis? The introduction of picosecond and then femtosecond lasers for kinetic measurements in mid 1970s to 90 s was a revolution in basic photosynthesis research that vastly improved our understanding of the energy conversion processes in photosynthetic reactions. Until recently, the use of this technology in the ocean was not feasible due to the complexity of related instrumentation and the lack of picosecond lasers suitable for routine operation in the field. However, recent advances in solid-state laser technology and the development of compact data acquisition electronics led to the application of picosecond fluorescence lifetime analyses in the field. Here, we review the development of operational ultrasensitive picosecond fluorescence instruments to infer photosynthetic energy conversion processes in ocean ecosystems. This analysis revealed that, in spite of the high production/biomass ratio in marine phytoplankton, the photosynthetic energy conversion efficiency is exceptionally low—on average, ca. 50% of its maximum potential, suggesting that most of the contemporary open ocean surface waters are extremely nutrient deficient.

Keywords Fluorescence kinetics · Phytoplankton · Variable fluorescence · Solar-induced fluorescence

Introduction

Phytoplankton in the ocean account for less than 1% of the global photosynthetic biomass, but contribute about 45% of the photosynthetically fixed carbon on Earth (Field et al. 1998). In striking contrast to aquatic photosynthetic organisms, terrestrial plant biomass includes, by far, tree trunks, stems, and roots, which are mostly woody and thus photosynthetically inert (Bar-On et al. 2018). The amazing production/biomass ratio in phytoplankton may imply a very high photosynthetic efficiency. But, how efficiently is the light used in oceanic photosynthesis?

The photon budget in photosynthetic reactions can be derived from three fates of absorbed energy: photochemical conversion, fluorescence emission back to the environment, and dissipation to heat. Measurement of the two of these processes closes the photon budget of photosynthesis. The quantum yields of the first two processes can be accurately measured by using two independent techniques, namely chlorophyll variable fluorescence and picosecond fluorescence lifetime measurements (Falkowski et al. 2017). Variable fluorescence from chlorophyll *a* has been used for decades to infer quantum yields of photochemical conversion in phytoplankton and plants (Gorbunov and Falkowski 2022). Recent development of solid-state picosecond lasers, compact data acquisition systems, and ultrasensitive detector technology allowed one to construct compact instruments for picoseconds lifetime measurements in the ocean (Lin et al. 2016). Here, we review the development of picosecond lifetime fluorometers and their applications to infer photosynthetic energy conversion processes in ocean ecosystems.

This mini-review is dedicated to Vladimir A. Shuvalov, one of the most famous biophysicists who revolutionized our understanding of the picosecond and femtosecond kinetics and function of primary photosynthesis processes in nature (Shuvalov and Klimov 1976; Klimov et al. 1977; Shuvalov

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et al. 1978; Shuvalov and Parson 1981; Shuvalov and Duysens 1986 and papers in this issue).

Theoretical bases of fluorescence quantum yields and fluorescence kinetics

The relationship between photosynthesis and chlorophyll fluorescence derives from the three possible fates of light energy absorbed by any photosynthetic organism. These fates are photochemical reactions, heat dissipation, or fluorescence emission back to the environment (Butler 1978). The rates (and kinetics) of these processes (k_p, k_t, and k_f, respectively) determine the quantum yields and, hence, the budget of absorbed photons. The rate k_p is, to first order, proportional to the fraction of open or active reaction centers. The rate k_t is the sum of a dark component (k_D) and a light-activated component (k_{NPQ}); the latter comprises a set of non-photochemical quenching processes (NPQ).

The quantum yield of fluorescence (ϕ_f) is defined as the ratio of the photons reemitted to those absorbed. In a dark-adapted state or under low irradiance, the quantum yield of chlorophyll fluorescence, $\phi_f(= k_f/(k_p + k_D + k_f))$, is inversely related to the quantum yield of photochemistry in PSII, $\phi_p = k_p/\left(k_p + k_D + k_f\right) = F_v/F_m$:

$$\phi_f = \phi_{fm}(1 - F_v/F_m) \tag{1}$$

where $\phi_{fm}\left(= k_f/\left(k_t + k_f\right)\right)$ is the maximum fluorescence yield obtained when the quantum yield of photochemistry is nil (e.g., all reaction centers are closed). This simple first-order model assumes that PSII units are excitonically uncoupled and predicts a linear relationship between fluorescence yield and the fraction of closed PSII reaction centers and F_v/F_m. The presence of excitonic coupling can be described by a more complex formalism, using a "connectivity factor," which defines the probability of energy transfer between PSII centers (Joliot and Joliot 2003; Oja and Laisk 2020).

Exposure to high irradiance generates a suite of thermal dissipation mechanisms (collectively called non-photochemical quenching, NPQ: Falkowski et al. 1986; Schreiber et al. 1986) that markedly decreases the quantum yield of chlorophyll fluorescence at high irradiance. As NPQ phenomena play an increasingly larger role in energy dissipation at high background light, the relationship between fluorescence yield and photochemistry becomes non-linear (Gorbunov and Falkowski 2022).

The sum of the quantum yields (ϕ) of photochemistry, heat dissipation, and fluorescence is equal to 1.0 (Butler and Strasser 1977):

$$\phi_p + \phi_f + \phi_t = 1.0 \tag{2}$$

Hence, measuring two of these three processes closes the photon energy budget. The development of ultrasensitive, seagoing chlorophyll variable fluorescence instruments, and picosecond fluorescence lifetime instruments has allowed independent closure on the first two terms (ϕ_p and ϕ_f, respectively; Falkowski et al. 2017). With this closure we can understand how phytoplankton respond to the environment on time scales of minutes to months, and over longer time scales, to changes in climate.

Using fluorescence lifetimes to derive quantum yields of fluorescence

Fluorescence is delayed emission of absorbed light, which is described by one or more exponential decay functions which can be parameterized by the lifetime or the e-folding time of the decay function. The average fluorescence lifetime is directly proportional to the absolute quantum yield of fluorescence (Lakowicz 2006):

$$\phi_f = \tau/\tau_n \tag{3}$$

where τ is the observed lifetime and τ_n is the intrinsic (or "natural") lifetime, which is a constant for the molecule. Analytical measurements of the quantum yield of fluorescence are among the most complicated measurements to obtain in applied optics (Falkowski et al. 2017). The measurement of fluorescence lifetimes offers a convenient, practical tool to directly infer the quantum yield.

The "natural" lifetime (τ_n) is that which would be observed if fluorescence emission would be the only path of dissipation of excited state energy. This number cannot be measured directly. For chlorophyll *a*, τ_n is 15 ns (Brody and Rabinowitch 1957). The actual measured lifetimes are inevitably shorter than the "natural" lifetime due to intramolecular conversion (i.e., energy dissipation as heat) and triplet state formation. The actual measured lifetimes of isolated chlorophyll *a* molecules range from ca. 3.0 to 5.1 ns, depending on solvent polarity. These measured lifetimes correspond to quantum yields ranging from 20 to 32%. Fluorescence lifetimes in live cells are even shorter (ca. 0.3 to ~ 1.5 ns), as a significant fraction of the absorbed energy is used in photochemical reactions (Holzwarth 1986); fluorescence lifetimes in vivo reflect the physiological state of the cells.

In isolated antenna complexes, the measured lifetimes are as long as 2.5 to 4 ns (Moya et al. 2001; Morales et al. 2001), but the lifetimes decrease to less than 200 ps when these complexes are energetically coupled to active reaction centers (Croce and van Amerongen 2011). Fluorescence lifetimes of isolated PSII core complexes without external

antennae are extremely fast, ranging from 100 to 155 ps (Croce and van Amerongen 2011).

Technology to measure fluorescence lifetimes

Phase shift fluorometry

Phase shift fluorometry does not measure fluorescence lifetimes directly, but rather records the phase difference between sinusoidally modulated optical excitation and the emitted fluorescence that is delayed in phase relative to the excitation (Lakowicz 2006). The fluorescence lifetime is then calculated from the phase difference and the modulation frequency. In order to determine the fluorescence lifetime accurately, the modulation frequency must be comparable to the rate of decay of the fluorescence. A single frequency phase fluorometer measures the average fluorescence lifetime. In order to resolve individual fluorescence decay components in complex multi-component fluorescent systems such as live cells, the fluorometer must operate at several excitation modulation frequencies; that is technically difficult. Furthermore, phase shift lifetime instruments exhibit inherently low sensitivity, which makes them relatively ineffective in the open ocean where chlorophyll concentrations average ca. 0.05 µg/L (Falkowski and Raven 2007).

Time-correlated single photon counting (TCSPC)

Time-Correlated Single Photon Counting is one of the best ways to measure multi-component fluorescence decay kinetics (O'Connor and Phillips 1984; Becker 2005). The TCSPC measurement relies on the concept that the probability distribution for emission of a single photon after an excitation with a short (pico- or femtosecond) flash yields the actual intensity against time distribution of all the photons emitted as a result of the excitation. By sampling the single photon emission after a large number of excitation flashes, the experiment constructs this probability distribution that describes the decay kinetics of fluorescence (Fig. 1). The TCSPC method has much better sensitivity, accuracy, and resolution over phase shift technologies, and resolves multiple components in fluorescence kinetics, which is crucial for lifetime analysis in live algal cells (Holzwarth 1986).

Picosecond lifetime measurements in the ocean

To measure fluorescence lifetimes in the ocean, we constructed a compact, seagoing picosecond lifetime instrument, PicoLIF, that employs the TCSPC method. This instrument

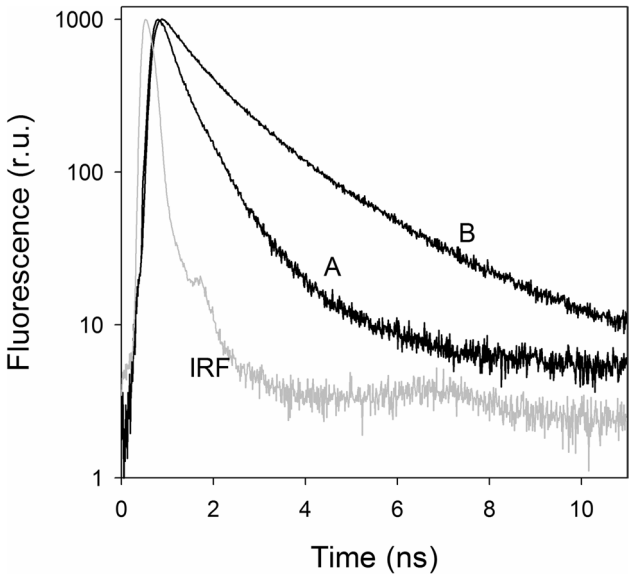

Fig. 1 Picosecond fluorescence kinetics of phytoplankton at different physiological states, including nutrient-replete cells (**A**) and iron-limited phytoplankton (**B**) from the Southern Ocean. Both profiles were recorded in dark-adapted cells with open reaction centers (at F_o level). The instrumental response function (IRF) is shown in gray

uses a picosecond diode laser (BDS-640-SMN or similar, Becker & Hickl GmbH, Berlin) with a pulse duration of ca. 100 to 150 ps. The laser excitation wavelength is 640 nm or 450 nm, depending on the laser used (BDS-640-SMN or BDS-450-SMN, respectively). The induced fluorescence photons are recorded by a Peltier cooled, ultra-fast single photon counting detector (Becker & Hickl GmbH, Berlin). The instrumental response function of the instrument has the half bandwidth of ~ 200 ps (Fig. 1), which allows resolution of lifetime measurements down to ca. 20 ps. The PicoLIF instrument has extremely high sensitivity; it can measure lifetimes in samples as low as 0.01 mg chlorophyll/m^3—effectively the lowest concentration of chlorophyll observed anywhere in the upper ocean (Falkowski et al. 2017).

Measured kinetic curves (Fig. 1) are fitted to a sum of two or three exponential components, depending on the chlorophyll concentration and thus fluorescence signal. The average lifetime given in this paper represents an average over the resolved individual components:

$$\tau = \sum_i f_i \tau_i \tag{4}$$

$$f_i = \frac{A_i \cdot \tau_i}{\sum_j A_j \cdot \tau_j} \tag{5}$$

where τ_i, A_i, and f_i are the lifetime constants, amplitudes, and relative quantum yields of each component. The average lifetime constant (τ), therefore, corresponds to a single

exponential decay with the amplitude and the integral area equal to the total amplitude and the area under the multiple exponentials.

The PicoLIF fluorometer operates in two regimes and records fluorescence kinetics in open or closed photosynthetic reaction centers (F_o and F_m levels, respectively). The measurements are conducted in a flow-through cuvette. The kinetic signal at the F_o level (τ_o) is induced by laser excitation with low average optical power density (ca. 0.1 mW·cm^{-2}), which is achieved by using a laser beam expander and neutral density filters. In contrast, measurements of the τ_m kinetics are conducted under maximum optical power density (ca. 100 mW·cm^{-2}) in a collimated laser beam to ensure full closure of the reaction centers in the measured volume. To minimize the effects of NPQ on fluorescence during the daytime, the flow-through measurements are conducted after a period (3–5 min) of low-light acclimation, which permits NPQ to relax.

Variability and controls of fluorescence lifetimes and quantum yields in the ocean

Using paired PicoLIF and mini-FIRe (Fluorescence Induction and Relaxation; Gorbunov and Falkowski 2021) instruments, we obtained > 300,000 discrete measurements of chlorophyll fluorescence lifetimes and photosynthetic characteristics from the Pacific, Atlantic, Arctic, and Southern (Antarctic) Oceans. These comprise the global map of in situ measurements of lifetimes and quantum yields of chlorophyll fluorescence from phytoplankton in the upper ocean (Fig. 2).

The night-time in situ lifetimes ranged from 0.5 to 2.7 ns with a mean of 1.13 ± 0.33 ns (Lin et al. 2016). These values span (and exceed) the entire range of published lifetimes from cultured phytoplankton and reflect the extraordinary variability in phytoplankton energy conversion efficiency in the global ocean.

The general pattern of the fluorescence lifetimes in the central gyres of the global ocean is rather featureless (Lin et al. 2016), although phytoplankton growth is subject to both macro- and micronutrient limitation (Falkowski and Raven 2007). The shortest fluorescence lifetimes (< 1 ns) were observed along continental margins, in the Antarctic convergence, the Subtropical Atlantic Ocean, and iron-replete regions of the Pacific Ocean. The shortest lifetimes were consistent with the highest Fv/Fm values (> 0.45), indicating the absence of nutrient stress in these regions (Lin et al. 2016). These lifetime distributions support the hypothesis that phytoplankton in the central gyres are acclimated to broad scale and persistent nutrient limitation (Moore et al. 2013). The kinetic analysis of variable fluorescence yields on micro- and millisecond time scale allows us to quantify the impact of macronutrient (i.e., N and/or P) limitation on growth rates and net primary production (Gorbunov and Falkowski 2021). The reduction in growth rates can accurately be deduced from the reduction in photosynthetic turnover rates retrieved from fluorescence kinetic analysis (Gorbunov and Falkowski 2021).

In contrast, the longest fluorescence lifetimes were observed in high-nutrient-low-chlorophyll (HNLC) regions of the equatorial Pacific Ocean and the Southern Ocean where primary production is limited by iron (Boyd et al. 2007; Behrenfeld et al. 2006), a micronutrient that is critical for the function of photosystem II (Vassiliev et al. 1995). The exceptionally long fluorescence lifetimes (> 2.0 ns) in these regions are consistent with extremely low F_v/F_m values (< 0.20) and suggest that there is a significant fraction of non-functional PSII reaction centers and/or energetically uncoupled antenna pigment–protein complexes (Vassiliev et al. 1995; Schrader et al. 2011). Fluorescence lifetime analysis allows us to distinguish between these two phenomena and to quantify the amount of uncoupled antenna complexes in natural phytoplankton populations (see below).

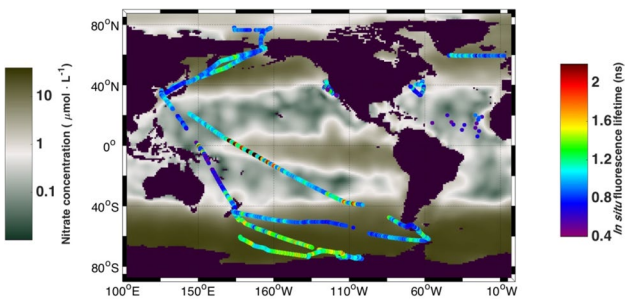

Fig. 2 Distribution of in situ measurements of chlorophyll fluorescence lifetimes in the upper ocean superimposed on the climatological map of surface nitrate concentrations in the world ocean. Measurements were conducted on low-light adapted phytoplankton [Data from Lin et al. 2016]

Effect of iron limitation on the uncoupling of light-harvesting complexes from reaction centers in oceanic ecosystems

The range of lifetimes observed in the ocean exceeds that in laboratory cultures of algae. The exceptionally long fluorescence lifetimes (> 2.0 ns) exceed the typical maximum lifetime values observed in algal cultures with fully closed reaction centers (Holzwarth 1986) and thus cannot, entirely, be explained by the presence of inactive PSII reaction centers alone. This result suggests a large pool of detached antenna complexes in natural phytoplankton populations exposed to nutrient limitation. The combination of variable fluorescence

and lifetime analyses allows us to determine the fraction of uncoupled antenna complexes in iron-limited regions of the ocean, such as the Southern Ocean (Park et al. 2017; Sherman et al. 2020) and the equatorial Pacific Ocean (Lin et al. 2016).

The portion of detached antenna complexes can be estimated from the dependence between F_v/F_m and fluorescence lifetimes (Park et al. 2017; Sherman et al. 2020). In the absence of detached antenna complexes, this dependence follows an inverse relationship between F_v/F_m and the lifetime (Butler 1978; Lin et al. 2016). This model dependence is calculated using the Butler equation (modified from Eq. 1):

$$\tau = \tau_m(1 - F_v/F_m) \quad (6)$$

and the following fluorescence lifetimes parameters: 0.5 ns for fully open reaction centers and 1.5 ns for fully closed reaction centers. When a portion of the detached antennae with a very long fluorescence lifetime (4.0 ns; Morales et al. 2001) is present, the observed lifetime becomes longer than that predicted by Butler's model (Park et al. 2017). A large portion of detached antenna complexes would ultimately lead to very long lifetimes, which may exceed the values observed for fully closed reaction center (~ 1.5 ns). The fluorescence lifetime analysis revealed that in iron-limited regions of the Amundsen Sea (Park et al. 2017) and the Western Antarctic Peninsula (Sherman et al. 2020), about 25% of the antenna complexes are energetically uncoupled. In severely iron-limited regions, up to 40% of the antenna complexes are uncoupled (Park et al. 2017).

The use of in situ fluorescence lifetime measurements for validation and calibration of satellite-based solar-induced fluorescence yields

Variable chlorophyll fluorescence is the most sensitive technique to infer instantaneous phytoplankton physiology and photosynthetic rates in the upper ocean (Falkowski and Kolber 1995; Kolber et al. 1998; Gorbunov and Falkowski 2022). However, variable fluorescence signals cannot be recorded from space without high-power pulsed lasers—which is not practical. An alternative approach to assess phytoplankton physiology is based on measurements of the absolute quantum yields of solar-induced chlorophyll fluorescence (Lin et al. 2016; Falkowski et al. 2017). Solar-induced fluorescence is detected from space as a red peak (Fig. 3) in the spectra of water-leaving radiance (Neville and Gower 1977; Gordon et al. 1988; Gower et al. 1999). This signal is recorded as a "fluorescence line height" (FLH) (Fig. 3), which is observed over the background

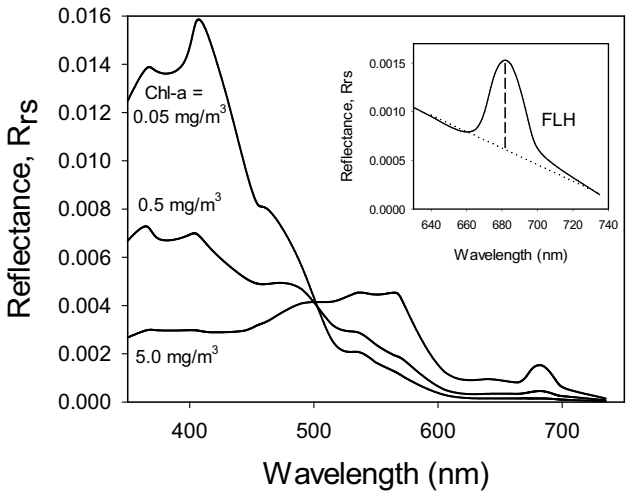

Fig. 3 Spectra of remote-sensing reflectance (i.e., normalized water-leaving radiance) recorded at different concentrations of chlorophyll a in water. The insert shows solar-induced fluorescence from phytoplankton, which can be recorded as Fluorescence Line Height (FLH). Data adapted from https://www.oceanopticsbook.info/view/atmospheric-correction/normalized-reflectances

of the water-leaving radiance (Abbott and Letelier 1999). Although the presence of phytoplankton in natural waters alters the entire visible spectrum of water-leaving radiance (Fig. 3), solar-induced fluorescence is the only signal emitted from the ocean and detectable from space that can be unambiguously ascribed to phytoplankton physiology (Cullen et al. 1997; Behrenfeld et al. 2009).

With the launch of satellite-based sensors capable of detecting solar-induced chlorophyll fluorescence signals from the ocean, it became theoretically possible to estimate the quantum yield of chlorophyll fluorescence from space (Behrenfeld et al. 2009; Huot et al. 2013). The satellite-based analytical algorithms retrieve the quantum yields of chlorophyll fluorescence from the ratio of two independently obtained variables, namely the magnitude of solar-induced fluorescence and the number of quanta absorbed by phytoplankton (Behrenfeld et al. 2009; Huot et al. 2005; 2013). The latter is proportional to chlorophyll concentration, which is based on the ratio of green to blue water-leaving radiances (Fig. 3).

Remote sensing analysis and in situ measurements revealed that the quantum yield of solar-induced fluorescence is highly variable in the ocean (Cullen et al. 1997; Letelier et al. 1997; Abbott and Letelier 1999; Maritorena et al. 2000; Morrison 2003; Huot et al. 2005). While the apparently huge variability of chlorophyll fluorescence yield (ca. tenfold) is often correlated with environmental factors, such as nutrient availability and photoacclimation (Letelier et al. 1997; Behrenfeld et al. 2009; Huot et al.

2005; Lin et al. 2016), the mechanisms and interpretation of this relationship remain to be elucidated.

The development of remote-sensing algorithms for interpreting the quantum yields of solar-induced chlorophyll fluorescence crucially depends on comparison with accurate in situ measurements of the quantum yields. The quantum yields cannot be measured by using variable fluorescence instruments, but can be measured by using picosecond fluorescence lifetimes (Lin et al. 2016). Such measurements are the only way to validate and calibrate satellite-derived estimates of the quantum yields of solar-induced chlorophyll fluorescence (Lin et al. 2016).

Conclusions

The combination of variable fluorescence and picosecond lifetime measurements revealed that, in spite of the high production/biomass ratio in marine phytoplankton, the photosynthetic energy conversion efficiency is exceptionally low. While there was significant spatial and temporal variability, F_v/F_m in the upper ocean averaged ca. 0.35 at night, when non-photochemical quenching is nil. Moreover, the chlorophyll fluorescence lifetime at night averaged ca. 1.1 ns, which is approximately twice as long as that in phytoplankton cultures under optimal growth conditions. These results indicate that, on average, phytoplankton photosynthesis in the ocean operates at ca. 50% of its maximum potential, suggesting that most of the contemporary open surface waters are extremely nutrient deficient. Assuming a natural lifetime of 15 ns, these average observed lifetimes suggest that approximately 7% of the absorbed photons are reradiated as fluorescence. Combining the two measurements suggests that approximately 60% of the absorbed excitation energy in natural phytoplankton is converted to heat (Falkowski et al. 2017), while under optimal growth conditions for unicellular algae in culture ca. 60% of the absorbed excitation energy can be used in photosynthetic energy conversion. The reduction in photosynthetic performance in the open ocean is primarily due to the uncoupling of absorbed excitation energy from photosynthetic use, which is, in turn, related to nutrient limitation.

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Author contributions M.G. and P.F. designed this research, conducted analysis, and wrote the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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