

## New method for the determination of extracellular production of superoxide by marine phytoplankton using the chemiluminescence probes MCLA and red-CLA

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### Abstract

A new rapid and highly sensitive microplate-based chemiluminescence method for the detection of extracellular production of superoxide by phytoplankton cultures has been developed. Replicates of the sample, blank, and three standards were placed into 96-well plates and the chemiluminescence was detected with a microplate luminometer. The method was tested on *Trichodesmium erythraeum* cultures using two superoxide-specific chemiluminescent probes, MCLA and the closely related compound red-CLA, which emit light at 460 and 610 nm, respectively, in the presence of superoxide. Calibration of the chemiluminescent signal is performed individually for each sample using the xanthine/xanthine oxidase system by adding a fixed concentration of xanthine (50  $\mu\text{M}$ ) and variable concentrations of xanthine oxidase (0.001–0.5 units  $\text{L}^{-1}$ ). The method is selective for superoxide, and the detection limits are as low as 1.41 pmol/s for MCLA and 76 fmol  $\text{s}^{-1}$  for red-CLA, with limits of quantification of 4.70 pmol/s for MCLA and 253 fmol  $\text{s}^{-1}$  for red-CLA. Application of the new method to the determination of extracellular superoxide production by the prolific superoxide-producing phytoplankton *Chattonella marina* yielded results comparable to those obtained using an existing flow injection analysis method. The use of microplates offers several advantages over existing methods, including a short analysis time of 10 min for triplicates of blank, sample, and standards; good reliability of signals; and use of small sample volumes.

### Introduction

Superoxide, a reduced form of dioxygen, is produced in natural waters by both abiotic and biotic processes. It is a highly redox-active species owing to the presence of an unpaired electron but somewhat selective in its reactions due to its resonance stabilized bonding structure (Neuman 1934), which plays an important role in the biogeochemical cycles of several trace metals and in the degradation of several organic pollutants in natural waters (Goldstone and Voelker 2000; Rose and Waite 2005; Voelker et al. 2000).

Superoxide can be produced biologically by both prokaryotes and eukaryotes and is an important product of many biochemical reactions occurring in the photosystems, mitochondria, and endoplasmic reticulum of cells (Lesser 2006), making it one of the most abundant radicals produced in biological systems (Auchère and Rusnak 2002). Superoxide is potentially

biologically toxic and can react either directly via reactions with molecules such as polyphenols, small sugars, or ascorbate by autoxidation (Auchère and Rusnak 2002; Mashino and Fridovich 1987) or indirectly, as a precursor for more potent oxidants such as hydroxyl radicals (Auchère and Rusnak 2002; Mashino and Fridovich 1987).

Many marine phytoplankton species including raphidophytes (Lee et al. 1995; Marshall et al. 2005), dinoflagellates (Marshall et al. 2005; Yamasaki et al. 2004), heterotrophic bacteria (Henry and Vignais 1980), cyanobacteria (Rose et al. 2005), and diatoms (Kustka et al. 2005) produce reactive oxygen species (ROS), including superoxide, in their surrounding environment. The raphidophyte *Chattonella marina*, the most prolific extracellular superoxide producer of these species, is well known for its contribution to toxic red tides in Australia, Canada, Japan, and New Zealand and is recognized to be responsible for mass fish deaths. The precise mechanism of toxicity from this and other red-tide organisms remains unclear, but the production of ROS is suggested to be one of the key factors contributing to fish mortality (Lee et al. 1995; Marshall et al. 2005). However, living organisms can potentially

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also take advantage of ROS chemistry by using superoxide as a mediator through the immune system to kill invading microorganisms (Lamb and Dixon 1997; Segal et al. 1992). Additionally, recent evidence exists that superoxide, under some conditions at least, facilitates the acquisition of the trace nutrient iron by phytoplankton (Garg et al. 2007; Rose et al. 2005).

Several methods exist for determining the production of extracellular superoxide from phytoplankton cells. Most of them are based on the chemiluminescence (Campbell et al. 1998) of MCLA (2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3(7H)-one), which is elicited only from the reaction of MCLA with either superoxide ( $O_2^-$ ) or the short-lived singlet excited state of dioxygen ( $^1O_2$ ) (Campbell et al. 1998). Two particular techniques have been widely used. The first method consists of measuring the  $O_2^-$  produced by cells collected in cuvettes (Kim et al. 2000; Marshall et al. 2005; Marshall and Ross 2005)—basically, MCLA is added to the culture and the mix is analyzed with a luminometer. The second technique consists of analyzing the  $O_2^-$  production from cells transported in a flow-type system. In this case, MCLA and the sample are simultaneously pumped into the spiral flow cell (Kustka et al. 2005; Lee et al. 1995). The chemiluminescence (CL) signal recorded is the signal produced by the mix between the sample and the CL probe, constantly delivered to a spiral flow cell. MCLA is known to strongly associate with cell membranes because of its hydrophobic moieties (Nakano 1998), however, which introduces a cell density dependency to the  $O_2^-$  trapping properties of MCLA. A variant of the above techniques is used by Garg et al. (2007) in which the cells are retained on an inline filter positioned upstream of the spiral flow cell. The cells are washed with a clean and DTPA-enriched medium that transports the  $O_2^-$  produced by the cells to the spiral flow cell, where it is mixed with MCLA to generate the CL signal. This method permits the analysis of the  $O_2^-$  produced by the cells but without the inconvenience of the association of MCLA with phytoplankton membranes. When using this method, the CL signal is calibrated by generation of known steady-state concentrations of  $O_2^-$  using the enzymatic xanthine/xanthine oxidase system (Garg et al. 2007; Rose et al. 2008).

Although the methods described above have been found to provide a reasonable measure of  $O_2^-$  production rate under particular circumstances, they have some disadvantages that decrease their efficiency. For the method of Garg et al. (2007), the cells housed on the filter may be damaged by the pressure applied by the peristaltic pump, resulting in an increase in  $O_2^-$  production rate due to stress, or even killed if the pressure is too high or the measurement time too long. In addition, the method requires a long analysis time: each replicate is analyzed for approximately 5–10 min. Considering the preparation time and the need for analysis of three replicates of the sample, blank, and three standards, the total analysis time is approximately 2.5 h for one sample (Table 1). Recently, our knowledge of superoxide production by *C. marina* as a func-

tion of growth conditions has been improved using this method (Garg et al. 2007; Rose et al. 2008). However, the long analysis time limits the number of samples that can be analyzed. For the method involving in situ measurement of luminescence, difficulties exist in calibrating the signal intensity in terms of superoxide concentration. Additionally, MCLA emits light at wavelengths similar to those absorbed by chlorophyll, with the result that signal intensity may be influenced by the cell density present.

One approach to reducing the impact of cell density on chemiluminescence intensity is to make use of a series of new CL probes that have been developed recently (Teranishi 2007). The probes have been synthesized by covalently linking MCLA with a sulforhodamine 101 moiety, such that the CL energy resulting from reaction of MCLA with superoxide is transferred to the conjugated fluorophore, causing red light to be emitted. This phenomenon, called chemiluminescence resonance energy transfer (CRET), improves the efficiency of the CL reaction and enables emission at a higher wavelength (Teranishi 2007). One such commercially available probe compound is red-CLA ([2-[4-[4-[3,7-dihydro-2-methyl-3-oxoimidazo[1,2-a]pyrazin-6-yl]phenoxy]butyramido]ethylamino]sulforhodamine101), which emits CL that is more intense than that emitted by MCLA and occurs in the red region of the visible spectrum (Teranishi 2007). This effect is potentially advantageous since the wavelength of the emitted light is well above the peak absorbance of chlorophyll *a* (Chl *a*) and other organic compounds typically encountered in natural waters, which might otherwise absorb the emitted light.

In this study, we propose a new method for the determination of extracellular superoxide production based on the emplacement of both samples and standards into multiwell microplate wells containing the CL reagent with subsequent direct analysis of superoxide production rates from the sample using a reader capable of detecting emitted light from each well. This analysis is performed without any pretreatment (such as filtration) of the sample before conducting measurements in a manner similar to that used by Lee et al. (1995). Use of 96-well plates significantly decreases sample volume and analysis time compared to the approaches used by Garg et al. (2007) and Lee et al. (1995) and enables measurement of three to five replicates of blank, standards, and sample within 10 min. The use of two superoxide-specific CL probes, namely MCLA and the closely related red-CLA (Nakano et al. 1986; Teranishi 2007), is examined in this study with the expectation that use of red-CLA may reduce the extent of absorption by the organisms of light emitted by the chemiluminescing agent.

### Experimental method

**Reagents**—Stock solutions of 125  $\mu$ M MCLA (TCI scientific), 5 mM xanthine (X) (Sigma-Aldrich), 3 units  $L^{-1}$  (reported as real activity; see Eq. 1 below and associated explanations) xanthine oxidase from bovine milk (Sigma-Aldrich), 3  $kU mL^{-1}$

**Table 1.** Comparison of characteristics of various superoxide analysis methods for analysis of triplicates of blank, sample, and three standards.

Method	Working range	Detection limit	Matrix	Time of analysis	Sample volume, mL	Probe used	Standardization
Microplate method	0.185–45 pmol s <sup>-1</sup> <sup>a</sup>	0.076 pmol s <sup>-1</sup>	Culture	10 min	4.2	Red-CLA or MCLA	Unique standards for each sample; standards analyzed at same time as sample
FIA <sup>b</sup>	ND	ND	Filtered seawater	2.5 h	30–75	MCLA	One series of standards for a series of samples
Flow analysis <sup>c</sup>	200–5000 cells mL <sup>-1</sup>	ND	Culture	≈45 min	ND	MCLA	
Luminometer <sup>d</sup>	ND	ND	Culture	≈30 min	≈45	MCLA	

ND, not determined.

<sup>a</sup>Varies with pH.

<sup>b</sup>Asai et al. 1999; Rose et al. 2005; Garg et al. 2007; Kustka et al. 2005.

<sup>c</sup>Lee et al. 1995.

<sup>d</sup>Kim et al. 2000; Oda et al. 1997.

superoxide dismutase (SOD) from bovine erythrocytes (Sigma-Aldrich), 3 mM diethylenetriaminepentaacetic acid (DTPA) (Sigma-Aldrich), and 1 μM and 100 nM H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich) were prepared in 18.2 MΩ cm<sup>-1</sup> MilliQ water (MQ). A stock solution of 250 μM red-CLA (TCI scientific) was prepared in 50% (vol/vol) ethanol/MQ. Secondary stock solutions of XO were prepared before each experiment by diluting the primary XO stock solution with MQ to concentrations of 90 mU L<sup>-1</sup> (standard 1), 480 mU L<sup>-1</sup> (standard 2), and 1.2 units L<sup>-1</sup> (standard 3). MCLA, red-CLA, SOD, and XO were stored at -80°C, H<sub>2</sub>O<sub>2</sub> and xanthine were stored at 4°C, and DTPA was stored at room temperature.

*Algal cultures*—Batch cultures of *Trichodesmium erythraeum* IMS101 (CCMP; Bigelow) were maintained in exponential phase in YBCII artificial seawater medium (Chen et al. 1996) containing 400 nM total Fe and 2 μM EDTA (Fe' = 8 × 10<sup>-10</sup> M, calculated using the equilibrium speciation program MINEQL+), at 27°C under a 14-/10-h light/dark cycle with a light intensity of 52 μEinstein m<sup>-2</sup> s<sup>-1</sup>. Batch cultures of *Chattonella marina* were maintained in GSe medium (Blackburn et al. 1989) containing 100 nM total Fe at 18°C under a 14-/10-h light/dark cycle with a light intensity of 100 μEinstein m<sup>-2</sup> s<sup>-1</sup>.

*Method*—Experiments with *Trichodesmium* were performed at 27°C, the temperature to which the organism was most suited, whereas experiments with the hardier *C. marina* were performed at room temperature (approximately 22°C). All reagents used for the experiments, except MCLA and/or red-CLA, were directly added to microplate wells immediately before addition of the sample as indicated in the detailed procedure below. After subsequent addition of MCLA or red-CLA, superoxide produced by the cells reacted with the CL probe, and the CL emitted was detected by a Fluostar Optima microplate reader (BMG Labtech). Continuous production of superoxide by the cells resulted in continuous CL emission by the CL probes at an approximately constant rate. CL emission was followed over 10 min, and the stable, quasicontant part of each signal was used to calculate superoxide production rates. As a calibration method, the X/XO system was used by exploiting the fact that XO enzymatically mediates the reduction of oxygen by xanthine to produce superoxide (Fridovich 1970; Garg et al. 2007). A fixed concentration of 50 μM X was used with different concentrations of XO added to yield different rates of superoxide production (see "Detailed procedure" below). Rates of production of superoxide by the X/XO system were determined as a function of pH using the equation determined by Rose et al. (2008):

$$\frac{d[\text{O}_2^-]}{dt} = \frac{0.618}{10^{\text{p}K_1 - \text{pH}} + 1 + 10^{\text{pH} - \text{p}K_2}} \text{ nmol/unit XO/s} \quad (1)$$

where pK<sub>1</sub> = 6.6 is the pK<sub>a</sub> of the enzyme XO and pK<sub>2</sub> = 8.2 is the pK<sub>a</sub> of xanthine. Note that the enzyme concentration used in this equation and elsewhere in this article is based on the real activity of XO when assayed according to manufacturer's

instructions by spectrophotometrically (292 nm) measuring the formation of urate in time when XO was added to a phosphate buffered X solution. The slope of the line (in absorbance units  $\text{min}^{-1}$ ) determines the rate of uric acid production; given that the molar extinction coefficient for uric acid is  $11000 \text{ M}^{-1} \text{ cm}^{-1}$ ,

$$\varphi = 90.9 \times \text{slope} \quad (2)$$

where  $\varphi$  is the rate of production of uric acid in  $\mu\text{mol L}^{-1} \text{ min}^{-1}$ . The activity of the XO (units  $\text{L}^{-1}$ ) is then equivalent to the rate of uric acid production since one unit of XO is defined as the amount of XO that converts  $1 \mu\text{M X}$  to uric acid per minute. This value is typically less than that specified by the manufacturer (probably due to the handling and storage procedures used). To quantify the net production rate of superoxide by the standards or the samples, the output from Eq. 1 is multiplied by the number of units of XO added to the wells.

**Detailed procedure**—In this method, the common matrix used for the sample, the blank, and the three standards was the phytoplankton culture itself. For the blank, SOD was added so that all the  $\text{O}_2^-$  produced by the cells was scavenged before reacting with the CL probes. For the standards, both X and XO were added to the culture: in this case, the difference in the signal between the sample and the standard represents the signal of the added standard.

Some precautions in sample preparation must be followed to obtain maximum sensitivity from the method and minimum deviation between replicates. In particular, samples and reagents must be added in the specific order described below:

1. Dispense  $10 \mu\text{L DTPA}$  ( $[\text{DTPA}]_{\text{final}} = 100 \mu\text{M}$ ) and  $3 \mu\text{L xanthine}$  ( $[\text{X}]_{\text{final}} = 50 \mu\text{M}$ ) at the bottom of 15 wells (five columns of three wells) of an acid-washed white 96-well plate without touching the well sides. This procedure allows for three replicates of each, the blank, sample, and three standards.
2. To the first column, add  $5 \mu\text{L SOD}$  ( $[\text{SOD}]_{\text{final}} = 50 \text{ kU L}^{-1}$ ).
3. To the third, fourth, and fifth columns add  $5 \mu\text{L XO}$  secondary standard 1, standard 2, and standard 3, respectively, on the wall of the wells (to prevent mixing of X and XO before adding the culture, which results in spurious results) to achieve final XO concentrations of 1.5, 8.0, and  $20 \text{ mU L}^{-1}$  for each standard, respectively.
4. Immediately before inserting the microplate into the reader, add  $280 \mu\text{L culture}$  to each well in such a way that the XO drop is mixed with the other contents of the well.
5. An initial reading of the plate is undertaken in the absence of the CL probe, then  $12 \mu\text{L red-CLA}$  solution ( $[\text{red-CLA}]_{\text{final}} = 10 \mu\text{M}$ ), which is maintained in an ice bath, or  $6 \mu\text{L MCLA}$  ( $[\text{MCLA}]_{\text{final}} = 2.5 \mu\text{M}$ ) maintained at room temperature, is injected into each well and the CL emission from the plate is read for 10 min using an acquisition time of 1 s for each well.

Considering the concentrations of X and XO in standards 1 and 3, and the fact that, at pH 8.31, the XO activity is equal

to  $7.3 \text{ nmol of urate produced/s/units XO}$  (Rose et al. 2008), we can determine the time when all the X is used by the XO. This time is  $>11 \text{ h}$  for the highest XO standard (standard 3 =  $20 \text{ mU L}^{-1}$ ) and  $>146 \text{ h}$  for the lowest standard (standard 1 =  $1.5 \text{ mU L}^{-1}$ ). The amount of X used during the time experiment (10 min) is 1.5% and 0.1% for standard 3 and standard 1, respectively.

## Results and discussion

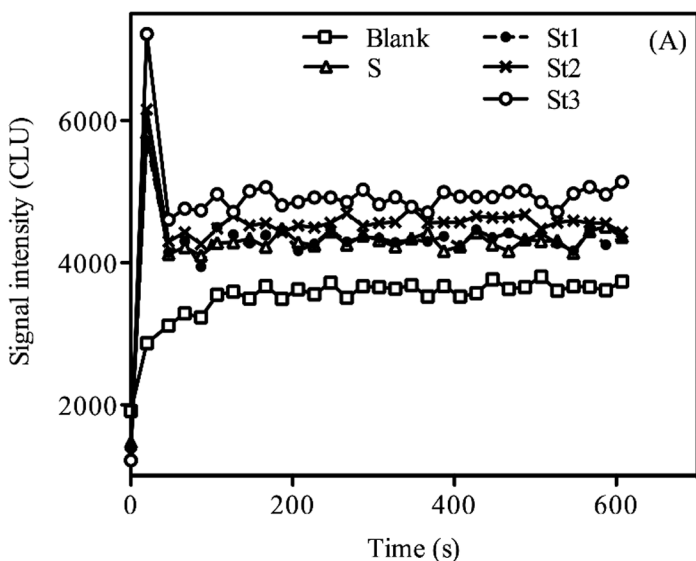
**Chemiluminescence signal**—As shown in Fig. 1A, injection of MCLA or red-CLA into the wells produces an initial CL signal peak followed, after a few seconds, by a signal of lower intensity which then remains stable for  $>10 \text{ min}$ .

As seen in Fig. 1B, the blank corrected average value of the steady signal (in this instance recorded from 250 to 600 s) is directly proportional to the concentration of XO added and thus the rate of superoxide generation.

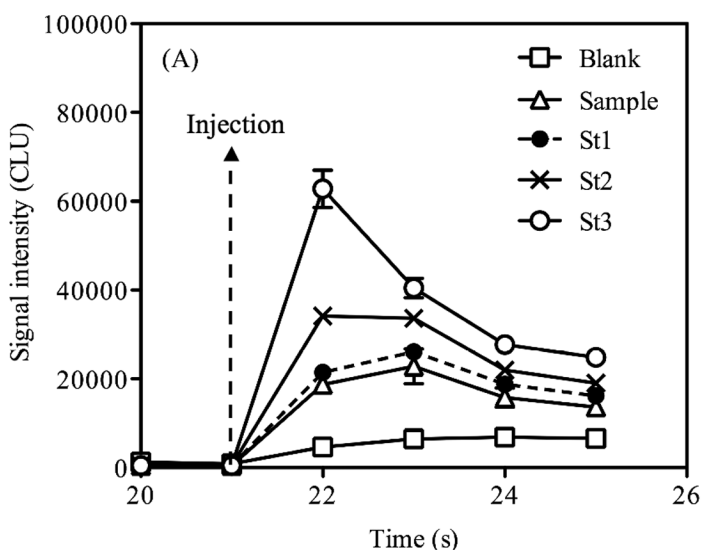
To obtain insight into the cause of the initial peak, samples and standards were prepared as indicated above and the CL measured at 1-s intervals for 5 s with the CL probe added 1 s after readings commenced (about 20 s after adding the culture) (see Fig. 2A). Time series analyses were obtained for the blank, a sample, and standards containing final XO concentrations of 5, 20, and  $100 \text{ mU L}^{-1}$ , with an increase in the initial peak signal observed as the XO concentration (and thus  $\text{O}_2^-$  production rate) increased. Superoxide would be expected to accumulate after addition of X and XO until reaching a steady-state concentration given by the balance of its production and decay rates. This suggests that the initial peak in the CL signal is due to the reaction of the CL probe with  $\text{O}_2^-$  that has accumulated in the medium at the time of injection of the probe. To further investigate this hypothesis, injection of the CL probe into each well, accompanied by CL analysis, was initiated at different times (20, 50, and 100 s) after addition of cells into wells along with  $100 \text{ mU L}^{-1} \text{ XO}$ . A linear increase in the intensity of the initial peak with increasing time between mixing of reagents and injection of red-CLA was observed, consistent with accumulation of  $\text{O}_2^-$  in the wells over time (Fig. 2B).

As seen in Fig. 2A, the accumulated  $\text{O}_2^-$  reacts with the CL probe in about 3 s. After this accumulated  $\text{O}_2^-$  has been consumed, the residual steady-state signal intensity represents CL due to the ongoing reaction between the CL probe and the  $\text{O}_2^-$  produced in real time by the cells and/or the X/XO. This steady-state signal is therefore used as the basis of determining superoxide production rates in the remainder of this work.

**Effect of red-CLA, MCLA, and DTPA concentration on CL**—To measure superoxide production from *Trichodesmium* cells, defined volumes of culture were transferred as previously described to microplates in the presence of DTPA, a metal complexant used to bind metals such as iron and copper present in the medium which otherwise catalyze the disproportionation of superoxide (Goldstone and Voelker 2000; King et al. 1995) and might thus be expected to decrease the CL signal intensity. Various concentrations of MCLA (2.5 and  $10 \mu\text{M}$ ),

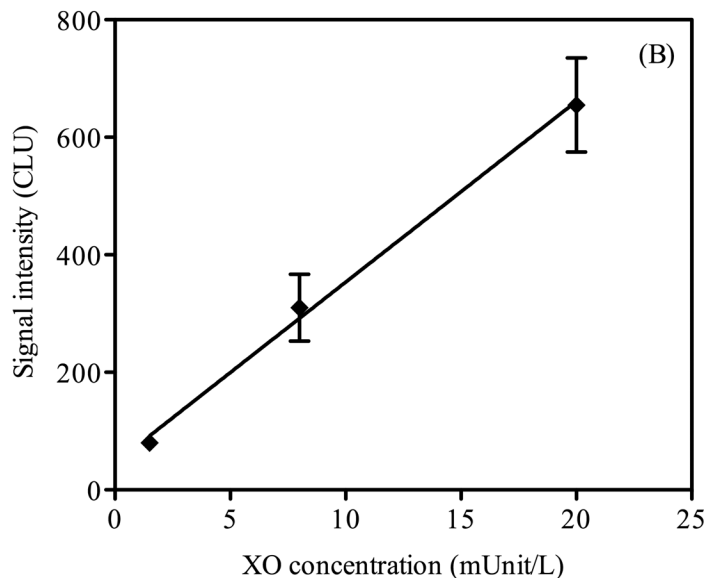


**Fig. 1A.** Typical instrument output for detection of red-CLA chemiluminescence due to extracellular superoxide production by *Trichodesmium erythraeum*: blank (SOD), sample (S), standard 1 (St1, 1.5 mU L<sup>-1</sup>), standard 2 (St2, 8.0 mU L<sup>-1</sup>), and standard 3 (St3, 20 mU L<sup>-1</sup>). The average rate of superoxide production was calculated from the plateau after subtracting the blank signal from the sample signal.

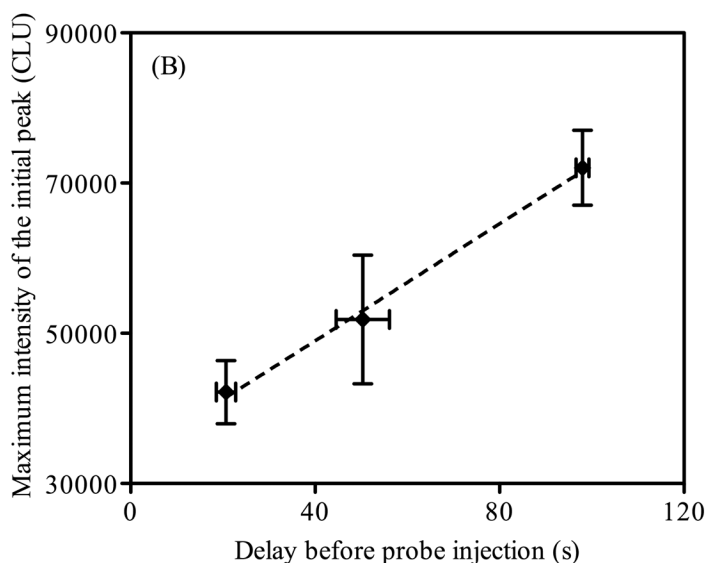


**Fig. 2A.** Analysis of CL signals from a blank, sample, and three standards before and after injection of red-CLA. The standards were added to the sample at concentrations of 5, 20, and 100 mU L<sup>-1</sup> for standards 1, 2, and 3, respectively. The signal from the blank represents the background signal due to the autoxidation of the CL probe.

red-CLA (2.5, 5, and 10 μM), and DTPA (0, 5, 10, 50, 100, 200, and 500 μM) were tried to optimize the signal to CL probe concentration ratio. The addition of DTPA (data not shown) showed no significant difference for the CL signal emitted by the MCLA probe. However, addition of DTPA showed a smooth but significant increase in the CL signal for the red-CLA probe



**Fig. 1B.** Calibration curve drawn with the “blank corrected” values of the three standards indicated in Table 2 ( $r^2 = 0.99$ ); error bars are SD of five replicates.



**Fig. 2B.** Maximum CL signal observed for triplicate analysis of the CL signal produced by standard 3 performed at different times before addition of the CL probe to the mixture. Error bars are SD of triplicates ( $r^2 = 0.99$ ).

between 0 and 100 μM DTPA, and a dramatic decrease in the signal when the DTPA concentration was 200 or 500 μM. These results were observed for all red-CLA concentrations, with a slight but significant increase in the CL intensity when the concentration of red-CLA was increased to 10 μM.

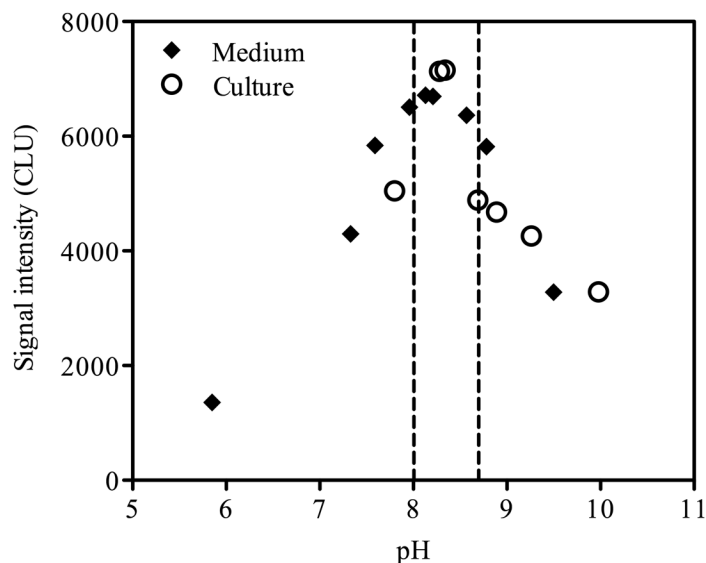
Because DTPA solutions are acidic, it was recognized that reaction mixtures containing different concentrations of DTPA may exhibit different pH. In view of this observation, a

study of the effect of pH on signal intensity was undertaken by adding DTPA solutions with pH 2.17 (DTPA dissolved in MQ), 8.47, 10.15, and 12.23 (pH adjusted with NaOH), in either YBC-II medium or *Trichodesmium* culture. The results reported in Fig. 3 show that the CL signal is strongly dependent on pH of the medium, suggesting that the decrease of CL signal observed in the previous experiment was likely due to the substantial pH change associated with addition of >100  $\mu\text{M}$  DTPA.

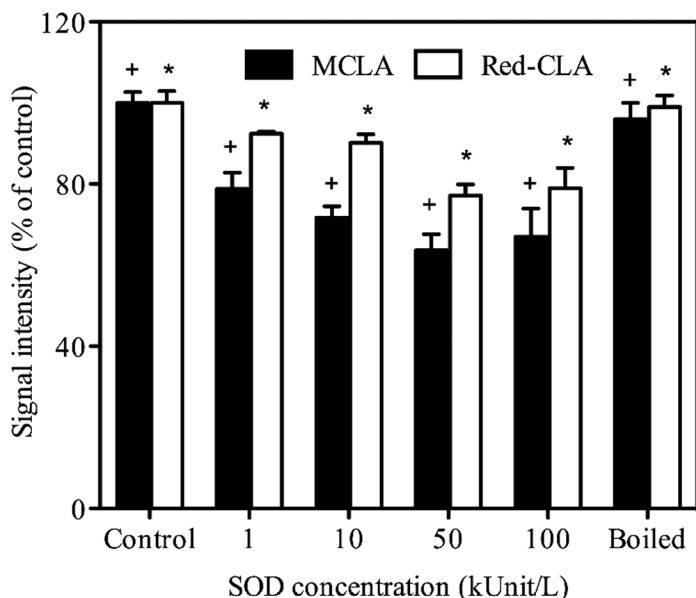
The protocol described in “Detailed procedure” under “Experimental method” above is based on conditions that produced the maximum signal intensities using YBCII medium and *Trichodesmium* cells, namely 100  $\mu\text{M}$  DTPA when using 10  $\mu\text{M}$  red-CLA, but no DTPA when using 2.5  $\mu\text{M}$  or 10  $\mu\text{M}$  MCLA.

**Quantitative determination of the CL signal**—Unlike the FIA method used for *Chattonella marina* (Garg et al. 2007) and *Lynghya majuscula* (Rose et al. 2005), in the microplate method the matrix is essentially the same as the medium of the culture, with addition of SOD or X/XO to generate the blank and the standards (see “Detailed procedure” under “Experimental method”). Different concentrations of SOD were added to the culture to verify that the CL signal was indeed due to superoxide production by the *Trichodesmium* cells. The results presented in Fig. 4 show a significant decrease in the CL signal when 1  $\text{kU L}^{-1}$  SOD was added ( $P < 0.01$ ) and an even greater inhibition when more than 50  $\text{kU L}^{-1}$  SOD was added. In contrast, adding 50  $\text{kU L}^{-1}$  SOD that had been boiled at 100°C for 30 min (to inhibit the enzymatic activity without changing the molecule conformation) resulted in a CL signal that was equivalent to that obtained without SOD addition for both MCLA and red-CLA. These results therefore confirm that the SOD-inhibitable part of the signal was due to reaction between the CL probe and  $\text{O}_2^-$ . The signal that was not inhibited by addition of SOD has previously been shown to result from autoxidation of the CL probes (Teranishi 2007). Thus, to account for the background signal due to CL probe autoxidation, wells containing the culture plus 50  $\text{kU L}^{-1}$  SOD were defined as the blanks, and the CL signal measured in these wells was subtracted from the CL signals measured in sample and standard wells during data analysis.

For each experiment, replicates of the blank (+ SOD), three standards (+ X/XO), and sample were prepared and analyzed almost simultaneously by the microplate reader, before and after injection of the CL probe. As shown in Fig. 1A, the CL signals exhibited a plateau for about 10–15 min after stabilization of the system (representing the CL signal produced in real time as the CL probe reacts with freshly produced  $\text{O}_2^-$ ). After this time, the CL signal of the standards decreased as xanthine was consumed and superoxide was no longer generated with quasi-zero order kinetics. The signal then further decreased to background levels after about 20–25 min of analysis, likely due to consumption of the CL probe. The net superoxide production rate was calculated by averaging the values of the



**Fig. 3.** Variations in the CL intensity of 10  $\mu\text{M}$  red-CLA injected in YBC-II medium or *Trichodesmium* culture versus the pH while DTPA solution is added. The dashed lines represent the working pH range with *Trichodesmium* cultures.



**Fig. 4.** Typical suppression of CL signal by the addition of SOD (1–100  $\text{unit mL}^{-1}$ ) into a *Trichodesmium erythraeum* culture. The inhibition is significant ( $P < 0.01$ , SOD test significantly different from sample marked with \* for red-CLA samples and with + for MCLA samples) with more than 1  $\text{kU L}^{-1}$  SOD for red-CLA and MCLA. Addition of 50  $\text{kU L}^{-1}$  SOD denatured by boiling does not result in a CL intensity significantly different from that of the blank ( $P > 0.9$ ).

CL signal obtained during the plateau phase, and converted from equivalent units of XO to an actual superoxide generation rate using Eq. 1 (see Fig. 1A and B and Table 2). The *Trichodesmium* culture analyzed contained 8100 trichomes  $\text{mL}^{-1}$  (= 2270 trichomes  $\text{well}^{-1}$ ), yielding an  $\text{O}_2^-$  production rate of

**Table 2.** Calibration of the signal obtained in Fig. 1A from a culture of *Trichodesmium erythraeum* IMS101 at pH 8.31 based on the linear calibration curve constructed in Fig. 1B.

	Signal from plateau	Blank corrected signal	O <sub>2</sub> <sup>-</sup> production rate (equivalents of mUnit XO/L)	O <sub>2</sub> <sup>-</sup> production rate (pmol/s) <sup>a</sup>
Blank	3653	—	—	—
Standard 1	4353 (762)	80 (14)	1.3	0.11
Standard 2	4583 (843)	310 (57)	6.7	0.54
Standard 3	4927 (1000)	655 (133)	16.7	1.36
Sample	4273 (593)	620 (86)	12.4 (7)	1.00 (0.57)

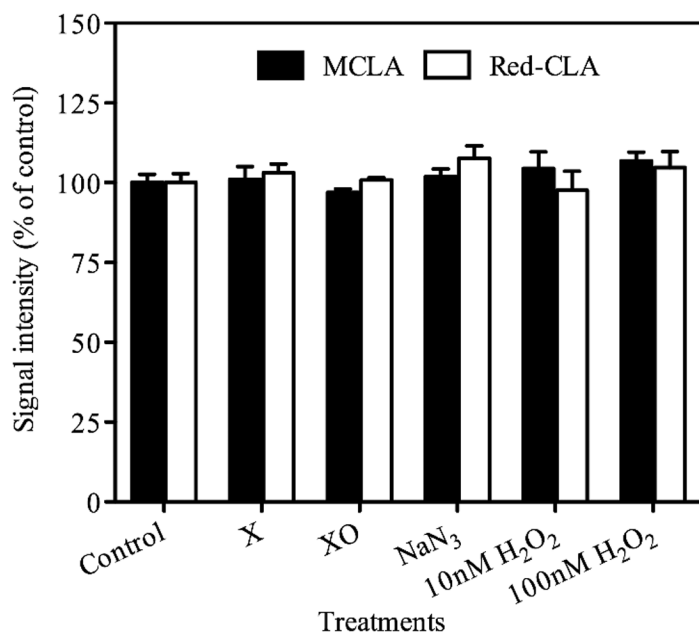
The blank correction of the signals of the sample and the standards is described in "Quantitative determination of the CL signal." Data are average (SD).  
<sup>a</sup>Determined by applying the factor calculated from Eq. 1 (81 pmol/s/unit XO in this case)

1.59 (± 0.09) pmol trichome<sup>-1</sup> h<sup>-1</sup>. The average number of cells per trichome determined using light microscopy was 88, giving a cell-normalized superoxide production rate of 18.10 (± 0.99) fmol cell<sup>-1</sup> h<sup>-1</sup> (or 7.5 × 10<sup>-20</sup> mol O<sub>2</sub><sup>-</sup> μm<sup>-2</sup> h<sup>-1</sup> assuming a cell surface area of 242 μm<sup>2</sup> cell<sup>-1</sup>). In comparison, *C. marina* produces between 0.29 and 4 pmol of O<sub>2</sub><sup>-</sup> cell<sup>-1</sup> h<sup>-1</sup> (Garg et al. 2007; Oda et al. 1998) (or 4.2 × 10<sup>-16</sup> mol O<sub>2</sub><sup>-</sup> μm<sup>-2</sup> h<sup>-1</sup>) (Kustka et al. 2005), whereas the diatom *Thalassiosira weissflogii* produces about 0.84 ± 0.14 fmol of O<sub>2</sub><sup>-</sup> cell<sup>-1</sup> h<sup>-1</sup> (or 1.8 × 10<sup>-18</sup> mol O<sub>2</sub><sup>-</sup> μm<sup>-2</sup> h<sup>-1</sup>) (Kustka et al. 2005).

**Interferences for superoxide detection**—Studies were conducted with potentially interfering compounds including X, XO, H<sub>2</sub>O<sub>2</sub>, and singlet oxygen (<sup>1</sup>O<sub>2</sub>). CL signals obtained when different treatments were applied to *Trichodesmium* cultures are shown in Fig. 5. X and XO alone were added directly to the DTPA-treated culture at their maximum concentrations used during the experiments (50 μM X and 0.5 units L<sup>-1</sup> XO), whereas H<sub>2</sub>O<sub>2</sub> was added at typical concentrations found in marine waters of 10 and 100 nM. No significant influence of these chemicals on the CL signal was evident (*P* > 0.8), suggesting that they do not interfere with either the CL probes or the organisms themselves.

Because singlet oxygen (<sup>1</sup>O<sub>2</sub>) is also known to react rapidly with MCLA (Lee et al. 1995; Suzuki et al. 1991), 100 μM of its quencher NaN<sub>3</sub> was added to the culture to test for a possible influence of singlet oxygen during analysis. Because NaN<sub>3</sub> is toxic to living organisms, the superoxide production rate was determined immediately following NaN<sub>3</sub> addition. As the rate constant for reaction of NaN<sub>3</sub> with <sup>1</sup>O<sub>2</sub> is 1.3 × 10<sup>8</sup> M<sup>-1</sup> s<sup>-1</sup>, and the reaction rate constant of MCLA with <sup>1</sup>O<sub>2</sub> is 2.9 × 10<sup>9</sup> M<sup>-1</sup> s<sup>-1</sup>, 100 μM of NaN<sub>3</sub> is sufficiently high to outcompete MCLA. The observation of no decrease in the CL signal intensity on addition of NaN<sub>3</sub> indicates that singlet oxygen is not produced by *Trichodesmium* in sufficient quantities to interfere with superoxide determination.

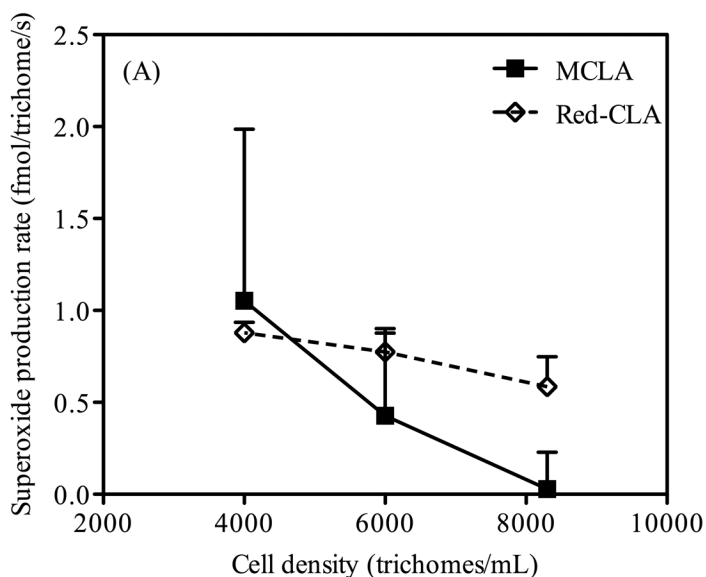
Other potential interfering agents found in natural waters [Cr(III), Se(IV), NO<sub>2</sub><sup>-</sup>, Cu(I), Sb(III), V(III), V(IV), Mo(V), I<sup>-</sup>, Mn(II), Co(II), Fe(II), and As(III)] examined by Rose et al. (2008) did not show any significant effects at concentrations typical of natural waters. The only significant contribution would be the amount of O<sub>2</sub><sup>-</sup> that these entities could generate



**Fig. 5.** CL intensity obtained from a *Trichodesmium erythraeum* culture using MCLA or red-CLA with addition of X alone (50 μM), XO alone (0.5 units L<sup>-1</sup>), NaN<sub>3</sub> (100 μM) or H<sub>2</sub>O<sub>2</sub> (10 or 100 nM). No treatments had a significant effect on the CL signal (*P* > 0.8).

by reaction with O<sub>2</sub> when they are present in high concentrations in freshwaters or coastal seawaters. Because red-CLA reacts with superoxide via a mechanism identical to that of MCLA (except that the CL energy is transferred to the conjugated sulforhodamine 101 moiety before light emission), these agents would similarly not be expected to interfere with superoxide determination using red-CLA.

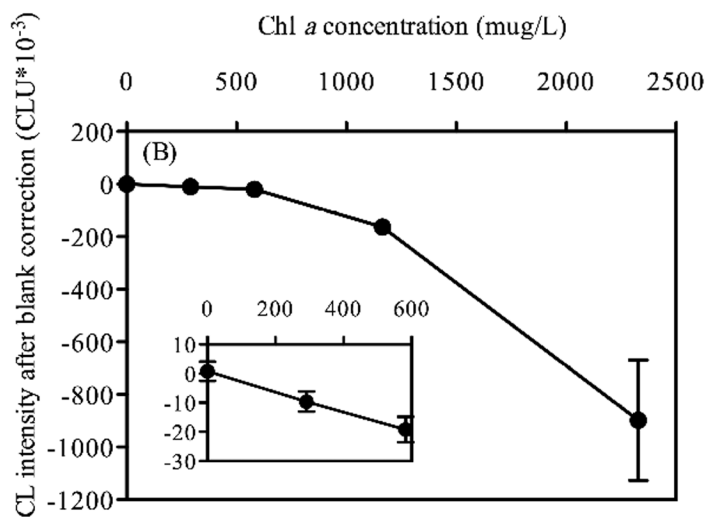
**Effect of cell density on CL intensity**—Superoxide production rates were studied in *Trichodesmium* cultures containing different cell densities. This was achieved by diluting a particular culture with its own medium (which was obtained by filtration of the culture using a 0.22-μm Millex filter). Although there was no significant influence of cell density in the medium on the CL intensity when red-CLA was used (*P* > 0.01; Fig. 6A), significant quenching of signal intensity resulted with increasing trichome density when MCLA was used. These



**Fig. 6A.** Effect of cell density on apparent (measured) rate of extracellular superoxide production with MCLA and red-CLA. Error bars are SD of triplicates.

results are consistent with previous work by Marshall and Ross (2005), in which the relationship between cell density and CL intensity when MCLA was added directly to cultures was non-linear, with the apparent rate of CL emission per cell decreasing at high cell densities.

Chl *a*, a major pigment in cyanobacteria, absorbs light at wavelengths up to 460 nm and, as such, may quench CL emitted by MCLA, which emits light (following reaction with superoxide) between 400 and 550 nm with a maximum intensity between 430 and 480 nm (Teranishi et al. 1998). In comparison, red-CLA emits CL between 560 and 650 nm and has a maximum intensity at 610 nm (with a very sharp peak), a wavelength where Chl *a* as well as other pigments such as the phycobilins and some other carotenoids exhibit very poor light absorption (Subramaniam et al. 1999). These results are, therefore, consistent with an expectation that the CL intensity of MCLA would be strongly quenched at high cell density. To analyze the effect of Chl *a* concentration in the medium on MCLA CL intensity more closely, we added pure Chl *a* in various concentrations to cell-free medium. As seen in Fig. 6B, much stronger quenching of the CL signal is observed with pure Chl *a* than *Trichodesmium* cells alone. This is perhaps not surprising since the extracted chlorophyll is in direct contact with MCLA and will thus capture emitted light very efficiently whereas chlorophyll within *Trichodesmium* cells will be much less effective at capturing light. Indeed, it is possible that much of the light emitted by MCLA will be captured by photosystem-II, the bilayer lipid membrane, or even by some metals present in the cytoplasm or in the medium rather than by chlorophyll directly (Campbell et al. 1998; Phillip et al. 1996).



**Fig. 6B.** Effect of pure Chl *a* on CL intensity of MCLA. Inset, CL from 0 to 600 µg L<sup>-1</sup> Chl *a*. Error bars are SD of triplicates.

Despite this strong quenching, the same degree of quench should apply to both sample and standard signals such that the calibrated superoxide production rate should not be affected. However, it is clear from Fig. 6A that this is not the case, suggesting that one or more processes other than CL quenching must also be occurring when using MCLA as the probe. MCLA is known to strongly associate with cell membranes because of its hydrophobic moieties (Nakano 1998), which introduces a cell-density dependency to the O<sub>2</sub><sup>-</sup> trapping properties of MCLA. MCLA is much smaller (molecular weight 291.73 g mol<sup>-1</sup>) and contains less hydrophilic/polar moieties than red-CLA (molecular weight 958.11 g mol<sup>-1</sup>). As such, it is reasonable to expect that red-CLA will show less tendency to associate with cell membranes and would thus be expected to yield more reliable results at high cell densities, as observed. Therefore red-CLA appears preferable for use at high cell densities, and results obtained using MCLA in direct contact with cells at high cell densities should be interpreted with caution. These results indicate that the red-CLA method is very well suited for laboratory and field work on seawater-based sample analysis, as the optimum working pH range corresponds to the pH of the seawater (between 8 and 8.5) and the red-CLA probe is not influenced by the presence of living organisms such as phytoplankton cells. The fact that the MCLA probe CL signal is partially quenched by high phytoplankton cell densities may limit its application for field studies. Although medium conditions (e.g., Fe concentration and the type and concentration of organic ligands) are constant in the present study, these conditions may vary considerably in the field depending on the sample source (e.g., open ocean or coastal waters) and may also affect the method's sensitivity.

**Method comparison and figures of merit**—To validate the reliability of the microplate method against an existing method, superoxide generation rates by the raphidophyte *C. marina*

**Table 3.** Figures of merit for the microplate method.

CL probe	CL wavelength, nm	Matrix	Probe concentration, $\mu\text{M}$	Working range, $\text{pmol s}^{-1}$	Detection limit, $\text{pmol s}^{-1}$ <sup>a</sup>	Limit of quantification, $\text{pmol s}^{-1}$ <sup>b</sup>
Red-CLA	610	Seawater phytoplankton culture	10	0.25–45	0.076	0.25
MCLA	465	Seawater phytoplankton culture	2.5	4.7–45	1.4	4.7

<sup>a</sup>Defined as 3SD of the blank.

<sup>b</sup>Defined as 10SD of the blank.

were measured using both the established FIA method (using MCLA as the CL probe) and the microplate method (using red-CLA as the probe). Superoxide production rates of  $9.4 \pm 7.8$   $\text{pmol cell}^{-1} \text{ s}^{-1}$  by the FIA method and  $20 \pm 6.3$   $\text{pmol cell}^{-1} \text{ s}^{-1}$  by the microplate method were obtained. These results are not significantly different ( $P > 0.8$ ), but the microplate method gave more reliable results with a smaller sample quantity and in a shorter time. Figures of merit for the microplate method using both MCLA and red-CLA as CL agents and using an acquisition time of 1 s are given in Table 3.

This acquisition time was found to represent a good compromise between the signal intensity, sensitivity, and time between each signal measurement per well. Obviously, the detection limits and the limits of quantification shown in this table can be optimized depending on the sample analyzed by changing instrument settings. The characteristics of other methods used for detecting the rate of  $\text{O}_2^-$  generation from phytoplankton cells are compared in Table 1 to those of the microplate analysis method described in this work. As noted, the time of analysis using the microplate method is much shorter than any other method; in particular, the analysis time of the FIA method is excessive (2.5 h for the analysis of one condition). Knowing that some phytoplankton cultures have growth rates of  $>1.5 \text{ d}^{-1}$ , culture conditions will have most likely altered between analysis of the first and the last samples. In addition, use of the FIA method in field studies is problematic when stations are located close to each other, since storing samples for more than few minutes will likely result in changes to living organisms.

The small sample volume required in the microplate method (4.2 mL) is particularly attractive in laboratory studies where culture volumes are typically limited. Additionally, this method enables use of unique standards for each sample and exhibits very low detection limits with quite a large working range compared to the other methods available. It is also important to reiterate that the FIA and flow analysis methods are not well suited to use with fragile cells since both cell stress and cell breakage may lead to anomalously high superoxide generation rates.

During this study, we used only 15 wells of the plates for the measurement of  $\text{O}_2^-$  production rate from *Trichodesmium*

and *Chattonella* cultures due to the design of the experiment. The instrument settings may have to be changed if the whole plate is used, and are also dependent on the nature of the sample and the average superoxide production rate. To keep maximum reliability in the results, the exact same protocol as the one described in this manuscript has to be applied: each sample would have its own blank and standards. In this case, the maximum number of wells used in a plate would be 90 ( $6 \times 15$  wells). The total time of analysis would still be 10 min, but the time between two measurements for each well would increase from 16 to 90–95 s: the time for the plate reader to read the whole plate before coming back to the first well. These settings are satisfactory as the CL signal is very stable over the 10-min analysis (Fig. 1A).

### Conclusion

The application of microplate-based measurements to phytoplankton cultures shows considerable promise due to significant decreases in sample volume and processing time. In particular, application of microplate technology to the determination of extracellular superoxide production by phytoplankton using the superoxide-specific CL probes MCLA and red-CLA is shown here to be a reliable method that offers advantages over existing methods including direct analysis of samples with minimum manipulation of the cells and a significant decrease in analysis time (by a factor of 15 compared to FIA). The use of 96-well plates allows near simultaneous measurement of a series of blanks, sample and standards, with each sample individually calibrated using X/XO. The results obtained are more accurate than any other existing method and specific to each sample analyzed. The microplate method we propose here is selective for superoxide detection, with low detection limits and large working ranges adaptable to field studies. Although the method may be applied using MCLA or red-CLA as the CL probe, MCLA has been shown to have some limitations at high cell densities. Thus the choice of using MCLA or red-CLA will depend largely on the type of sample to be analyzed.

Similar estimates of superoxide production rate by the prolific superoxide producer *Chattonella marina* have been obtained by both a filtration/flow injection approach and

the microplate method described here. Application of the new method to *Trichodesmium erythraeum* IMS101 cultures shows that this organism produces extracellular superoxide in environmentally relevant amounts, which is consistent with previous work by Marshall et al. (2005) showing that extracellular superoxide production is widespread among phytoplankton species.

### Comments

Although the method may be applied to most phytoplankton species, two factors could hamper the analysis of some phytoplankton species or limit its application during field analysis. First, as the enzyme solution XO is unstable at room temperature, it is necessary to calibrate the stock solution after preparation (and to recalibrate if the stock solution is not fresh). Also, the red-CLA is dissolvable in alcohol which, under our culture conditions, does not appear to influence the results, as the final concentration of ethanol in the sample is less than 2%. This method could possibly be improved by changing the calibration method to a chemical superoxide generation system instead of an enzymatic reaction (or by using a more stable enzyme), and by dissolving the red-CLA powder in an aqueous solvent.

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