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RESEARCH ARTICLE

Optode use to evaluate microbial planktonic respiration in oligotrophic ecosystems as an indicator of environmental stress

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Abstract The suitability of O_2 optodes to resolve the effects of global-change stressors on respiration of microbial planktonic assemblages from oligotrophic ecosystems was tested. With this aim, we first evaluated how O_2 measurements with optodes on closed flasks depended on delayed temperature equilibration (hysteresis), which can bias actual measurements on samples not subjected to constant temperature. This must be addressed when using optodes for in situ measurements. Thus, we provide the mathematical tools to correct the effects of hysteresis on O_2 measurements hence removing the constraints of maintaining a constant temperature over the long incubations required to measure respiration in oligotrophic ecosystems. Optodes proved suitable to resolve the effects of stressors such as CO_2, temperature, thermal stratification, nutrient input, and ultraviolet radiation in different oligotrophic aquatic ecosystems. These experiments resulted in significant differences in microbial planktonic respiration for all stressors tested. From these results, we conclude that (1) optodes constitute a useful tool to make realistic measurements on samples subjected to natural (or experimental) ranges of temperature variability, and (2) microbial planktonic respiration measured with O_2 optodes has the potential to be used as an ecological indicator for assessing the effects of environmental stress, even in oligotrophic aquatic ecosystems, where higher sensitivities are needed.

Keywords Optode · Microbial respiration · Indicator · Environmental stress · Global change · Oligotrophy

Introduction

Respiration fuels most metabolic pathways and thus determines the pace at which organisms and ecosystems work, providing information on the possible consequences of environmental stress at different levels of biological organization (del Giorgio and Williams 2005; Staehr et al. 2012). Consequently, microbial respiration, mainly from prokaryotes and protists, has the potential of being a functional indicator for environmental stress in aquatic ecosystems. In fact, respiration alone or together with other variables has been used previously to assess eutrophication and organic pollution (Garnier and Billen 2007), heavy-metal contamination (Nwachukwu and Pulford 2011), ecosystem state and functioning (Oyonarte et al. 2012), inputs of biogenic detritus (Rowe et al. 1994), the effects of herbicides (Rocha et al. 1998), and changes in nutrient availability, temperature, and salinity (Faxneld et al. 2010).

Despite its importance for ecosystem functioning or as an environmental indicator, respiration measured as O_2 consumption poses a methodological challenge. Ever since Winkler (1888) developed his technique to measure oxygen concentration, many direct and indirect techniques have been used to measure respiration: electrochemical sensors (Carignan 1998; Briand et al. 2004; Revsbech et al. 2009), electron-transport system (Packard 1971; Kenner and Ahmed 1975; del Giorgio 1992; Martínez-García et al. 2009, 2013), CO_2 release (Cimbleris and Kalff

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1998; Carvalho and Eyre 2012), and mass spectrometry (Kana et al. 1994; del Giorgio and Bouvier 2002), including changes in the ^{18}O isotope such as the ^{18}O:^{16}O ratio (Bender and Grande 1987; Grande et al. 1989) or added ^{18}O-enriched tracers (Luz et al. 2002; Holtappels et al. 2014). However, these techniques are often too expensive, difficult to manipulate under in situ conditions or lack temporal resolution or analytical precision to measure very low rates of microbial planktonic respiration. In such cases, techniques with high stability and sensitivity are needed, since long incubation times for microbial communities are not recommended because of the risk for changes in community composition and substrate concentration (Pomeroy et al. 1994; Massana et al. 2001; Gattuso et al. 2002; Warkentin et al. 2007). O_2 optodes, an inexpensive technique with both robustness and resolution (Wikner et al. 2013; Wang and Wolfbeis 2014), have been used previously to study different oxygen-related processes in aquatic systems with good results (e.g., Warkentin et al. 2007; Marchand et al. 2009; Gregg et al. 2013; Holtappels et al. 2014). This technique enables non-invasive measurement with high temporal resolution and without oxygen consumption or gas exchange (Warkentin et al. 2007; Table 1). However, according to previous studies that measured microbial respiration, optode use requires thermal stability in order to avoid potential variations in O_2 measurements (Warkentin et al. 2007; Marchand et al. 2009). In fact, Warkentin et al. (2007) specifically mentioned that "temperature-induced changes in oxygen saturation will interfere with any respiration rate measurement of environmental samples. Thus, temperature control is essential when using optodes". These authors also discussed how mathematical correction for the temperature dependence of oxygen saturation (i.e., the temperature-compensation system) resulted in oscillating oxygen concentrations when measurements were made on closed flasks under temperature cycles (with a thermostat), and pointed out that this signal-oscillation problem can be avoided by very tight temperature control (Warkentin et al. 2007). Because keeping the temperature constant may not be feasible for many applications (e.g. for in situ measurements), the question of the reliable use of optodes under temperature variation remains open. This topic is relevant in order to achieve more realistic measurements of microbial respiration rates subjected to the natural temperature variations of ecosystems, rather than to constant temperature (i.e., laboratory conditions), particularly during the several hr incubations required to register the respiratory O_2 consumption over time in oligotrophic environments. In addition, the internal temperature-compensation system may not be suitable when measurements are made in closed flasks because the O_2 gas-exchange linked to changes in O_2 solubility due to temperature variations is prevented. Therefore, quantifying the potential hysteresis of the measurements due to external temperature variations and time span in closed flasks where making temperature measurements inside the flasks is difficult or unfeasible, would enable the correction of respiration rates from this effect.

Most stressors related to global change, such as increasing CO_2, rising temperatures, strengthened stratification, greater nutrient inputs, and ultraviolet radiation (UVR) can affect the metabolism of microorganisms in different ways. For example, one mechanism by which CO_2 can affect plankton metabolism is through acidification. Compensation of the acid-base imbalance implies the use of active acid-base equivalent ions across cell membranes (Fabry et al. 2008), which are energy dependent. High CO_2 concentrations can also augment the fixation of inorganic carbon, i.e., primary production, which in turn can result in increased excretion of organic carbon (EOC) by algae, favouring the heterotrophic activity (Riebesell et al. 2007). The relationship between temperature and respiration, as

Table 1 Sketch of the most commonly used respiration-measurement techniques with a brief list of advantages, disadvantages, and observations

	Advantages	Disadvantages	Observations
Winkler	Widely used	No live data	Many technique modifications
Electrochemical sensors	Widely used	Drift, fragile	New guard cathodes with low O_2 consumption
ETS (electron transport system)	High precision	Potential data, data depend of intracellular e^- donors	R:ETS ratio variable
CO_2 release	Same units as primary production (no need of conversion factors)	pH dependent, different physiological processes for respiratory O_2 consumption and CO_2 release	Less developed than oxygen methods
Mass spectrometry	High precision, high throughput, small sample size	Mass spectrometer plus additional components, drift, no live data	High analysis speed (>100 samples day)
O_2-Optodes	High-precision, non-invasive, non-consuming, continuous measurement	2-year sensor stability (use dependent)	Linear relationship with Winkler

opposed to CO_2, has a strong background. The dependence of respiration on temperature is described by the Van't Hoff-Arrhenius relation at the subcellular and individual levels of organization (Gillooly et al. 2001). This relationship has been the foundation for prevailing ecological theories (Brown et al. 2004), and several influential studies (López-Urrutia et al. 2006; Vázquez-Domínguez et al. 2007; Mahecha et al. 2010; Yvon-Durocher et al. 2012). However, at the ecosystem level, this dependence between temperature and respiration can vary due to the interaction with other factors related to global change (Yvon-Durocher et al. 2012). The effects of nutrient inputs have been extensively studied, and greater nutrient availability for planktonic communities improves their nutritional status (Sterner and Elser 2002), particularly in oligotrophic ecosystems. This improvement stimulates primary and secondary production, and thus the growth of algae and bacteria. Consequently, it increases not only the amount of biomass in the trophic food web, but also the catabolic metabolism of these organisms (Obernosterer et al. 2003; Smith and Kemp 2003; Roberts and Howarth 2006). Light can be a limiting resource for primary production, but intensified irradiance can also raise community and bacterial respiration (Roberts and Howarth 2006). Changes in light quality (PAR:UVR ratios) can also affect respiration. UVR harms organisms either by directly damaging macromolecules such as DNA, proteins, or lipids (Hessen et al. 1997; Häder and Sinha 2005) or indirectly by boosting the production of reactive oxygen species (ROS) (Lesser 2006). To overcome this damage, cells start different metabolic processes. The activation of photorepair and photoprotective metabolisms can thus be expected to accelerate respiration rates, but these have barely been studied and the results remain inconsistent (Harrison and Smith 2009; Carrillo et al. 2015a, b) as well as taxon dependent (Hörtnagl et al. 2011). Finally, global warming indirectly shoals the upper mixed layer (UML stratification), causing organisms to become trapped in near-surface layers and to be exposed to higher mean UVR irradiance. Hence, strengthened stratification can also affect the metabolic rates of microbial plankton in the epilimnion.

We tested a laboratory methodology to quantify and correct variations in the O_2 measurements due to hysteresis that, we hypothesize, will occur linked to temperature variations and time. We further tested effects of different global-change stressors on microbial planktonic respiration in six oligotrophic ecosystems which cover a wide range, from high-mountain lakes to marine ecosystems, with and without the application of the corrections from fitted equations as functions of temperature variations and time span. We hypothesize that stressors will generally increase respiration rates under stress, due to the expected rise in metabolic costs.

Materials and methods

O_2 measurements with optodes

The measurements were performed using O_2 sensor-spot optodes (SP-PSt3-NAU-D5-YOP; PreSens GmbH, Germany) and an optic-fibre oxygen transmitter equipped with a temperature-compensation system (Fibox 3; PreSens GmbH, Germany) together with a computer to collect the data. New sensor spots were glued to the inner wall of 25-mL quartz-glass flasks with transparent silicone (RS Components) of high permeability to atmospheric gases (Wang and Wolfbeis 2014; Wolfbeis 2015). The photoluminescence lifetime was measured by pointing the optic fibre towards the sensor spot through the hole drilled through PVC squares glued to the outer side of the flask. The hole had the same diameter of the fibre, allowing it to fit tightly and point towards the spot at consistently the same angle. This provided more accurate measurements because different angles of the optic fibre can add noise to measurements (Marchand et al. 2009). Before each measurement, the optodes followed a two-point calibration (0 and 100%). The 0% oxygen saturation was calibrated by adding sodium sulphite (Na_2SO_3) to distilled water to a final concentration not exceeding 0.1 mg mL^{-1}. For 100% oxygen saturation, wet cotton wool was placed into the closed flask; according to the optode handbook, this allows the air inside the flask to be saturated with water vapour and this vapour to be 100% O_2-saturated. All respiration rates (µM h^{-1}) were calculated as the slope of linear regression of oxygen concentration (after a thermal equilibration period of 20 min) vs. time.

Optimizing the optode laboratory setup

Effect of temperature fluctuation on O_2 measurements in closed flasks

Firstly, we assessed the time required for the thermal equilibration of samples to constant temperature in order to make non-biased O_2 measurements. For this, 25-mL quartz-glass flasks (in triplicate), filled without bubbles with sterilized double-distilled water at 15 °C, were completely closed, sealed and incubated in darkness in a thermo-regulated bath (RC 25, Lauda GmbH, Germany) at a constant temperature of either 15 or 20 °C (±0.01 °C), and oxygen dynamics were measured every 10–15 min for 1 h.

We secondly determined to what extent the temperature-compensation system might affect O_2 measurements in closed flasks, where O_2 gas-exchange linked to changes in O_2 solubility due to temperature variations is prevented, and then we assessed the potential of optodes to provide precise measurements in oligotrophic waters subjected

to non-constant temperatures. Thus, we set temperature cycles spanning a thermal range of either 5 °C (gradually from 15 to 20 °C and back to 15 °C every 12 h) or 20 °C (gradually from 5 to 25 °C and back to 5 °C every 12 h) on closed 25-mL quartz-glass flasks filled with sterilized double-distilled water (for controlling any abiotic O_2 consumption), incubated in darkness in the thermo-regulated bath, equipped with a temperature-ramp program (RC 25, Lauda GmbH, Germany). The O_2 measurements were made every 5 min, after the thermal-equilibration period determined previously. Therefore, the thermal range of 0.4 °C reported by Warkentin et al. (2007) was extended to reduce the noise signal and achieve a better fitting of the equations that describe the responses of the optodes to temperature.

Temporal stability under controlled temperature

The signal variation over a 24-h period at constant temperature was also tested to evaluate the possible effects of photobleaching due to light pulses on the sensor. For this, the bath temperature was set at constant temperature of 15 ± 0.01 °C and measurements were made every 5 min for 24 h, corresponding to 288 light pulses. The measurements of sterilized double-distilled water were not expected to significantly change during incubation.

Sensitivity of microbial planktonic respiration to environmental stress

We evaluated the potential of optodes to detect differences in the respiration of microbial planktonic assemblages exposed to five different environmental stressors: increasing CO_2 (ΔCO_2), temperature (ΔT), strengthened thermal stratification, nutrient enrichment, and UVR.

Experimental setup and stressors tested in the ecosystems

The common experimental set up for all experiments was a complete factorial design with 'stressor' and 'control' treatments (each performed in triplicate). Microcosms, made of polyethylene bags (which transmitted 90% of the photosynthetic active radiation, 75% of the UVA, and 60% of the UVB; Plásticos Andalucía, Spain), were filled with the appropriate volume of water sample (5–20 L) to make unbiased measurements in relation to organism size and incubation time. The experimental water was previously filtered through 45 µm to remove zooplankton. Incubations were performed for 5 days in situ in the upper layers (~0.5 m depth) for the experiments in lakes, or in tanks (on deck of an oceanographic ship) to avoid possible damage due to waves. The tanks were painted black and surrounding water was used for continual renewal to preserve the light and temperature conditions of the sea during the incubations (as in Carrillo et al. 2015b). After the experimental incubations, water from each treatment (with or without the stressor) was used to completely fill (without bubbles) 25-mL quartz-glass flasks used for O_2 measurements, which were placed in darkness in a bath connected to a continuous-flow water system from the ecosystem, to keep in situ temperature and its natural variation during the O_2 measurements. For the temperature experiment, the flasks were placed in a thermo-regulated baths (see below). After 20 min of thermal equilibration, the O_2 measurements were made at time intervals over the incubation period in the bath (8 to 48 h, depending on the experiment and ecosystem), gathering sufficient data (at least 6–12 measurements per replicate) to ensure a linear decrease of oxygen concentration vs. time ($R^2 \geq 0.9$ for most of the regressions, although a few regressions with $R^2 \sim 0.7$ linked to the experiments of strengthened stratification and UVR were also included as they did not unduly enlarge the standard deviations; see "Discussion" below).

The experiment of ΔCO_2 was conducted with water taken from Cabo de Gata Natural Park, a markedly oligotrophic area in the eastern Alborán Sea (southern Spain; Fig. 1; Table 2). Three microcosms were air-bubbled with current atmospheric CO_2 concentration (380 ppm; control), and another three microcosms were bubbled with air enriched in CO_2 (~1050 ppm, stress treatment), as in previous studies (Riebesell et al. 2007).

The experiment of ΔT also used water from Cabo de Gata, but in this case, the O_2 measurements were performed in thermo-regulated baths at 15 °C (control treatment) and 20 °C (stress treatment).

The experiments of strengthened stratification were made in the lakes Enol (Picos de Europa National Park, northern Spain), La Conceja (Ruidera Lakes Natural Park, central Spain), and Las Yeguas and La Caldera (Sierra Nevada National Park, southern Spain; Fig. 1; Table 2). In these experiments a microbial planktonic community was enclosed directly in the 25-mL quartz-glass flasks which were split into two trays. The first tray (mixed control) simulated a mixing regime in the water column, and was moved up and down from surface to 3 m deep at 25 cm min^{-1}, as reported by Helbling et al. (2013). The second tray (static treatment) remained static at a depth of between 1.3 and 1.4 m (according to lake k_{PAR}) to receive the mean irradiance of the upper 3 m of the water column (Helbling et al. 2013), simulating the stratification regime in the water column. Incubation lasted 4 h with a total of ten up-down cycles.

The experiments of nutrient enrichment (intended to mimic atmospheric nutrient loads) were conducted in the lakes Enol (Picos de Europa National Park, northern Spain), La Conceja (Ruidera Lakes Natural Park, central Spain) and La Caldera (Sierra Nevada National Park,

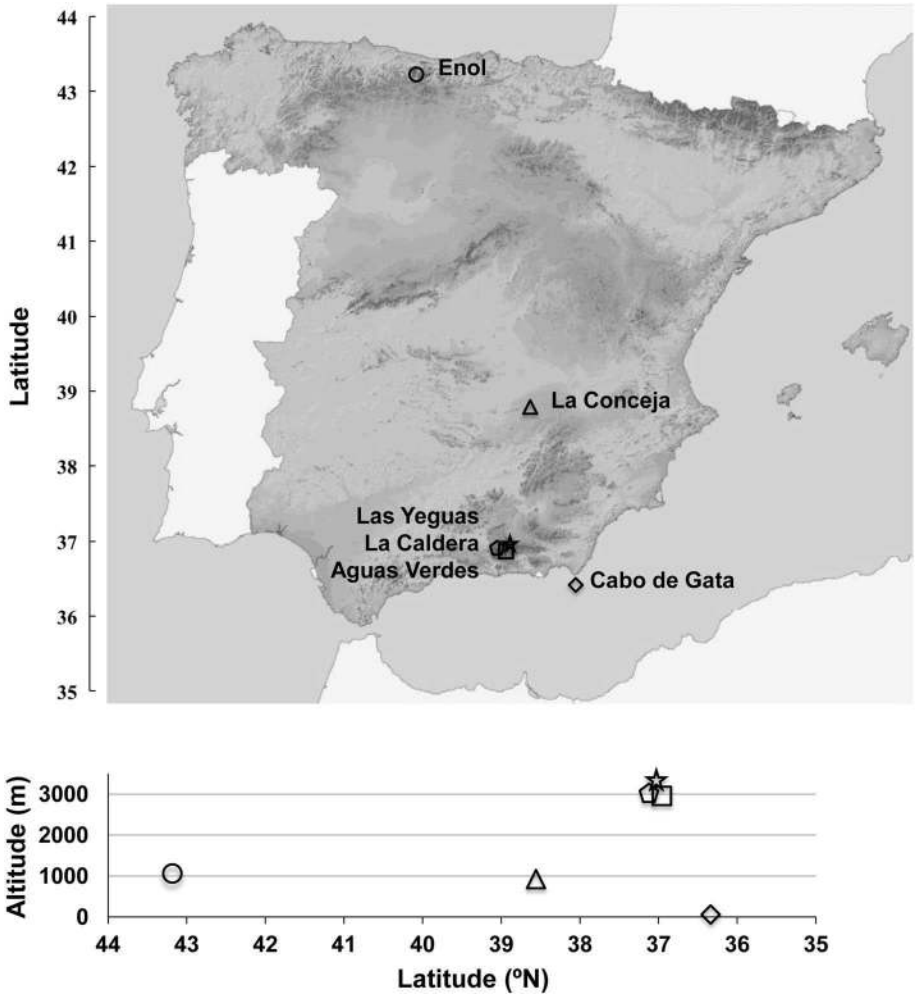

Fig. 1 Map of study sites and their altitudinal and latitudinal location

southern Spain; Fig. 1; Table 2). For each experiment, P was added as Na_2HPO_4 to three microcosms (P-enriched treatments) at a final concentration of 30 µg P L^{-1} to simulate natural nutrient inputs similar to those found by Morales-Baquero et al. (2006) for wind deposition by Saharan dust in southern Spain. To avoid N depletion, NO_3NH_4 was added to maintain a N:P molar ratio of 30; the other three microcosms without nutrient enrichment served as control.

Finally, the UVR experiments were conducted in the lakes Enol (Picos de Europa National Park, northern Spain), La Conceja (Ruidera Lakes Natural Park, central Spain), and Las Yeguas, La Caldera, and Aguas Verdes (Sierra Nevada National Park, southern Spain), as well as in the marine ecosystem (Cabo de Gata; Fig. 1; Table 2). Microcosms were exposed to different light conditions: UVR (UVR + PAR), and PAR (control). For the PAR treatment, enclosures were covered with UVR-filter foil (UV-Process Supply Inc., IL, USA) which transmits 85% of PAR but blocks UVR below 390 nm.

We performed a physical and chemical characterization of the ecosystems where the experiments were conducted. Vertical diffuse-attenuation coefficients (k_d) for the UVR (305, 320, 380 nm) and visible [photosynthetic active radiation (PAR), 400–700 nm] spectral regions were determined from water-column profiles of downwelling irradiance measured with a submersible BIC Compact 4-Channel Radiometer (Biospherical Instruments Inc. CA, USA) at noon. Temperature (T) was measured in the epilimnion with a YSI MPS-556 probe (YSI Incorporated, OH, USA). For soluble reactive phosphorus (SRP) and nitrate (NO_3^-) measurements, samples were filtered through GF/F Whatman filters before the determinations. SRP was measured following the acid molybdate technique, and NO_3^- following the UV spectrophotometric screening (APHA 1992). For total phosphorus (TP) and total nitrogen (TN) measurements, samples were first digested with potassium persulphate at 120 °C for 30 min and then analysed as SRP or NO_3^-, respectively, as described above.

Table 2 Characterization of the ecosystems studied

	Enol	La Conceja	Las Yeguas	La Caldera	Aguas Verdes	Cabo de Gata
Latitude	43°16′20″N	38°55′33″N	37°03′18″N	37°03′17″N	37°02′54″N	36°33′5″N
Longitude	4°59′29″W	2°48′51″W	3°22′34″W	3°19′45″W	3°22′06″W	2°16′8″W
Altitude (m)	1075	850	2850	3050	3050	0
k_{305} (m^{-1})	2.43	4.64	0.62	0.34	2.00	0.27
k_{320} (m^{-1})	2.26	2.61	0.53	0.27	1.78	0.25
k_{380} (m^{-1})	1.26	0.44	0.31	0.16	0.83	0.10
k_{PAR} (m^{-1})	0.43	0.29	0.21	0.18	0.47	0.06
T (°C)	12.5	20.1	14.5	13.5	12.5	21.1
DOC (mg C L^{-1})	2.24 ± 0.45	1.67 ± 0.07	1.44 ± 0.05	0.86 ± 0.19	1.16 ± 0.02	1.72 ± 0.23
TP (µg P L^{-1})	3.39 ± 2.17	4.74 ± 0.44	4.79 ± 0.82	3.36 ± 1.83	6.42 ± 1.22	46.5 ± 13.9
SRP (µg P L^{-1})	2.28 ± 0.26	2.02 ± 0.04	0.96 ± 0.41	0.73 ± 0.26	1.38 ± 0.29	3.10 ± 1.86
TN (mg N L^{-1})	0.38 ± 0.01	14.91 ± 0.04	0.13 ± 0.03	0.32 ± 0.10	0.10 ± 0.01	0.06 ± 0.01
NO_3^- (mg N L^{-1})	0.39 ± 0.01	17.94 ± 2.23	0.15 ± 0.02	0.20 ± 0.10	0.02 ± 0.01	0.01 ± 0.00
Chl a (µg L^{-1})	4.00 ± 1.29	1.30 ± 0.03	2.34 ± 0.34	1.75 ± 0.02	1.25 ± 0.14	0.99 ± 0.23
AA (x10^3cell mL^{-1})	3.46 ± 0.20	1.5 ± 0.5	5.0 ± 0.6	20.4 ± 3.7	0.5 ± 0.1	7.9 ± 1.49
BA (x10^6cell mL^{-1})	2.10 ± 0.79	0.28 ± 0.03	3.4 ± 0.9	1.4 ± 0.3	2.4 ± 0.8	2.04 ± 0.7
Stressors studied	SS, NE, UVR	SS, NE, UVR	SS, UVR	SS, NE, UVR	UVR	ΔCO_2, ΔT, UVR

Mean values of water column (±SD) for different variables from the ecosystems studied

K_{305}, K_{320}, K_{380}, and K_{PAR} vertical diffuse-attenuation coefficients for different wavelengths, *T* temperature, *DOC* dissolved organic carbon, *TP* total phosphorus, *SRP* soluble reactive phosphorus, *TN* total nitrogen, *NO_3^-* nitrate, *Chl a* chlorophyll *a, AA* algal abundance, *BA* bacterial abundance; *ΔCO_2* CO_2 increase, *ΔT* temperature increase, *SS* strengthened stratification, *NE* nutrient enrichment, *UVR* UV-radiation

DOC values were determined by filtering the samples through pre-combusted (2 h at 500 °C) glass-fibre filters (Whatman GF/F) and acidifying them with HCl. Samples were then measured in a total organic carbon analyser (TOC-V CSH/CSN Shimadzu).

Chlorophyll *a* (Chl *a*) was quantified fluorimetrically by concentrating cells by filtration (<100 mmHg in Whatman GF/F 25 mm diameter), grinding the glass-fibre filters and extracting pigments in 90% acetone at 4 °C for 24 h. Chl *a* fluorescence was transformed to Chl a concentrations from a calibration curve drawn from a Chl *a* standard (spinach Chl *a*, Sigma).

Samples for identification and enumeration of phytoplankton were preserved in 250-mL brown glass bottles containing Lugol alkaline solution (1% vol vol^{-1}). A volume of 50 mL was allowed to settle for 48 h in Utermöhl chambers (Hydro-Bios GmbH, Germany) and species were counted and identified using an inverted microscope (Leitz Fluovert FS, Leica, Wetzlar, Germany). Bacterial abundance (BA) was determined by a flow-cytometry technique (FACScanto II, Becton Dickinson Biosciences, Oxford, UK) from water samples (three replicates and two controls for each stratum considered of the water column) fixed with 1% paraformaldehyde and stained with SYBR Green I DNA stain (Sigma–Aldrich) to a 1:5000 final dilution of initial stock. Stained microbial cells were discriminated on bivariate plots of particle side scatter vs. green fluorescence. Yellow-green 1-µm beads (Fluoresbrite Microparticles, Polysciences, Warrington, PA, USA) were used as an internal standard of particle concentration and fluorescence (Zubkov et al. 2007; Dorado-García et al. 2014).

Data analysis

The dependence of O_2 measurements on abiotic processes (i.e., non-respiratory) linked to temperature variations and time span were assessed by non-linear regression analysis of O_2 concentration vs. temperature, and vs. time, respectively. Then, respiration rates determined from the stressor experiments were corrected for non-respiratory hysteresis by subtracting from each O_2 datum the variation of O_2 due to temperature shift (*f*) and time span (*f′*) according to the expression which generates the derivative of a function:

$$O_2 \text{ corrected} = O_2 \text{ measured} - [f(T + h) - f(T)] - [f'(t + h') - f'(t)], \quad (1)$$

where *f* is the fitted function (2) or (3) (see "Results") for temperature shift, *f′* is the fitted function (4) (see "Results") for time span, T is the temperature value, t the time span for each O_2 measurement, and h and h′ are the variation intervals of T and t, respectively, with respect to the previous O_2

measurement. Then, the corrected O_2 data served to calculate the corrected respiration rates.

A one-way ANOVA was used to evaluate differences in microbial respiration rates between corrected and uncorrected measurements, as well as between the treatments of each stressor in each ecosystem, after testing normality and homoscedasticity assumptions.

Results

Optimizing the optode laboratory setup

Effect of temperature fluctuation on O_2 measurements in closed flasks

In the thermal-equilibration experiment, the water at the same temperature as the bath showed no variations in the O_2 measurements, as concentration (µM) or as oxygen saturation (%), whereas the water exposed to 5 °C warmer showed a decrease of ca. 10 µM (3.2% oxygen saturation) over the first 20-min interval (during the thermal equilibration), but without changes afterwards (Fig. 2).

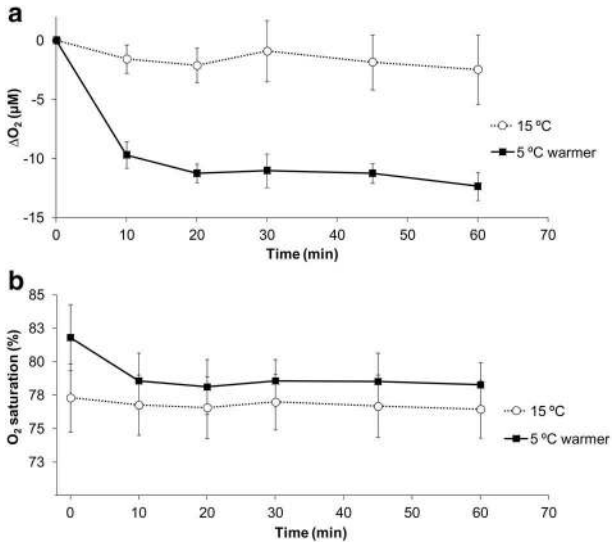

Fig. 2 Time course of O_2 variation (sterilized double-distilled water) of samples filled with sterilized double-distilled water at a temperature equal to that of the bath (15 °C) and subjected to a bath temperature 5 °C warmer, measured with new sensor spots and expressed as **a** O_2 concentration (µM), **b** O_2 saturation (%)

Figure 3a displays the oxygen concentration variations during the warming and cooling cycles. Because flasks were hermetically sealed and no visible bubbles formed inside the flasks during the measurements or after the experiments ended (O_2 concentration: 266 µM at 5 °C, ~72.6% saturation), the variation found in the O_2 measurements was presumably not due to real changes in dissolved oxygen. At the beginning of any temperature ramp, an acute oxygen variation was found, after which oxygen dynamics remained linear until the next temperature ramp started (Fig. 3a). Different non-linear relationships were found for O_2 concentration vs. temperature (T) increase or decrease ramps, respectively, and the best fit to the data were cubic equations with different parameters for each temperature ramp (Fig. 3b):

$$\text{Temperature increase: } \left[O_2\right] = 317.4875 - 15.4386(T) + 0.7632\,(T)^2 - 0.0132(T)^3, \quad (2)$$

$$\text{Temperature decrease: } \left[O_2\right] = 292.8275 - 7.3013(T) + 0.3722\,(T)^2 - 0.0086(T)^3. \quad (3)$$

For a whole temperature cycle (e.g. from 5 to 25 °C and back to 5 °C), the ratio between oxygen concentration (µM) and temperature (°C) remained constant at the end of each cycle (e.g. $[O_2]/T \approx 52.8$ at 5 °C), indicating the convergence of the measurements at the turning points.

Temporal stability under controlled temperature

The mean values of O_2 concentration measured in sterile water in closed flasks during the 24-h incubation at constant temperature showed a non-linear relationship with time (t), and the best fit to the data was a smoothed quasi-logistic equation, as the O_2 concentration followed an upward trend to a plateau, with a maximum variation <2.5 µM (<1.5%; Fig. 4):

$$\left[O_2\right] = 3.7305[1 + \exp(-0.1564t)]^{-1} + 164.75. \quad (4)$$

Sensitivity of microbial planktonic respiration to environmental stress

No significant differences between corrected and uncorrected respiration rates were found for any of the experiments ($p > 0.05$; Fig. 5), where the absolute variation of in situ temperature was below 3 °C. By contrast, all stressors exerted significant effects on the respiration of the microbial planktonic community. The metabolic response of the microbial planktonic assemblage under different stressors in the ecosystems studied is shown in Fig. 5 and Online Resource 1.

CO_2 addition (Cabo de Gata) significantly increased respiration by ca. 88% ($p < 0.05$; Fig. 5a; Online Resource 1). In the temperature experiment, a rise of 5 °C boosted

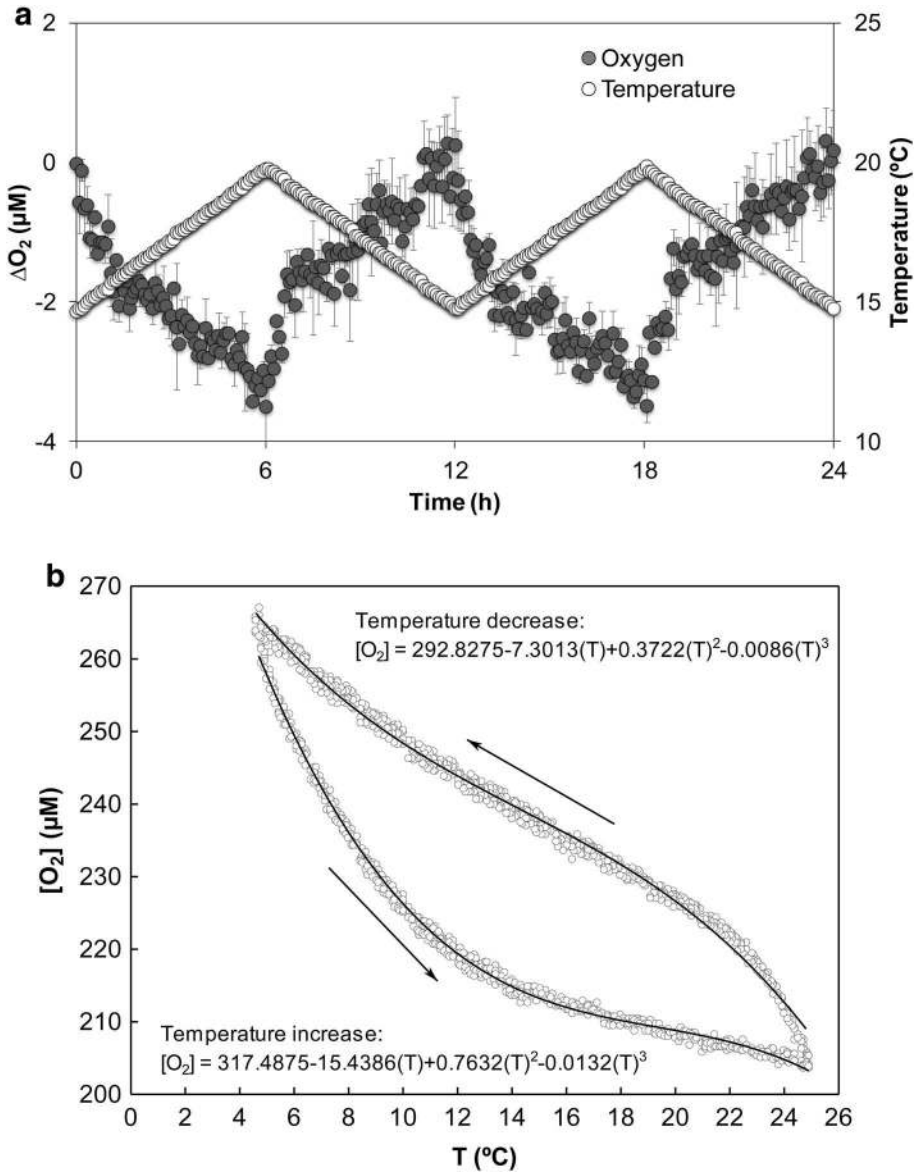

Fig. 3 Oxygen changes with temperature. **a** Temperature cycles of 5 °C (from 15 to 20 °C and back every 12 h for 24 h) in double-distilled water and associated O_2 concentration changes. *Dark spots* represent oxygen (μM), *white spots* represent temperature (°C). **b** Non-linear relationships between O_2 concentration (μM) and temperature (°C) in the temperature cycles of 20 °C (from 5 to 25 °C every 12 h for 24 h)

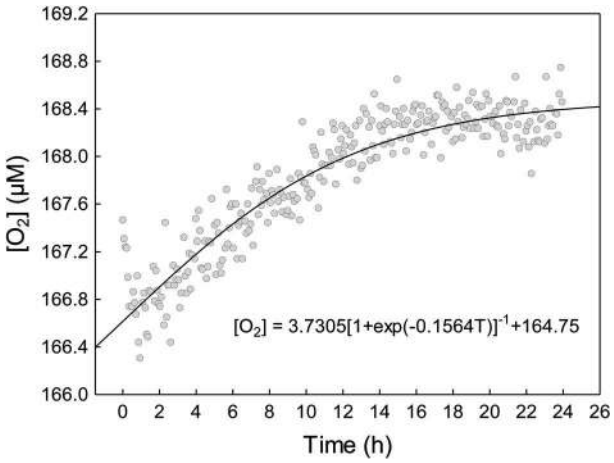

Fig. 4 Temporal stability test. Time-course of O_2 concentration (μM) in double-distilled water at 15 °C for 24 h

the respiration rates by >214% ($p < 0.01$; Fig. 5b; Online Resource 1).

Strengthened stratification affected respiration rates depending on the lake considered (Fig. 5c; Online Resource 1). In the mid-altitude lakes, Enol and La Conceja, effects on respiration rates were not significant ($p > 0.5$ for both lakes). By contrast, in the high-mountain lakes of Sierra Nevada, respiration rates were >2.6-fold (Las Yeguas) or ca. twofold (La Caldera) higher under stratified than under mixed conditions ($p < 0.05$ for both lakes).

Nutrient enrichment augmented respiration rates in Enol, La Conceja, and La Caldera lakes (Fig. 5d; Online Resource 1). Enol was more sensitive than La Conceja or La Caldera, because values increased by >120% ($p < 0.001$), compared with an increase of 60–75% ($p < 0.05$) in La Conceja and La Caldera, after nutrient enrichment.

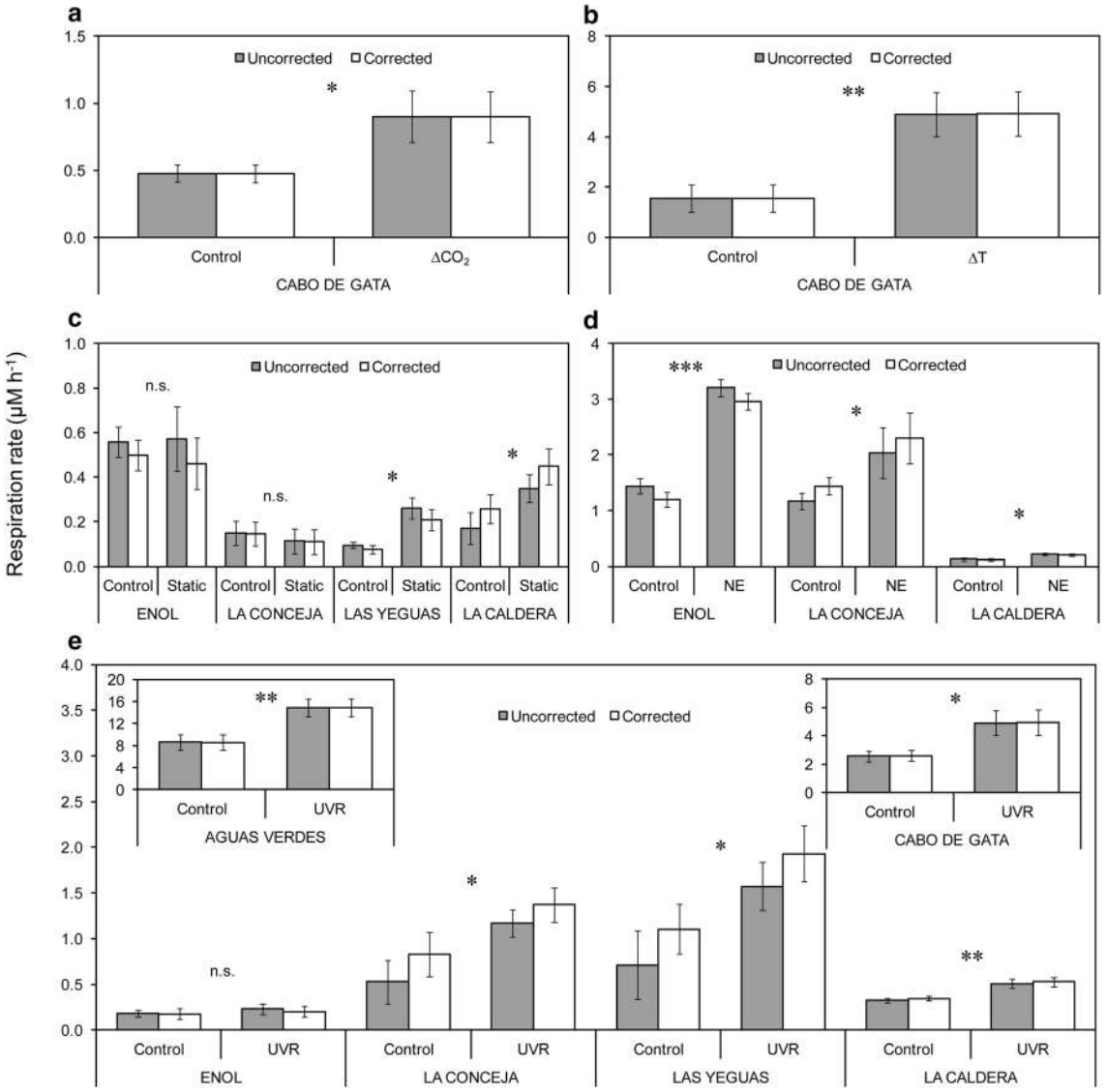

Fig. 5 Microbial-community respiration (μM h^{-1}) in the ecosystems studied under different stressors: **a** ΔCO_2 (control: 380 ppm CO_2; ΔCO_2: 1050 ppm CO_2), **b** ΔT (control: 15 °C; ΔT: 20 °C), **c** strengthened stratification (control: mixed; static: stratified), **d** nutrient enrichment (control: ambient nutrient concentration; *NE* nutrient enriched), **e** UVR (control: PAR, UVR: UVB + UVA + PAR). *Asterisks* represent significant differences in microbial respiration under control and stress conditions: *p < 0.05; **p < 0.01; **p < 0.001; *ns* not significant

UVR exposure resulted in higher respiration rates in five of the six ecosystems tested (Fig. 5e; Online Resource 1). In Enol, UVR did not affect to respiration rates ($p > 0.05$), whereas values significantly increased in La Conceja (>65%; $p < 0.05$), Las Yeguas (>75%; $p < 0.05$), La Caldera (>51%; $p < 0.01$), Aguas Verdes (>72%; $p < 0.01$), and Cabo de Gata (>90%; $p < 0.05$) under UVR.

Discussion

Our results show that O_2 optodes are useful tools to quantify the effects of environmental stress on the respiration of microbial planktonic assemblages inhabiting oligotrophic ecosystems (Fig. 5). This is supported by the high sensitivity and stability of optodes (Table 1), which were capable of accounting for differences in O_2 measurements in the oligotrophic ecosystems studied. However, optodes are not perfect, and temperature variations affect O_2 measurements. This work provides the mathematical procedure [general Eq. (1), linked to the Eqs. (2), (3), and (4)] to correct the O_2 measurements made in closed flasks of the non-respiratory hysteresis dependent on temperature variations and time span, hence removing the constraints of keeping the temperature constant during the measurements. This makes the optode methodology

practicable for further applications and environments (e.g., in situ measurements, filtered or unfiltered fresh- or salt-waters, etc.) enabling realistic measurements on samples subjected to natural or experimental ranges of temperature variability.

We evaluated how 5 °C of temperature change affected the oxygen measurements, which needed a thermal-equilibration time of at least 20 min until providing stable data (Fig. 2). Secondly, temperature cycles revealed that oxygen measurements are strongly affected by temperature changes. The conspicuous changes in the oxygen concentration with each temperature ramp (hysteresis) could be explained by a lag in the thermal equilibrium between the quartz-glass surface in contact with the surrounding water of the bath and the flask content, because temperature was measured within the bath, but outside the flask, i.e., separated from the sensor-spot optode by the flask wall. Thus, the time necessary to reach the thermal equilibrium depends on the material and thickness of the flask wall and volume. In addition, other abiotic processes affecting optode measurements, such as the temperature-dependence of the luminophore quantum yield, the oxygen permeability of the matrix to which it is attached, or even the oxygen permeability of the optical isolation layer of the sensor spots (Wang and Wolfbeis 2014), may also help explain these patterns. Therefore, the precise value of parameters of the given equations may not be universally applicable, but can easily be determined for different experimental settings (i.e., different types of flasks or optode brands). The possible photobleaching of the sensor was also assessed. Our results indicated that, with temperature kept constant, a modest stability was achieved throughout the 24-h period, with a maximum variation <1.5% (Fig. 4), this adding to findings by Warkentin et al. (2007) of a lesser variation (±0.2%) in their oxygen measurements at constant temperature (20 °C) for 30 min. Probably, the longer incubation period (24 h) in our experiment generated the greater variation found. Nevertheless, this temporal variation was also accounted for in the mathematical procedure (Eqs. 1, 4) to correct the O_2 measurements in the field experiments.

The absence of significant differences between corrected and uncorrected respiration rates for all experiments (Fig. 5) indicated that optodes directly provided realistic and reliable measurements of microbial planktonic respiration in oligotrophic ecosystems when the samples were subjected to slight temperature variations (<3 °C). Temperature correction is necessary to make reliable measurements of microbial respiration rates in applications or environments subjected to a faster or wider range of temperature variation than that of the oligotrophic sites studied. Examples of these applications may come from laboratory experiments with induced quick or pronounced temperature shifts to in situ profiling through the water column of aquatic ecosystems subjected to sharp thermal gradients (strong thermal stratification, cold-water upwelling, etc.; e.g., Uchida et al. 2008).

The stressors assayed were those that may differentially affect each type of ecosystem (i.e., ΔCO_2, ΔTemperature, and UVR in marine environments; strengthened stratification, nutrient enrichment, and UVR in freshwater ecosystems), in agreement with previous reports (Stocker et al. 2013). In the coastal ecosystem, higher CO_2 levels significantly increased microbial respiration by ca. 88%. This result might be supported by metabolically expensive mechanisms such as greater transport of ions or CO_2 from the cells, as suggested by Fabry et al. (2008), which is interpreted as a mechanism to deal with the stress generated by the higher $[H^+]$ (lower pH) that has the potential to affect the metabolic rates of algae (Hurd et al. 2009) and other organisms. Closely related to the increase in atmospheric CO_2 is the rise in global temperature. The sharp increase in microbial respiration at higher temperatures (>threefold, $Q_{10}=9.9$) is consistent with the temperature dependence of metabolic activity reported in many studies that evaluate the warming effects on microbial food webs of the marine environment (López-Urrutia and Morán 2007; Vázquez-Domínguez et al. 2007; Sarmento et al. 2010). In a future scenario of global change, a higher temperature might cause shoaling of the epilimnetic layer of lakes, increasing the stratification of the water column and having consequences for planktonic metabolism, such as exposure to higher UVR fluxes and decreased nutrient inputs from deep waters (Hiriart-Baer and Smith 2005; Häder et al. 2011). Previous studies have shown contrasting results of the effects of mixing conditions on primary production (Neale et al. 1998; Helbling et al. 2003, 2013) and bacterial production (Durán et al. 2014; Carrillo et al. 2015a), and this is the first study to evaluate the effects of fixed vs. fluctuating irradiance on whole microbial planktonic respiration. It can be argued that oxygen measurements in darkness may not provide information on the short-term effects of those stressors (fluctuating irradiance, UVR) that were physically removed during the long incubations in darkness. In fact, dark respiration is not constant over time but is often highest just after light exposure and can decline with the pool of respirable organic compounds built up during the previous light period. Because this dynamic is not reflected by the given constant respiration rates, it may contribute to the relative weakness of some regressions of oxygen vs. time (those with $R^2<0.9$) linked to these experiments (strengthened stratification and UVR). Although we cannot rule out some amelioration of the impact of these stressors on respiration rates, our results showing significant effects suggest that their impact lasted—at least partially—over the dark incubations. Recent studies have also shown significant effects of stratification, UVR and their interactions

on respiration rates following a similar approach (Carrillo et al. 2015a, b). Our results reveal that static incubation (strengthened stratification) augmented respiration rates by more than 100% compared with mixing conditions in high-mountain lakes, which receive strong UVR irradiance due to altitude and are more transparent to UVR, whereas in the mid-altitude low-UVR-transparent lakes no significant effect was found. In the high-mountain lakes, the increased planktonic respiration rates under static conditions could provide sufficient energy to support the repair of photodamage by UVR under conditions of longer and more intense UVR-exposure. By contrast, under mixing conditions (fluctuating UVR regime), the photodamage inflicted in near-surface waters can be mitigated and reversed by photorepair (metabolically less costly) mechanisms in deeper waters with attenuated UVR irradiance and more optimal (UVA + PAR):UVB ratio for photoreactivation (Kaiser and Herndl 1997; Sinha and Häder 2002; Bertoni et al. 2011). In the mid-altitude lakes, the lesser transparency to UVR could minimize the differences between the two conditions because of the photo-protective role of DOM (Williamson and Rose 2010; Williamson et al. 2016; but see; Helbling et al. 2013; Carrillo et al. 2015a).

The nutrient enrichment in oligotrophic waters stimulated metabolic activity. The significant rise found in community respiration after nutrient enrichment was >50% in all the ecosystems studied, indicating that P-enrichment boosted metabolic activity, probably as the result of enhanced nutrient uptake and growth, but also increased metabolic expenditure, of these assemblages. Finally, our results are consistent with higher respiration rates found under UVR exposure in epilimnetic layers of high-mountain lakes, mid-altitude lakes, and coastal marine ecosystems, where a negative impact of UVR appears to be the general response (Harrison and Smith 2009; Häder et al. 2011).

Previous studies have shown that respiration could be used as a indicator (e.g. Garnier and Billen 2007; Nwachukwu and Pulford 2011; Oyonarte et al. 2012). However, most of these studies were performed in environments other than oligotrophic aquatic ecosystems, where the use of high-resolution techniques for measuring O_2 dynamics is needed. O_2 optodes proved to have sufficient sensitivity to resolve the effects of different global-change stressors on microbial planktonic respiration in oligotrophic ecosystems. Therefore, we propose that microbial planktonic respiration measured with O_2 optodes may be used as a potential indicator of environmental stress also (and especially) in oligotrophic environments. Microbial respiration may be applied as an early alarm system in water bodies subjected to stress risk, although interpretation of the results will require data under different environmental conditions and it will also depend on specific ecosystem characteristics.

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