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Development of a Handheld Submersible Chemiluminescent Sensor: Quantification of Superoxide at Coral Surfaces

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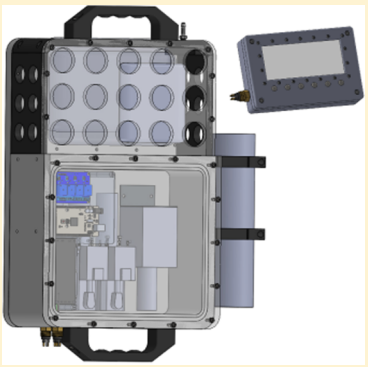
Supporting Information

ABSTRACT: Reactive oxygen species (ROS) are produced via various photochemical, abiotic, and biological pathways. The low concentration and short lifetime of the ROS superoxide ($O_2^{\bullet-}$) make it challenging to measure in natural systems. Here, we designed, developed, and validated a DIver-operated Submersible Chemiluminescent sensOr (DISCO), the first handheld submersible chemiluminescent sensor. The fluidic system inside DISCO is controlled by two high-precision pumps that introduce sample water and analytical reagents into a mixing cell. The resultant chemiluminescent signal is quantified by a photomultiplier tube, recorded by a miniature onboard computer and monitored in real time via a handheld underwater LED interface. Components are contained within a pressure-bearing housing (max depth 30 m), and an external battery pack supplies power. Laboratory calibrations with filtered seawater verified instrument stability and precision. Field deployment in Cuban coral reefs quantified background seawater-normalized extracellular superoxide concentrations near coral surfaces (0−173 nM) that varied distinctly with coral species. Observations were consistent with previous similar measurements from aquaria and shallow reefs using a standard benchtop system. In situ quantification of superoxide associated with corals was enabled by DISCO, demonstrating the potential application to other shallow water ecosystems and chemical species.



1. INTRODUCTION

Reactive chemical species, of both chemical and biological origins, are emerging as important compounds in the biogeochemistry and health of the ocean.[1] Due to their rapid production and consumption, these reactive compounds are short-lived and typically exist in low concentration; yet, they often may have a disproportionately large influence on the surrounding geochemical and biological environment and local redox chemistry.[2,3] One such reactive molecule is superoxide ($O_2^{\bullet-}$), a reactive oxygen species (ROS) that forms via the univalent reduction of molecular oxygen (O_2), that can be further reduced to hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$)[3,4]

$$O_2 + e^- \rightarrow O_2^{\bullet-} \quad (1)$$

$$O_2^{\bullet-} + 2H^+ + e^- \rightarrow H_2O_2 \quad (2)$$

$$H_2O_2 + e^- \rightarrow OH^- + HO^{\bullet} \quad (3)$$

$$HO^{\bullet} + e^- \rightarrow OH^- \quad (4)$$

These reactions represent a cascade of ROS dynamics with high reactivity toward a wide range of organic and inorganic compounds. Reactive oxygen species can be removed through various pathways including reactions with organic matter, metals, and antioxidant enzymes such as superoxide dismutase (SOD), catalase, and peroxidase.[12,28−31] Superoxide, specifically, is a radical species, exhibiting a half-life in seawater ranging from seconds to minutes,[28−31] depending on the environmental conditions (i.e., production and decay mechanisms, temperature, sunlight, metals, organic matter, etc.).

Extracellular production of superoxide has been observed in a wide diversity of organisms including fungi, phytoplankton, and bacteria.[5−13] At circumneutral pH, the radical anion superoxide ($O_2^{\bullet-}$) dominates ($HO_2^{\bullet}/O_2^{\bullet-}$; $pK_a = 4.8$),[2] yet is short-lived (μs) with limited diffusive distances (~100 s per nm). Superoxide is not membrane-permeable,[14] and it has been demonstrated that superoxide measured extracellularly is produced outside the cell.[11,15] Despite the perception that ROS are stress molecules, there is mounting evidence that both intracellular and extracellular superoxide play essential physiological roles within microorganisms, including cell

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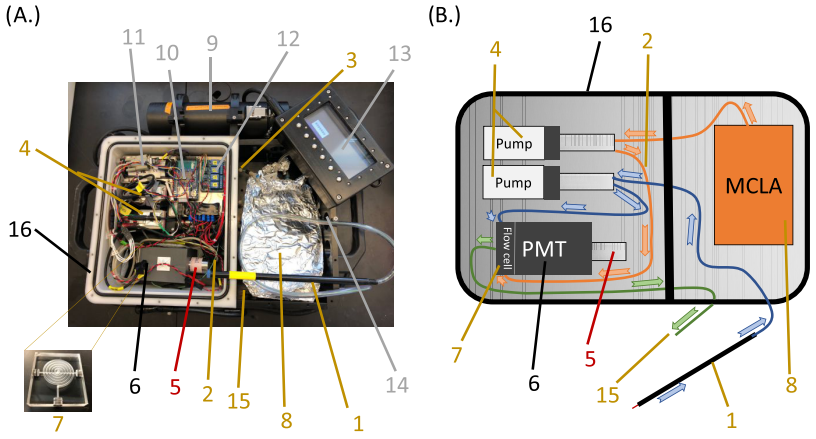

Figure 1. Picture (A) and illustration (B) of DISCO. Components are numbered with descriptions and details listed in Table 1. The colors of the labels correspond to the classification of the part (fluidics in gold, electronics in gray, sensor in red, and housing in black). The colors of the fluid flow pathways correspond to the analyte (blue), reagent (orange), and waste (green).

Table 1. Components of DISCO Listed with the Brand, Model, Details About the Part, and Function It Serves in the Instrument

	name	brand	model	details	function
1[a]	sampling wand	Made in-house	NA	Delrin tube with 1/32″ ID inlet tubing	intake of analyte fluids
2	tubing	IDEX Health and Science	PEEK Tubing	0.159 cm OD, 0.076 cm ID	transports fluids from the source through pumps and flow cell
3	flangeless ferrule fittings	IDEX Health and Science	P-235X	PEEK, 1/4-28 thread with a flat bottom	connect the tubing to ports of dive housing, pumps, and flow cell
4	microfluidic pumps	Global FIA	milliGAT high flow	chemically inert: PTFE, PAEK, Valcon H2, Viton, sapphire, ceramic	pumps analyte fluid and chemical reagents
5	photomultiplier tube (PMT)	Hamamatsu Photonics	Photon counting head H9319	2.54 cm diameter head-on PMT	measures chemiluminescent signal
6	pressure-baring housing	Made in-house	NA	pressure-bearing plastic housing	holds PMT against the faceplate of the flow cell to make chemiluminescent reading; blocks light
7	flow cell	Hellma Analytics	Glass Spiral Flow Cell	glass	mixes the analyte and reagent for PMT to measure
8	Labtainer bag	Thermo	BioProcess Container	plastic, 500 mL (MCLA) and 50 mL (SOD)	holds chemical reagents
9	battery	Light Monkey	15aH Conversion	24 VDC Li-ion	powers all units within the instrument
10	Arduino	Arduino	Mega 2560	256 KB, 16 MHz	microcontroller, stores data, and offloads to computer
11	power converter	Vicor Corporation	VI-200	MOD DC/DC 5 V 50 W	distributes instrument power
12	relay module	Songle	SRD-05VDC-SL-C	4 channel, 5 V, switch 120−240 V	switches pumps on and off
13	LED interface	Nextion	IM150416007	LED touch screen inside pressure-bearing case	displays data real time and allows for the control of pumps in situ
14	HOBO logger	Onset	MX2202	temp/light	records temperature and light intensity inside the instrument
15	waste outlet	IDEX Health and Science	PEEK Tubing	0.159 cm OD, 0.076 cm ID	discharges all fluids
16	dive housing	Made in-house	NA	Delrin housing, sealed watertight	protects electronics and mechanical parts against water and pressure

[a]The numbers are correlated with Figure 1, and the colors in Figure 1 correspond to the classification of the part (fluidics in gold, electronics in gray, sensor in red, and housing in black).

defense, micronutrient acquisition, cell signaling, and growth stimulation.[16−21]

The production of ROS, particularly superoxide and hydrogen peroxide, has also been implicated in both beneficial and detrimental effects to corals.[17,22−25] For instance, light- and heat-induced stress has been implicated in the overproduction of intracellular ROS within the endosymbiotic algae *Symbiodinium*, leading to oxidative stress and ultimately apoptosis (cell death) of the coral host.[26,27] In contrast, extracellular superoxide produced by the coral host has been linked to increased coral thermotolerance, prey acquisition, and protection against pathogens (e.g., *Vibrio shiloi*) and diseases (e.g., white band).[17,22,24,25] Further, recently, extracellular superoxide levels were found to vary widely with coral species, with a potential connection to coral bleaching resistance.[15] Thus, ROS clearly play important, yet currently unresolved, roles in coral function and health, pointing to a need for direct measurements of ROS within corals and reef ecosystems.

Quantifying extracellular ROS, however, is complicated by its reactivity and short lifetime. Superoxide, specifically, has a short lifetime (<1 min) within marine systems, precluding the

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ability to collect waters from point sources (e.g., coral surfaces) for later laboratory analyses. Instead, measurements need to be made at the source of superoxide before superoxide has degraded (to hydrogen peroxide and ultimately water and/or molecular oxygen). Traditionally, superoxide measurements have been completed using a benchtop flow injection chemiluminescent approach (for instance, using the commercially available FeLume system, Waterville Analytical, Waterville, ME[32]).[11,12,15,29,33] The chemiluminescent quantification is based on the specific reaction between superoxide and a chemiluminescent reagent (such as methyl *Cypridina* luciferin analogue (MCLA)).[34] This analytical approach involves two separate fluid lines for the analyte and reagent that join in a spiral mixing cell. An adjacent photomultiplier tube makes continuous measurements of the resulting chemiluminescent signal. This flow injection chemiluminescent approach has been used to successfully measure superoxide concentrations associated with a wide range of marine bacteria and eukaryotes in laboratory cultures.[8,10,12,29,35,36] Additionally, previous in situ measurements on coral reefs were made by pumping sample water from the coral surface directly into an FeLume system mounted on a small boat.[15] In these measurements, waters must enter the instrument before superoxide degrades but allow time for the chemiluminescent probe to react with superoxide within the mixing cell (sample residence times within the tubing have varied from 15 to 70 s in the previous studies (see, for example, refs 30, 33)). Thus, this approach is restricted to measurements in shallow waters (1−2 m) and stable reef environments (i.e., low wave action). Clearly, to expand research on in situ superoxide concentrations associated with coral reefs and other ecosystems, a submersible chemiluminescent sensor is required.

Here, the first submersible instrument designed and developed to measure superoxide in situ is presented: the DIver-operated Submersible Chemiluminescent sensOr (DISCO). DISCO was designed based on the operating premise of the FeLume with modified components optimized for underwater operation. Here, we describe the components of DISCO, which consists of precise fluidic and detection systems contained in a submersible, pressure-bearing housing, with a SCUBA diver-operated handheld LED display. Here, we present our evaluation of the instrument precision and accuracy through laboratory tests and calibrations, show results from initial field testing leading to the first in situ measurements of coral-associated superoxide, and provide suggestions for future development and improvement.

2. MATERIALS AND METHODS

2.1. Instrument Design. The overall premise of DISCO is based on the FeLume benchtop instrument (Waterville Analytical, Waterville, ME[32]) with additional features and modifications to allow for in situ underwater operation. DISCO contains two chemically inert fluidic pumps (Global FIA) that control separate fluid lines for analyte fluid and reagents that converge in a spiral glass mixing cell (Hellma Analytics) (Figure 1 and Table 1). Fluidic system components are connected using PEEK tubing (1/16″ OD, 0.030″ ID; IDEX Health and Science). The analyte is taken in through a rigid Delrin sampling wand, which has a 1/32″ inner diameter at the tip. MCLA reagent enters from a 500 mL Labtainer bag (Thermo Scientific) that is mounted external to the instrument and connected to the fluid line. The light emitted by the chemiluminescent reaction of MCLA with superoxide is quantified using a photomultiplier tube (PMT; Hamamatsu Photonics) enclosed in opaque plastic. For this study, photon count data were reported every 1.1 s and collected over an integration time of 90 ms at a PMT voltage of 1000 mV. To make a measurement, the analyte and reagent are simultaneously pumped into the spiral mixing cell where the PMT measures the ensuing chemiluminescent signal of the specific reaction between the analyte fluid and reagent (Figure 1). The fluid then exits through a waste line.

A 24 VDC Li-ion battery (Light Monkey) supplies power underwater to operate the internal control system, data logging, and storage (using an Arduino), and a handheld LED display module (Nextion) is packaged for underwater use. All of the components, except for the reagent containers, are contained within a submersible, opaque, pressure-bearing, O-ring sealed Delrin housing secured with 18 screws.

DISCO is operated manually by a SCUBA diver using the handheld underwater LED module. The module is configured with four pressure-sensitive buttons having the following functions: (1) PMT power on/off, (2) collection integration time, (3) inlet analyte pump on/off, and (4) reagent pump on/off. Data is displayed in real time, including a discrete readout of chemiluminescence signal intensity (related to the digital voltage signal output from the PMT), as well as a graphical display of signal intensity over time. In the present configuration, flow rates for the analyte and reagent pumps are preconfigured before deployment.

2.2. Chemical Reagents. While DISCO is capable of diverse chemiluminescent measurements depending on the chemical probe employed, this initial study optimized the system for superoxide measurements. Superoxide concentrations within the analyte fluid were measured through the specific reaction between superoxide and methyl *Cypridina* luciferin analogue (MCLA, Santa Cruz Biotechnology), a widely used chemiluminescent probe with high specificity for superoxide.[8,10,11,15,28,33] The MCLA reagent (4 μM MCLA) was prepared according to the previous protocols,[11,15,28,33] buffered with MES[11,15,28] (0.1 M MES, pH = 6), and amended with 50 μM diethylenetriaminepentaacetic acid (DTPA)[11,15,28,33] to sequester trace metals that would otherwise react with superoxide and significantly shorten its lifetime.[37] The reagent mixture is kept on ice until immediately before the dive. The PMT measures the light generated by MCLA chemiluminescent reaction,[38] which is then converted into superoxide concentration via daily benchtop calibration curves.

Superoxide dismutase (SOD) is an enzyme that rapidly degrades superoxide to hydrogen peroxide and molecular oxygen.[39] The addition of SOD (0.8 U mL^{-1}, final) to the analyte fluid was used to confirm the presence of superoxide in these measurements. Stock solutions of SOD were made by dissolving 4 kU mL^{-1} in deionized water and kept frozen at −20 °C until use.

2.3. Laboratory Tests and Calibrations. The instrument was leak-tested and modified as necessary to ensure the proper functioning of the fluidics, measurement, and interface systems while submerged. System buoyancy was determined and adjusted as needed to near-neutral for diver deployments. Initial instrument testing was conducted off the Woods Hole Oceanographic Institution pier (MA) in 19−21 °C seawater.

The fluidic configuration was optimized to minimize the sample fluid travel time within the instrument before being measured. Of primary concern for measurement precision is

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Table 2. Geographic and Environmental Data About the Sample Sites Analyzed for in Situ Coral Measurements in Jardines de la Reina, Cuba, Collected Aboard the M/V Alucia

sample date	site	latitude (N)	longitude (W)	temp (°C)	avg. depth	total coral indiv. sampled	species of individuals sampled, number sampled of each species[a] listed in parentheses
20-Nov-17	GQ2	21°18′12.0″N	79°35′27.84″W		9	4	*Dcyl* (1), *Mcav* (2), *Ppor* (1)
5-Nov-17	GQ4	21°03′49.5″N	79°25′38.1″W	28	11	4	*Past* (4)
6-Nov-17	GQ5	20°57′55.44″N	79°12′16.38″W	30	12	3	*Dlab* (1), *Mcav* (1), *Past* (1)
7-Nov-17	GQ6	20°50′36.72″N	79°00′35.76″W	28	12	4	*Acer* (1), *Ofav* (1), *Past* (1), *Ppor* (1)
11-Nov-17	GQ11	20°40′22.56″N	78°45′11.22″W	28	9	6	*Mcav* (1), *Ofav* (2), *Past* (1), *Ppor* (2)
14-Nov-17	GQ16	20°45′34.2″N	78°50′44.1″W		14	6	*Mcav* (3), *Past* (3)

[a]The coral species correspond with *Acropora cervicornis* (*Acer*), *Dendrogyra cylindricus* (*Dcyl*), *Diploria labyrinthiformis* (*Dlab*), *Montastraea cavernosa* (*Mcav*), *Orbicella faveolata* (*Ofav*), *Porites astreoides* (*Past*), and *Porites porites* (*Ppor*).

the ability to deliver both the analyte and reagent at constant and uniform rates. To this end, the stability of pumping rates was evaluated in the lab over a range of flow rates (1−10 mL min^{-1}). High-precision tuning of flow rates was conducted gravimetrically and adjusted by modifying the piston stroke length through a benchtop serial interface. No further adjustments were necessary after this initial assessment.

Daily calibration curves were conducted to determine the calibration factor for converting chemiluminescence intensity to superoxide concentration, as detailed previously.[10,12] Briefly, a primary standard of superoxide was generated by dissolving potassium dioxide (KO_2) into a basic solution (0.3 N NaOH, 50−100 μM DTPA, pH = 12.5). Superoxide concentration of the primary standard was quantified by the difference in absorbance at 240 nm before and after SOD addition. Absorbance readings were converted to molar units using the molar absorptivity of superoxide (2183 L mol^{-1} cm^{-1} at 240 nm, pH 12.5, corrected for the absorption of hydrogen peroxide formed during decay).[2] A secondary standard was created by diluting a 6 μL primary standard in a 30 mL aged filtered seawater, which consisted of a 0.22 μM filtered seawater from Vineyard Sound, MA, amended with 75 μM DTPA and left in the dark (>12 h) to complex trace metals. Throughout the study, all aged filtered seawater is amended the same way. Due to the short lifetime of superoxide, each standard stock was prepared immediately before use, and the time was noted to account for superoxide decay in the secondary standard. Chemiluminescent measurements of superoxide standards were quantified by first measuring a baseline of aged filtered seawater until a steady-state signal was obtained (the coefficient of variance < ∼3.1%; typically 2−3 min) and then measuring the secondary superoxide standard. The primary standard absorbance was quantified simultaneously. After 2−3 min, SOD was added (3 μM) to the remaining secondary stock (∼15 mL, 0.8 U mL^{-1}, final) to confirm that superoxide was being measured. The chemiluminescent decay of the superoxide standard exhibited pseudo-first-order decay kinetics, yielding a log-linear distribution. Based on the model fit of the decay kinetics and concentration of the standard added, a calibration factor (in lumens pM^{-1}) was calculated for converting superoxide concentrations from chemiluminescence.

2.4. In Situ Assessment and Data Collection. The first in situ testing of DISCO took place during a cruise on the M/V Alucia to Jardines de la Reina, Cuba, (Figure S1 and Table 2), in November 2017. For each dive, 200 mL of the MCLA reagent and 100 μL of SOD were preloaded into 500 and 250 mL Labtainer bags, respectively, wrapped in foil to minimize photodegradation and kept chilled. Immediately before the dive, the reagent bag was connected to the instrument and the SOD bag was carried in a diver pocket.

The instrument was calibrated daily in the ship laboratory immediately prior to each dive ("sample station calibration") using the same MCLA reagent used during the dive. These sample station calibrations were conducted in the same way as described above for the laboratory calibrations with the exception that aged filtered seawater was collected from each sample station, filtered, and allowed to age. As the MCLA reaction kinetics are temperature-dependent, the temperature of the laboratory was maintained at a similar temperature to the waters in situ.

DISCO was assessed in situ first by measuring background reef water and then by measuring superoxide concentrations at the surface of various coral species. All in situ measurements took place within the first 20 min of a dive, and the chemiluminescent signal of superoxide was continuously measured. Superoxide concentrations within background reef water were measured using DISCO in a sandy patch near a reef with the intake held >1 m above the seafloor. DISCO was further tested in situ through measurements of extracellular superoxide associated with 27 colonies belonging to seven coral species (*Porites porites*, *Porites astreoides*, *Orbicella faveolata*, *Montastraea cavernosa*, *Acropora cervicornis*, *Diploria labyrinthiformis*, and *Dendrogyra cylindricus*). These species were observed at six different reef sites (Figure S1 and Table 2) ranging between 28 and 30 °C; each colony was roughly similar in size (∼20 cm diameter) and exhibited pigmentation that suggested healthy condition (i.e., not bleached).

In situ measurements of superoxide associated with corals began with a collection of background seawater >30 cm away from any coral to obtain a steady baseline (typically 7−10 min). The intake was then held <0.5 cm away from the coral surface and moved slowly along the coral surface at a steady rate to minimize the entrainment of background seawater. Data were acquired until a stable signal was achieved (∼1−2 min). Background seawater was then measured again to ensure a similar background seawater measurement as observed before the coral. After a group of corals were measured (3−5 colonies per 3 m^2 section), SOD was injected to confirm superoxide measurements. While it was intended to have SOD directly injected into the fluidic line, this proved to be difficult in this present design. Therefore, 30 mL of the sample seawater from near the coral was injected with a syringe into the SOD Labtainer bag (prefilled with 100 μL of SOD, 16U mL^{-1}, final) and connected to the intake to obtain an SOD baseline value for the signal.

One liter of background seawater was collected >30 cm away from the coral at a reef depth in dark bottles during the dive

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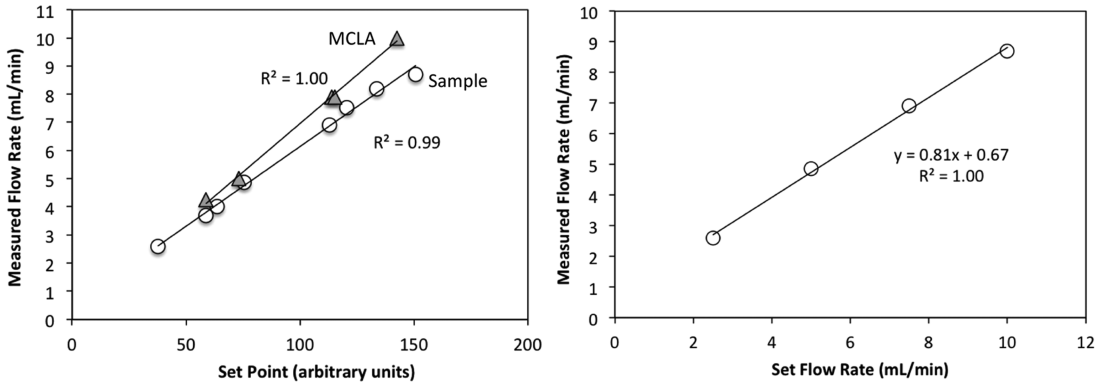

Figure 2. Flow rate variability for pumps in (left) isolation of DISCO and (right) assembled in DISCO. In isolation, both the MCLA (triangles) and sample (circles) pumps were tested. Fully assembled in DISCO, only the sample pump was tested. The *x*-axis represents the set point (left), which is in arbitrary units and the set flow rate (right) in mL/min. The *y*-axis on both is the measured flow rate (mL/min).

and filtered (0.22 μm) for use during lab analysis. Five hundred milliliters of this filtered background seawater was analyzed after every dive to test the instrument stability of measurements from the filtered seawater. The remaining 500 mL of the filtered background seawater was aged with 75 μM DTPA to make aged filtered seawater for calibrating the instrument the next day. Using site-specific aged filtered seawater, the daily calibration curves were conducted (as explained above) to determine a calibration factor specific to each site and then used to convert chemiluminescence to superoxide concentrations.

2.5. Processing Data. Measurements reported for each point of interest were expressed as an average plus or minus the standard deviation collected during these steady-state measurements. All in situ coral measurements were first corrected by subtracting the chemiluminescent signal from background seawater measured immediately before each coral (seawater-normalized superoxide concentrations), hereafter referred to as extracellular superoxide concentrations. Through this method, the steady-state concentrations of superoxide are measured, which represents a combination of simultaneous production and decay processes.

3. RESULTS AND DISCUSSION

DISCO represents the first diver-operated submersible chemiluminescent sensor, and here, we report the first measurements of superoxide concentrations collected during its underwater operation. As described below, through rigorous testing, we demonstrated its ability to reliably measure superoxide concentrations in the lab and in natural environments underwater up to a depth of 30 m. We show that the observed variance of in situ superoxide concentrations of background seawater and associated with corals greatly exceeds the variation of the instrument, thereby enabling accurate measurements of superoxide concentrations in reef water and associated with corals. Our tests and evaluations demonstrate that DISCO can be used in situ to measure superoxide.

3.1. Laboratory Characterization. Below, we discuss the results of the laboratory characterization of DISCO focusing on flow rate, signal stability, and instrument sensitivity.

3.1.1. Instrument Specifications. DISCO is watertight to depths 1.5× the maximum operation depth of 30 m (leak-tested to 45 m) (Figure 1). The instrument is 70 cm × 40 cm × 15 cm. It has a dry weight of 26.6 kg in air and a water weight of 0.9 kg in seawater (salinity 35 g/kg), giving it an ideally slight negative buoyancy during diver operation. DISCO has a battery life of ∼8 h during continuous operation. While the temperature limitations on superoxide detection by DISCO were not directly interrogated here, we confirmed that DISCO successfully operates over a range of temperatures between 19 and 30 °C. The primary temperature constraints on DISCO operation will be the slower reaction kinetics of MCLA at lower temperatures and the temperature range of the PMT (+5 to +50 °C). The background luminescence signal recorded by the PMT while running DI water through the system in the absence of MCLA was approximately 40 lumens, indicating negligible light interference within the sealed DISCO.

3.1.2. Instrument Stability and Variance. Testing of flow rates in the laboratory over three separate days indicated that the reagent and sample pumps run in isolation and together had a linear relationship between target set-point speeds and measured pump speeds (Figure 2). Continuous measurements over time and on separate days indicated high reproducibility. For instance, the coefficients of variation for the sample and reagent pumps ranged from 0.71 to 1.21% and 0.47 to 0.93% at a pump speed set to 8 mL min^{-1}. With the pumps working together, setting DISCO to a pump speed of 7.8 mL min^{-1} was reproducible to within ± 0.06 mL min^{-1} – or <0.8%. To decrease the residence time in the flow lines and reduce background seawater entrainment during coral analysis, all subsequent analysis and measurements were conducted at flow rates of 4.2 mL min^{-1}. All tested flow rates are within the range of the previously used flow rates with the FeLume, which ranged between 0.5 and 8 mL min^{-1}.[11,15,33] The flow rate for the analysis was routinely confirmed to have similar precision (coefficients of variance <1%). This flow rate allowed the analyte fluid to be measured on average within 16 s of entering the instrument, which is faster than or equivalent to the residence times for sample delivery in other studies.[30,33] While the half-life of superoxide changes based on environmental conditions, surveys of various marine environments (i.e., North Pacific, Southern Ocean, Kaneohe Bay, HI, Great Barrier Reef waters) found the half-life to range between 9.3 and 346.6 s.[15,28−31] With our detection speed, even the fastest decay rates reported can still be measured within two half-lives.

Signal stability was assessed in the ship laboratory by measuring filtered seawater and aged filtered seawater collected at various reef sites over the course of 2 weeks. The variance of the chemiluminescent signal was continuously monitored in

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filtered seawater and aged filtered seawater for periods of up to 15 min, exhibiting coefficients of variance ranging from 2.0 to 3.7% and 1.5 to 3.1%, respectively (Table 3). These values are consistent with the previous determinations of variance using benchtop chemiluminescent systems, including the FeLume instrument (see, for instance, Zhang et al. 2016; Diaz et al. 2016 that found coefficients of variance of ≤4%) (Table 3). The limit of detection (defined as 3 times the standard deviation of the blank) for deionized water and aged filtered seawater varied from 111 to 161 pM. Comparatively, this limit of detection is approximately twice that of values obtained in the laboratory using MCLA.[33]

Table 3. Summary of Percent Variance ((Standard Deviation/Average) × 100) for Aged Filtered, Filtered, and Background Seawater[a]

	% variance		
reef site	aged filtered seawater	filtered seawater	background seawater
GQ4b	1.5	3.7	2.6
GQ5b	2.1	3.4	2.6
GQ6a	1.6	2.1	1.9
GQ11a	1.0	2.0	1.4
GQ16a	3.1	NA	2.3

[a]Aged filtered seawater (amended with DTPA), filtered seawater (0.22 μM filtered), and background seawater (unfiltered) are collected at reef depth >30 cm away from any coral. The seawaters were measured for each sample on site.

3.1.3. Instrument Sensitivity. The sensitivity of DISCO to superoxide was tested using spiked superoxide additions within various marine and reef waters over several days. The concentration of superoxide added (as KO_2) ranged from 6.9 to 36.8 nM. Standard additions within aged filtered seawater illustrated the typical pseudo-first-order decay (see, for example, Figure 3), with half-lives ranging from 0.28 to 1.1 min and decay rate constants from 0.010 to 0.041 s^{-1}, which are similar to previous studies.[15,28−31] These standard addition results illustrated that DISCO has a high sensitivity, with calibration factors ranging from 0.342 to 0.571 counts per pM (mean 0.424 ± 0.116 counts/pM; n = 5), indicating that components specific to DISCO were not contributing to atypical sample decay. Further, the calibration and sensitivity values obtained are consistent with the previous observations using other previously vetted benchtop systems, including the FeLume system.[10,12,15]

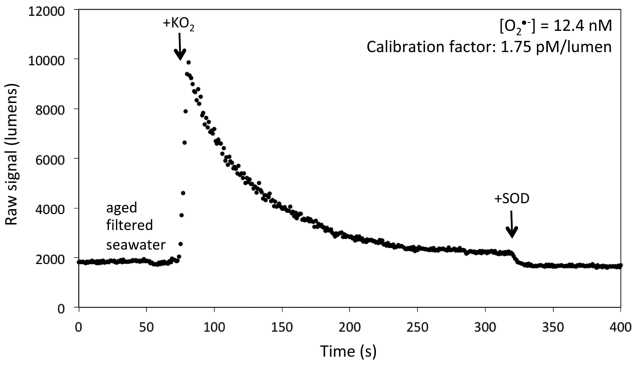

Figure 3. Representative superoxide decay curve in aged filtered seawater (amended with DTPA), used for calculating the site calibration factor. For all calibrations, first, the aged filtered seawater is measured as a baseline, then a superoxide spike is introduced as KO_2, and finally, SOD is added to confirm the signal is due to the reaction of MCLA with superoxide. Data from GQ4b.

3.2. In Situ Superoxide Concentrations Associated with Corals. DISCO was used to successfully measure superoxide in situ within six reefs in the coral reef system Jardines de la Reina, Cuba in November 2017. Background seawater levels measured for 1−2 min before each coral were similar in variability to that of the filtered seawater and aged filtered seawater assessed within the laboratory; background seawater had coefficients of variance ranging from 1.9 to 2.6%, depending on the site (Table 3). In contrast to filtered seawater and aged filtered seawater, however, the variance of chemiluminescence here represents the combined instrumental and environmental variance of superoxide concentrations. During an in situ test where DISCO was situated for 20 min over a sandy patch, background reef water had an average luminescence of 5397 ± 412 lumens, and the seawater plus SOD recorded 4292 ± 77 lumens yielding a signal of 1105 lumens for background reef water. This SOD-corrected signal equated to a superoxide concentration in the background seawater of ∼8.8 nM. However, MCLA autoxidation generates a small amount of superoxide that is eliminated by SOD;[40] thus, using the SOD baseline to correct for analyte signals can lead to an overestimation of true superoxide concentrations. With a more conservative approach that applies a baseline generated using DTPA-amended aged filtered seawater from the reef (4678 ± 89 lumens), we obtain an in situ superoxide concentration within the reef of ∼5.8 nM. This reef water concentration is consistent with the previous measurements of Hawaiian reef waters[15] and other productive marine systems.[11,31]

Chemiluminescent signals varied during each dive in response to the corals targeted (see, for example, Figure 4). Over the course of some dives, the baseline signal systematically increased slightly (as shown in Figure 4). Superoxide concentrations associated with corals ranged from <0 (i.e., below background seawater) to 173 nM and varied according to species (Figure 5). The highest extracellular superoxide concentrations were associated with *P. porites* exhibiting an average of 115 ± 40 nM (n = 4). Superoxide concentrations associated with *P. astreoides* were also elevated, but variable, with a range between 11 and 57 nM and an average of 24 ± 13 nM (n = 10). Superoxide concentrations associated with the one *D. cylindricus* (n = 1) measured was similar to *P. astreoides*, measuring 21 nM. The one *D. labyrinthiformis* (n = 1) and *A. cervicornis* (n = 1) that were assessed were associated with minimal superoxide, measuring 5 and 1 nM, respectively. *O. faveolata* (n = 3) and *M. cavernosa* (n = 7) had superoxide concentrations that were at or below background seawater levels.

Our superoxide measurements using DISCO are consistent with the only other study reporting measurements of superoxide associated with reef corals. A previous study[15] used a boat-mounted FeLume while floating over a reef to measure superoxide at the surface of various corals. Some coral species were associated with extracellular superoxide concentrations in the range between ∼25 and ∼120 nM (with *Pocillopora damicornis*, *Porites compressa*, and *Porites lobata*). Other coral species were associated with very low superoxide concentrations (*Fungia scutaria*, <5 nM) and concentrations slightly below background seawater (*Montipora capitata*, <0

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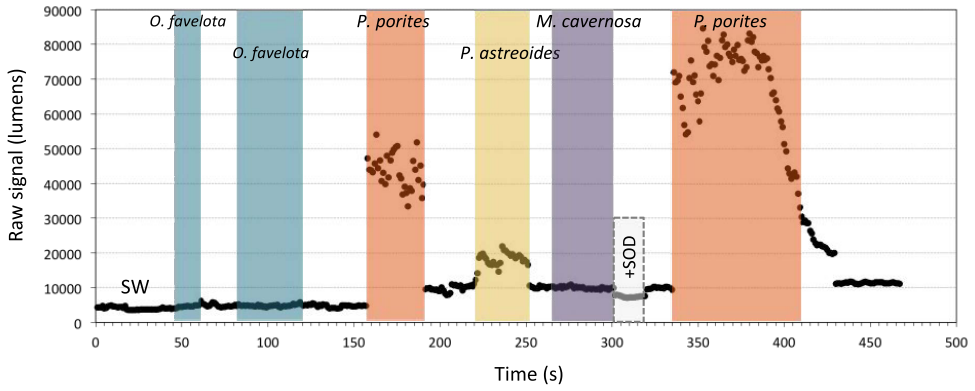

Figure 4. Representative DISCO chemiluminescent trace for background seawater and corals. Background seawater was measured directly before each coral measurement and used to calculate seawater-normalized superoxide levels associated with the coral surface. Each colored bar highlights coral measurements, and the coral species is listed above (the colors correspond to the same species colors in Figure 5). A decrease in signal due to the addition of SOD is also demonstrated (dashed square). Data from GQ11a.

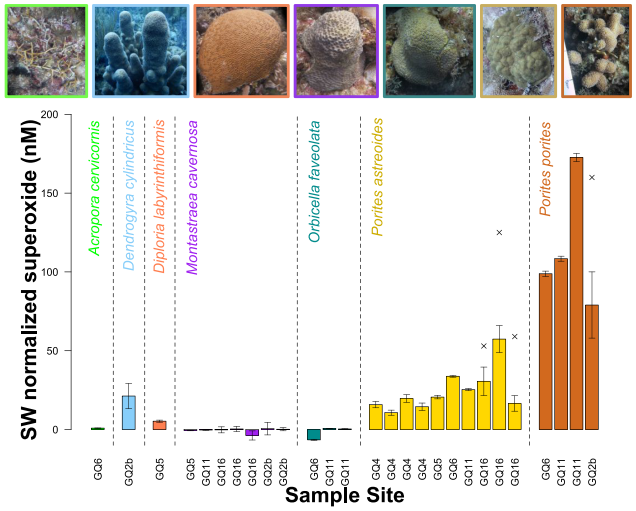

Figure 5. Seawater (SW)-normalized superoxide concentrations from seven coral species. Coral colonies are from six different sample sites, listed on the *x*-axis. Bars are the average of the data collected for one individual, and error bars represent the standard deviation of these measurements. The "*x*" represents the peak concentration for the few corals that exhibited peaks. Photographs were taken at the time of measurements. The measurements that are below zero represent superoxide concentrations that are less than that of background seawater.

nM). Here, using DISCO, we also observed similar species-specific superoxide levels. In particular, both *P. porites* and *P. astreoides* were associated with significantly higher levels of extracellular superoxide than the other coral species, while some species had superoxide levels at or below those of the surrounding seawater. Throughout each dive, these interspecies variations remained consistent, confirming the ability of DISCO to accurately measure superoxide associated with corals.

3.3. Ongoing Development. Several improvements are currently underway for DISCO to improve instrument performance and operation. First and foremost, the power demand from the high-precision pumps and electronics led to elevated temperatures and erratic performance under some circumstances, leading to baseline drift. Where observed in this study, calculations were corrected for drift using the baseline data collected immediately prior to any measurement.

While the current configuration for DISCO consisted of components that were rigorously tested and proved to serve their purpose, this design resulted in a large, heavy, and power-demanding instrument. A next-generation instrument is currently underway with a primary focus on miniaturization and reduction of power consumption. These are being accomplished primarily by using smaller peristaltic pumps and more compact reagent reservoirs. The redesigned user interface will also be more versatile and user-friendly by incorporating a custom underwater dive tablet. Furthermore, the current configuration had originally been designed to allow for SOD to be pumped in from a preloaded reagent bag to the sample line through an individual fluidic line and pump. However, this proved to be difficult with this fluidic system and therefore will be added in the next generation. Such improvements will help enhance deployment endurance and provide robust data over longer periods of time, thereby increasing the amount of data generated on each dive.

3.4. Future Research Within and Beyond Corals and ROS. Here, we confirmed previously observed species-specific trends in extracellular ROS associated with corals.[15] Additionally, measurements were made without direct contact with the coral, making DISCO a noninvasive tool to study aquatic organisms and ecosystems. Future investigations should employ DISCO to specifically monitor extracellular superoxide during stress events, such as bleaching and/or disease events, to better understand the linkage between ROS and coral health. Further development of the noninvasive DISCO to measure superoxide associated with corals is a necessary step in understanding the role of ROS in coral health.

Considering the broad relevance of ROS in ocean biogeochemistry and organismal health, DISCO can also be applied to a much wider range of systems. For instance, the ability to accurately measure in situ ROS concentrations could lend insight into phytoplankton bloom events, oxygen dynamics within seagrass beds, and wound repair in benthic macrofauna, to name a few. While DISCO was used primarily in this study in temperature ranges between 28 and 30 °C, initial testing was also successful at temperatures down to 19 °C and we expect this would apply for even colder environments. It is anticipated that the continued development of DISCO will provide a vital tool to survey the concentrations of ROS across different organisms and within various shallow ecosystems that will lead to a better understanding of its role

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within marine biogeochemistry. Currently, using DISCO as a foundation, we are also designing and developing a deep-sea sensor for submersible and rosette platforms, which will enable in situ measurements of ROS concentrations beyond the shallow depths attainable by DISCO.

Further, while the research here is centered on superoxide, DISCO will be amenable to the analysis of an extensive range of chemical species that have high-sensitivity chemiluminescent probes.[41] These include species with half-lives too short for ex situ analysis [e.g., nitric oxide (NO), adenosine triphosphate (ATP)] and species that are sensitive to even subtle changes in the redox environment [e.g., both aqueous ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron, and hydrogen sulfide (H_2S)].[32,42−44] DISCO may also enable the measures of other inorganic [e.g., phosphate (PO_4^{3+}), copper (Cu^{2+}), cobalt (Co^{2+}), bromide (Br^-), chromium (Cr^{3+})][45−48] and organic compounds [including key metabolites (e.g., riboflavin), contaminants, and pharmaceuticals]. Thus, this platform will open new scientific paths of exploration beyond ROS and corals, which remain out of reach by other approaches (e.g., mass spectrometers and electrochemical sensors) and will thereby pave the way for numerous applications for measuring chemical species within shallow ecosystems in situ.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b04022.

> Map with the location of sample sites in Jardines de la Reina, Cuba; sites are labeled and correspond to the sites where in situ extracellular superoxide measurements were conducted (Figure S1); ancillary geochemical data for background seawater (Table S1) (PDF)

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Notes

The authors declare no competing financial interest.

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