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Using resazurin as a reducing capacity indicator for analyzing physiological status of deep-sea bacterium *Photobacterium phosphoreum* ANT-2200

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Abstract

Resazurin (RZ) is a weak fluorescent blue dye and can be irreversibly reduced to highly fluorescent pink colored resorufin (RF), and further reversibly reduced to colorless dihydroresorufin (hRF) by photo-deoxygenation, chemical reaction and reductive organic compounds produced through cell metabolism. Because of the reliable and sensitive fluorescence-color change and noninvasive features, resazurin has been widely used as a redox indicator in cell viability and proliferation assays for bacteria, yeast and mammalian cells. However, resazurin is rarely used in physiological characterization of marine microorganisms. Here we developed a custom-made irradiation and absorption analysis device to assess reducing capacity and physiological status of marine bacterial cultures. We measured the absorption spectra of RZ, RF and hRF in presence of reducing compound Na_2S and under visible light irradiation. After establishing the appropriate parameters, we monitored the color changes of resazurin and its reduced derivatives to evaluate the coherence between reducing capacity, bioluminescence and growth of deep-sea bacterium *Photobacterium phosphoreum* ANT-2200 under various conditions. Emission of bioluminescence is an oxidation process that depends on cellular reducing capacity. We found that growth and bioluminescence of ANT-2200 cultures were progressively impeded with increased concentration of RZ, which suggests a competition for reducing molecules between RZ at high concentration with reductive metabolism. Therefore, it should be cautious when RZ is added directly in growth media to monitor the culture redox. Analysis of RZ reduction velocity of ANT-2200 cultures showed a detrimental effect of high hydrostatic pressure and revealed a high coherence between the reducing capacity and bioluminescence of the cultures. This study clearly demonstrates the potential of using resazurin to characterize microbial metabolism and physiology of marine bacteria.

Keywords: Oxic-reduction indicator, absorption spectra, bacterial growth, marine microorganism

1 INTRODUCTION

Resazurin (RZ) has a chemical structure as 7-hydroxy-10-oxidophenoxazin-10-ium-3-one and is also referred to as heterocyclic *N*-oxide or N-phenoxazine-3-one dye. Resazurin exhibits a blue color with weak fluorescence. As a donor in oxygen atom transfer reaction, RZ is irreversibly reduced to highly fluorescent pink dye resorufin (RF). Further reduction of RF results in the production of colorless dihydroresorufin (hRF), which is a reversible process (Fig. 1). Resazurin is water soluble, noninvasive, low or noncytotoxic. Therefore, resazurin and derivatives of N-phenoxzine-3-one dyes have been widely used as a simple, rapid, reliable, sensitive, safe and

cost-effective measurement of cell viability and proliferation, and determination of bacterial or yeast contamination in milk (Kowaltowski, 2019; Rampersad, 2012; Neumann, et al., 1996). Moreover, the anionic dye RZ is also appropriate probe for analytical determination of ions such as Se(IV) and Pb(II) (Safavi, et al., 1990; Afkhami, et al., 1991). However, the resazurin dyes remain poorly utilized in physiological studies of marine microorganisms.

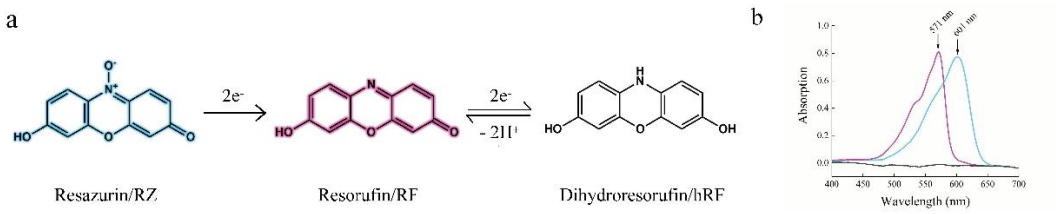

Fig. 1 Molecular structure and absorption spectra of resazurin, resorufin and dihydroresorufin. (a) illustrates reduction of resazurin (RZ, blue color) to resorufin (RF, pink) and further to colorless dihydroresorufin (hRF). (b) shows the absorption spectra of RZ (cyan curve), RF (red color) and hRF (black color). The absorption spectra were obtained in this study.

Reduction of the phenoxazine-3-one dyes, RZ and RF occurs through chemical reaction and metabolism process. In addition, irradiation in the presence of amines also leads to deoxygenation of RZ and its conversion to RF (Neumann, et al., 1996; Bueno, et al., 2002). Similarly, reduction of RF is a photosensitive reaction. Zhao et al., have reported that UV and visible light (λ>300 nm) irradiation leads to one-electron reduction of RF and produces a semiquinoneimide-type anion radical RF^{*-} under anaerobic conditions with reduced nicotinamide adenine dinucleotide (NADH) (Zhao, et al., 2011). Continuous irradiation further reduces RF^{*-} to its colorless form of dihydroresorufin (Fig. 1).

Bioluminescent bacteria have the peculiar capacity of emitting blue-green light (470~490 nm) through the luciferase-catalyzed oxidation of reduced flavin mononucleotide (FMNH) and fatty aldehyde (Fig. 2) (Dunlap & Kita-Tsukamoto, 2006). To continue the bioluminescent process, oxidized FMN must be reduced to FMNH by an enzyme using NADH as electron donor (Fig. 2). Most of the luminous bacteria dwell in marine environments from the shallow coast to deep-sea (Dunlap & Kita-Tsukamoto, 2006). The *Photobacterium phosphoreum* strain ANT-2200 (hereafter

called ANT-2200) was isolated at 2200 m depth from the Mediterranean Sea and capable of emitting bioluminescence (Al Ali et al., 2010; Martini et al., 2013). Genomic and physiological analyses revealed a versatile energy metabolic potential and growth capacity of ANT-2200 by deriving energy from fermentation of glucose or maltose, by respiration with formate as electron donor and trimethlyamine N-oxide (TMAO), nitrate or fumarate as electron acceptors, or by chemo-organo-heterotrophic growth in rich media (Zhang, et al., 2016). Interestingly, the light emission seems to be proportional with the growth rate. Continuous emission of bioluminescence depends on cell reductive capacity and the bio-luminous light might trigger the photo-reduction of RZ and RF. We wondered if it is feasible to use resazurin dyes to assess physiological status of ANT-2200 by analyzing the correlation among the reducing capacity, bioluminescence and growth. We set up an irradiation and spectrum-scanning device and showed the reliable coherence of RZ reduction with the growth and luminescence of ANT-2200. This study demonstrated using the heterocyclic *N*-oxide dyes in the physiological and metabolic investigation of marine microorganisms.

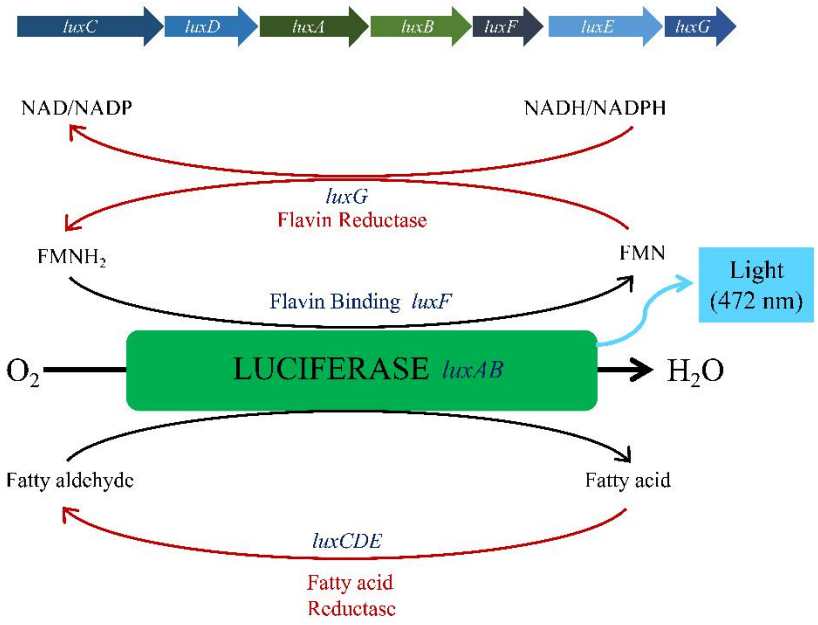

Fig. 2. **Bioluminescence of strain ANT-2200.** The *lux* gene cluster of ANT-2200 contains the genes coding the enzymes required for the bioluminescence process of ANT-2200 and regeneration of reduced substrates.

2 MATERIALS AND METHODS

2.1 Strain and bacterial cultures

Cultures of the strain ANT-2200 were performed in YPG media at 27 °C in serum bottles (Fig. S1), or in 5 ml syringes immerged in high-pressure incubators as described by Zhang et al. (Zhang, et al., 2016). When indicated, 20 MPa hydrostatic pressure was applied on high pressure incubators that contain the syringes. A stock solution of 20 mM resazurin was prepared in Milli-Q water and used at final concentrations as indicated.

To analyze effect of irradiation, resazurin solutions or bacterial cultures were exposed to tungsten bulb or 470 nm LED. Light densities were measured using Thorlabs S120vc sensor connected to Thorlabs powermeter.

Samples were taken at indicated time, and optical density at 600 nm was measured using spectrophotometer and luminescence was analyzed using microplate reader according to fabricant instructions.

2.2 Absorption spectra analysis

Absorption spectra of resazurin and its reduced derivatives were measured in serum bottles, directly as solution or in growth media at the concentrations as indicated. Alternatively for post-growth scanning, 7 to 8 ml cultures were transferred to serum bottles and resazurin was added at 0.01 mM final concentration, the bottles were sealed and scanned for the spectra every 5 min until the end of color change using Varian 50 or Agilent Technologies Cary60 UV-Vis spectrophotomers. Bottle holder was designed and fabricated by 3D-printing (Fig. S1).

3 RESULTS AND DISCUSSION

3.1 Spectral characteristics of resazurin and its derivatives

3.1.1 Set-up of a custom-made device to facilitate irradiation and spectrum scanning of cultures

Because RZ and RF are highly sensitive to oxidation, tight sealed containers should be used for bacterial cultivation and absorption spectra analysis. The 11 ml serum bottles are ideal for this study for following reasons: first of all, they can be easily tight sealed and addition of chemical solutions or exchange of the gases can be performed using needles piercing the rubber plugs; secondly, culturing marine bacteria, such as *Photobacterium phosphoerum* ANT-2200, with these bottles can provide enough biomass for further analysis; and most importantly, the bottle fits in

most standard spectrophotometers. To use these bottles with Varian 50 and Agilent Technologies Cary60 UV-Vis spectrophotomers, we designed a 3-D printed bottle holder for directly scanning the absorption spectra in these bottles (Fig S1). In addition, LED light bulbs can be easily mounted on the holders to irradiate the solution or bacterial cultures.

Using the bottle-Cary60 scanning device we observed a principal absorption at 601 nm and a shoulder peak at 550 nm of RZ in water solution saturated with nitrogen gas (Fig. 1b). Reduction of RZ to RF by Na_2S resulted in a shift of the spectrum with the major peak at 571 nm and shoulder peak around 530 nm (Fig. 1b). Further reduction to hRF led to disappearance of the absorption peaks. The absorption spectra of RZ, RF and hRF are similar with previous reports, although slight shift of absorption peaks that possibly caused by different media and spectrophotometers were observed. Therefore, this device allows us easily monitoring the reduction states of RZ, RF and hRF in sealed serum bottles, and peaks at 601 nm and 571 nm were used to represent RZ and RF, respectively, in this study.

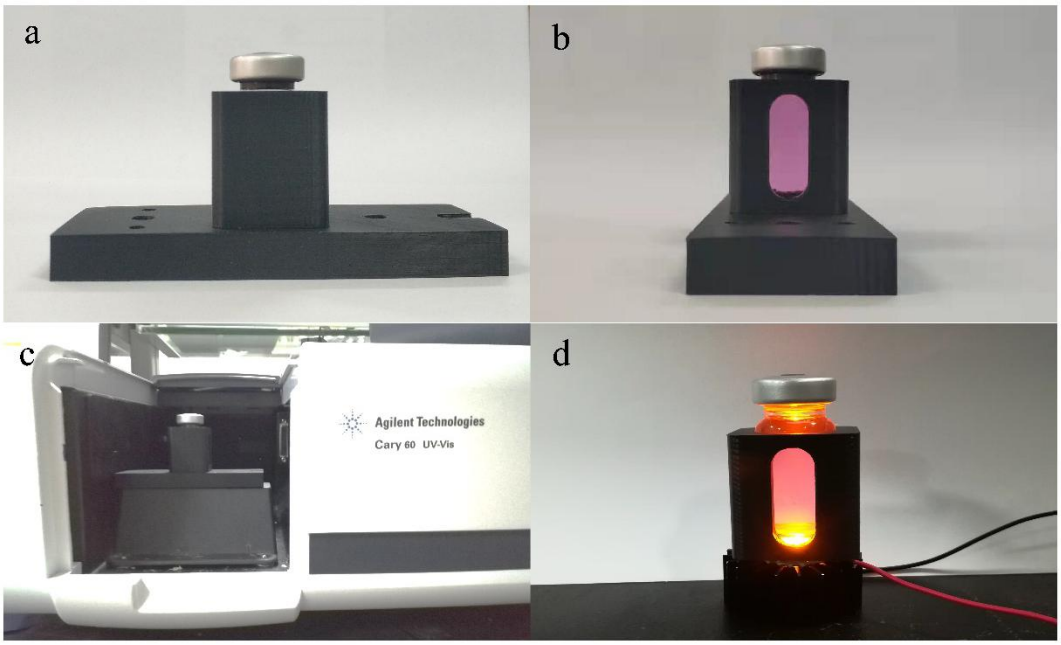

Fig S1 Bottle-scanning device for spectra scanning and irradiation in serum bottles. The 3-D printed bottle holders can be used for real-time scanning of the culture absorption spectra (a-c) and irradiation of cultures with LEDs at different wavelengths (d).

3.1.2 Effect of irradiation and Na_2S on the absorption spectra of resazurin and its derivatives

We first assessed the color change of RZ under different redox conditions in artificial seawater in a fermenter with controlled redox and pH. The solution had a blue color and remained unchanged after over-night bubbling with nitrogen gas. Changing the N_2 gas to carbon dioxide increased the redox potential from -90 mV to -53 mV and decreased pH of the solution from 8.9 to 7.9, but had no effect on the blue color. Therefore, the RZ solution is stable in the ranges of redox potential and pH under the conditions used. Decreasing solution redox along seems not sufficient to convert RZ to RF.

Other than pH and redox condition, irradiation has been reported leading to deoxygenation of RZ and reduction of RF under certain conditions (Porcal, et al., 2011). Consistently we found that when exposed to sunlight or illuminated with a tungsten light bulb, the solution became light-pink color, which implies the formation of RF from photochemical deoxygenation of RZ under this redox and pH condition in artificial seawater solution.

We then evaluated effect of irradiation and chemical reaction using the bottles-scanning devices. The 11 ml serum bottles were filled up with 8 ml water solution containing 7.2 μM RZ and bubbled with N_2 for 15 min. Then Na_2S were injected in the bottles to final concentrations at 72 μM (Fig. S2, bottle 1 and 6), 720 μM (bottles 2 and 7), 3600 μM (bottles 3 and 8) and 7200 μM (bottles 4 and 9). As a control the bottles 5 and 10 contained only RZ without Na_2S. The bottles were incubated in dark (bottles 1-5) or with illumination from tungsten light bulb (bottles 6-10, at 25 mW/cm^2). Visual observation showed that RZ remained blue color in the absence of Na_2S (Fig. S2a). At the 10-fold molar ratio of Na_2S/RZ (bottles 1 and 6) the solutions turned to pink color (RF), whereas all solutions became colorless (hRF) with the molar ratio of Na_2S/RZ higher than 100-fold.

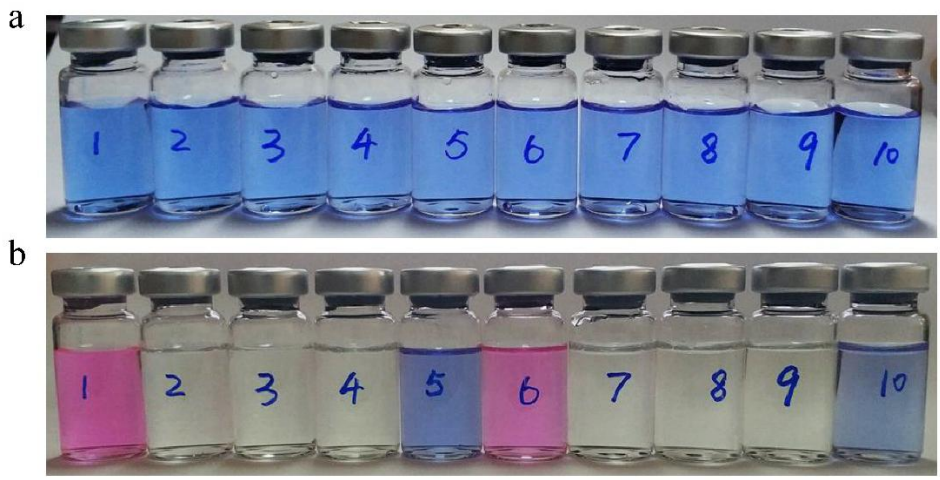

Fig. S2. Reduction of the phenoxazine-3-one dyes by chemical reaction and irradiation. The 11 ml bottles were filled up with 8 ml water solution containing 7.2 µM RZ and bubbled with N_2 for 15 min (a). Then Na_2S were injected in the bottles to final concentrations of 72 µM (b, bottle 1 and 6), 720 µM (b, bottles 2 and 7), 3600 µM (b, bottles 3 and 8) and 7200 µM (b, bottles 4 and 9). As a control the bottles 5 and 10 contain only RZ without Na_2S (b). The bottles were incubated in dark (1-5) or with irradiation (6-10, tungsten light bulb at 25 mW/cm^2).

We further quantified the effect of reductant and irradiation by monitoring the time-lapse of absorption spectra. As shown in Fig. 3, the spectra of RZ remained unchanged during 24 hours in the absence of Na_2S and light (Fig. 3a). However, the spectra diminished slightly or substantially after 12h and 24h irradiation with tungsten light, respectively (Fig. 3b). Notably, the scanning revealed spectral change, which was unperceivable by naked-eye. Therefore, the irradiation led to photobleaching and photo-deoxygenation of the RZ as reported by Porcal et al. (Porcal, et al., 2011).

With addition of Na_2S, decrease of RZ was concomitant with rise of RF spectra, and the decline of the latter afterwards under certain circumstances. The rates of spectral changing were in proportion with molarities ratio of Na_2S/RZ (Fig. 3c-f). When incubated in darkness, addition of ten-fold amount of Na_2S led to decrease of absorption at 601 nm from 0.78 to 0.33 and increase of that at 571 nm from 0.54 to 0.82 within 96 min (Fig. 3c and g). Notably, the absorption at 571 nm kept increasing under this condition (Fig. 3g dash-red curve), indicating RF was not further reduced into hRF within the analyzing time. The reduction process of RZ-RF-hRF can be

accelerated by irradiation. When exposed to irradiation, the peak at 601 nm decreased quickly to baseline level at 15 min (Fig. 3d and g, blue curve), when absorption at 571 nm reached its maximum and began to decrease gradually (Fig. 3g, red curve). By the end of analysis at 40 min, RF was partially reduced into transparent hRF that led to both lower absorption at 571 nm and a lighter pink color of the solution (Fig. 3d and Fig. S2, bottle 6).

As demonstrated above, the amount of reductant had great influence of the reduction of RZ. When excessive Na_2S (molar ratio of Na_2S/RZ of 500) was added, the 601 nm absorption decreased quickly to the baseline level in approximate 1 min (Fig. 3e, f and blue curves in h). The 571 nm absorption increased to the maximal level at 30 s, and then decreased to the approximate baseline level at 2.5 min after the addition of Na_2S, respectively. Irradiation did not change the reduction velocity (Fig. 3h), which indicates a dominant chemical reduction effect over the irradiation at excessive amount of Na_2S.

Altogether, these results show that Na_2S chemically reduces RZ to RF and hRF, and irradiation with visible light leads to photo-deoxygenation of RZ and accelerates the reduction process in the bottle-scanning systems. Evolution of the absorption peak at 571 nm was resulted from reduction of RZ in combination with further reduction of RF to hRF. In contrast, changes of absorption at 601 nm reflects the irreversible reduction of RZ alone, and therefore could be a better indicator of reducing capacity in the reaction system. In order to quantify and compare the reducing capacity between different samples, instantaneous reduction velocity was calculated using $V_n = d(A_{601\_n+1} - A_{601\_n})/d(t_{\_n+1} - t_{\_n})$, where A_{601_n} and $t_{\_n}$ are the absorption at 601 nm and the time at point n, respectively. As shown in Fig. 3, the instantaneous reduction velocity increased from 0.005 to 0.08 OD_{601}/min upon illumination at limited amount of Na_2S (g), whereas it reached the maximal 1.04 and 1.05 OD_{601}/min at excessive Na_2S independently of illumination (h). The V_n will be used to describe reducing capacity of the cultures in this study.

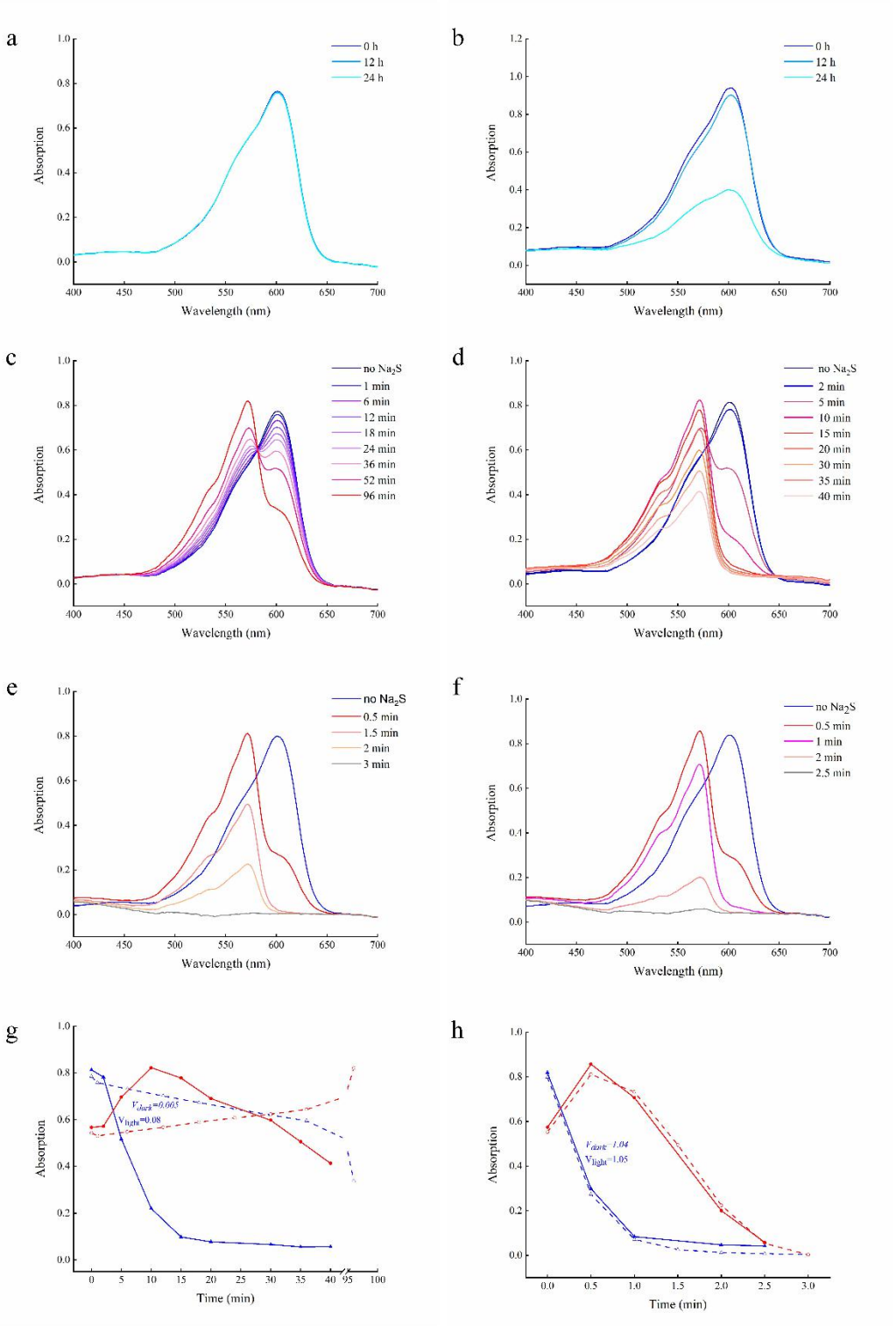

Fig. 3 Effect of Na_2S and irradiation on absorption spectra of resazurin, resorufin and dihydroresorufin. RZ solutions without Na_2S (a and b) or with Na_2S/RZ molar ratio of 10:1 (c and d) and 500:1 (e and f) were scanned with time interval as indicated. g and h show the changes of the RZ-601 nm (blue curves) and RF-571 nm (red curves) of RZ solutions with 10:1 (g) and 500:1 (h) Na_2S/RZ molar ratio without (dash lines) or with (lines)

irradiations. The maximal instantaneous reduction velocities are indicated in g and h.

3.2 Using reducing capacity indicator RZ to evaluate physiological status of ANT-2200

3.2.1 Effect of resazurin on the growth of ANT-2200

Bioluminescent bacteria emit light by a well conserved FMNH oxidation reaction (Fig. 2). To continue the emission, the oxidized FMN must be reduced back to FMNH by NADH or NADPH, which is catalyzed by flavin reductase LuxG in marine luminous bacteria (Nijvipakul et al., 2008). In parallel resazurin is converted to resorufin by reductant NADPH or NADH in the presence of the enzyme NADPH dehydrogenase or NADH dehydrogenase in living cells (Jong & Woodlief, 1977; Barnes & Spenney, 1980). Therefore, resazurin might compete with LuxG for the reductant NADPH/NADH and affect bioluminescence. To assess this hypothesis, we analyzed the effect of addition of resazurin on the culture of ANT-2200. Interestingly we observed that the growth of ANT-2200 was progressively hampered with increased resazurin in the growth media (Fig. 4a). Moreover, the bioluminescence was parallelly decreased (Fig. 4b).

We then analyzed the reducing capacity of these cultures. Since the resazurin in the growth media was irreversibly converted into RF during the growth of ANT-2200 cells, we followed the changes in characteristic RF absorption peak at 571 nm instead. By shaking the culture bottles, hRF generated during the growth was re-oxidized into RF by the air phase in the culture bottles. Then RF would be reduced into hRF by ANT-2200 cells. By measuring time-lapse absorption spectra we would be able to compare the rates and extents of RF reduction that reflect the physiological status of these cultures. As shown in Figure 4c, absorption at 571 nm of the culture with 0.01 mM RZ dropped to the baseline level 20 min after shaking. However, it took 30 min for the RF peak reduced to approximate half with cells cultured with 0.05 mM RZ (Fig. 4d). These results indicate that 0.05 mM RZ might be present at excessive amount to be completely reduced by the cells. The excessive amount of RZ in the media might also account for its inhibition effect of on the growth and bioluminescence. Therefore, caution should be taken when RZ is added in media to monitor the redox of cultures.

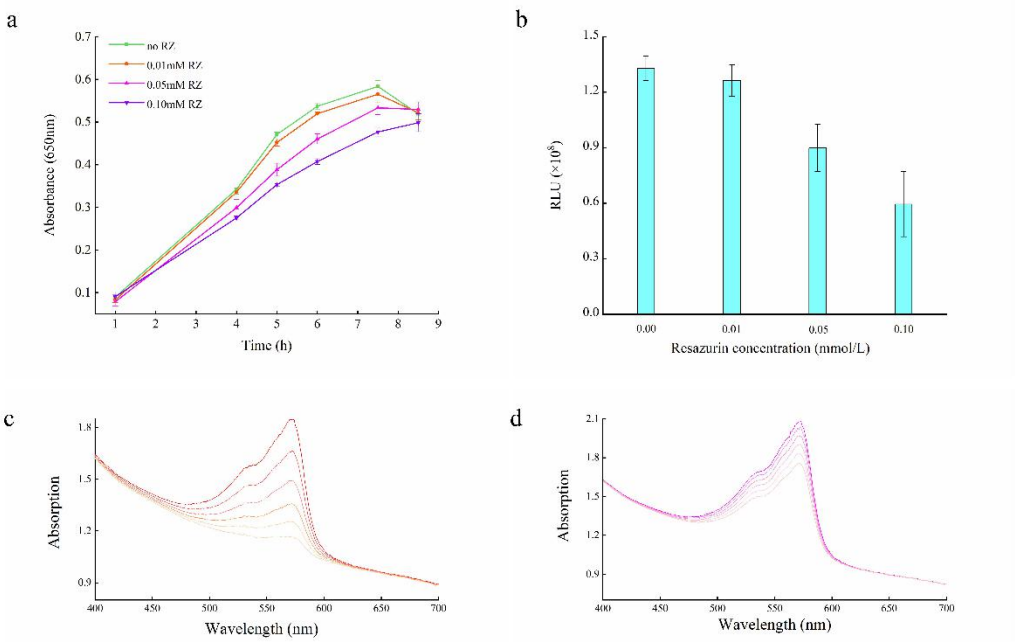

Fig. 4 Influence of RZ on ANT-2200 growth and bioluminescence. RZ was added in the cultures bottles at concentrations as indicated. The optical densities of the cultures (a) were measured at times indicated. At the end of the incubation bioluminescence was detected (b), and the reducing capacities of cultures with 0.01 mM (c) and 0.05 mM (d) RZ were analyzed by scanning the absorption spectra with 5 min intervals after shaking the bottles to re-oxidize hRF to RF.

3.2.2 Coherence of using bioluminescence and instantaneous reducing velocity to analyze physiological state of ANT-2200 cultures

The deep-sea luminous strain ANT-2200 has versatile energy metabolic potential (Zhang, et al., 2016) and its light emission seems to be proportional with growth rate. We thought to assess the proof of concept of using resazurin to evaluate the reducing capacity of marine bacterial cultures by inspection of growth, bioluminescence and RZ reducing velocity under different growth conditions. The growth of strain ANT-2200 under high hydrostatic pressure conditions was slightly slower than that at atmosphere pressure, and both cultures reached stationary phase after about 10h incubation (Fig. 5a). However, the cultures at atmosphere pressure exhibited a second growth phase and attained maximal optical density of 1.32 at 24h and remained the same after 50h incubation (Fig. 5a). Consistently the bioluminescence increased during exponential growth phase

and decreased in stationary growth phase, and the cultures at atmosphere pressure were more luminous than those under high hydrostatic pressure (Fig. 5b). Notably, the instantaneous RZ reducing velocity increased during the exponential growth phase and slowed down at the stationary phase, and those of the cultures at atmosphere pressure were higher than those under high pressure (Fig. 5c). Together these results showed that the instantaneous reducing velocity reflects truly the physiological state of cultures and is suitable for evaluating the marine bacterial metabolism.

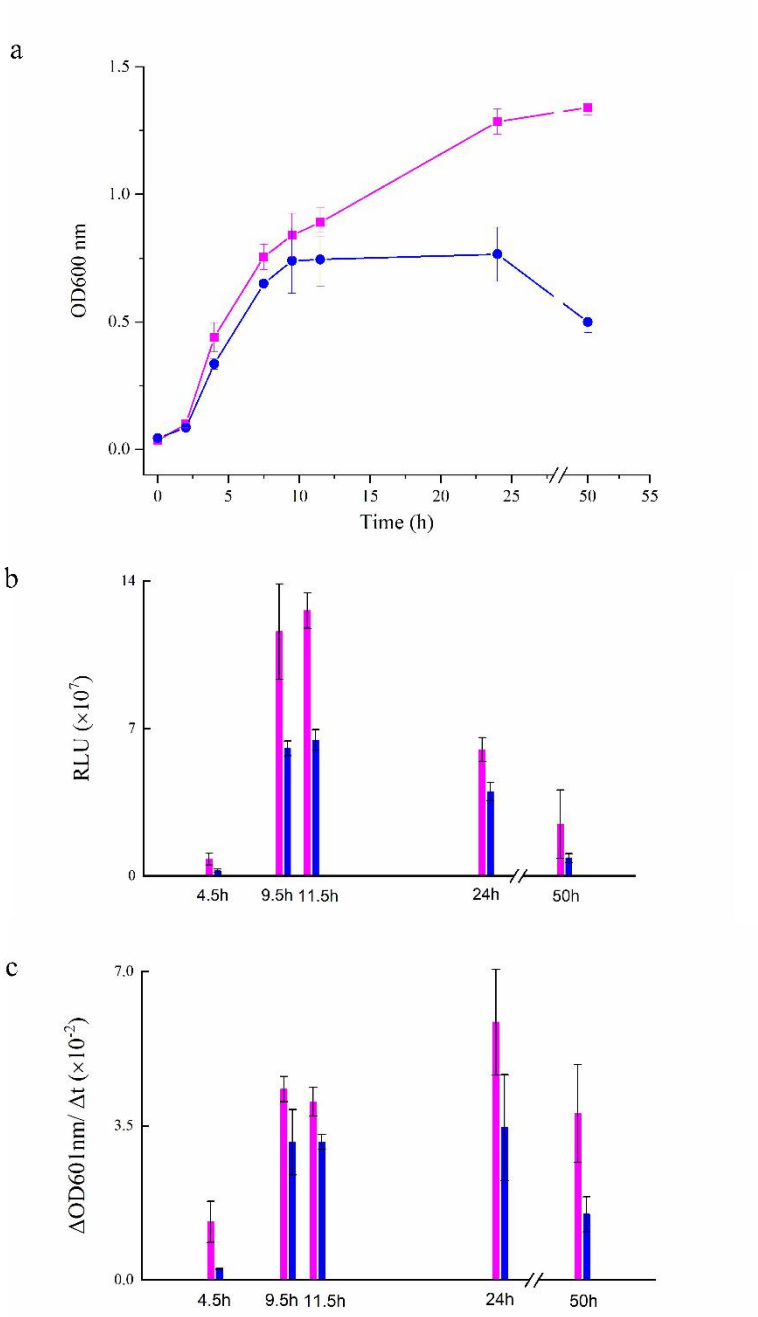

Fig. 5. Coherence of reducing capacity with growth and bioluminescence of ANT-2200 cultures. ANT-2200 were incubated at atmosphere pressure (red) or 20 MPa (blue). The optical density at 600 nm (a), bioluminescence (b) and the calculated RZ reducing velocity (c) were taken at times as indicated.

4 CONCLUSIONS

Resazurin is a sensitive reducing capacity indicator. In this study we assessed the feasibility of using resazurin as reducing capacity indicator to analyze physiology state of marine luminous

bacteria. RZ exhibits cytotoxicity at high concentrations and it should be cautious to use it directly in cultures. However, we found an obvious coherency between the reducing capacity of the cultures with their growth and bioluminescence. Resazurin can be used in physiological study of marine microorganisms.

5 ACKNOWLEDGMENTS

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