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Light-induced absorbance changes associated with photoinhibition and pigments in nitrifying bacteria

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ABSTRACT: Spectroscopic analysis of nitrifying bacteria revealed the presence of a porphyrin-like pigment with an absorption maximum at 408 nm. The photoresponsive pigment accumulated during the late exponential phase of growth. The photoreceptor was found at higher concentrations in NH_4^+ oxidizers than in NO_2^- oxidizers. When absorbance scans and action spectra of the nitrifiers were compared, it was found that the regression between the degree of photoinhibition and higher absorbances at 408 nm was significant ($r^2 = 0.7$). Reversible light-induced absorbance changes were observed *in vivo* and *in vitro*. Absorbance changes were maximally elicited by light in the 400 nm region for both types of nitrifiers, but the change was only significant ($p < 0.05$) for NH_4^+ oxidizers. This spectral sensitivity of the NH_4^+ oxidizing process suggests that the absorbance change observed is related to the blue light sensitivity of NH_4^+ oxidizers.

KEY WORDS: Photoinhibition · Nitrifying bacteria · Nitrification

INTRODUCTION

The damaging effects of light on microbes may impose constraints on some of their activities. For instance, the process of nitrification (NH_4^+ and NO_2^- oxidation) in nitrogen turnover has been repeatedly shown as light sensitive (Schön & Engel 1962, Smith 1977, Olson 1981, Yoshioka & Saijo 1984, Diab & Shilo 1988, Vanzella et al. 1989). Marine habitats offer a good system to study the effect of the repetitive diurnal variation in light intensity. Similarly, marine nitrifying bacteria offer a tentative test organism to look at how exposure to light can affect their activities. The differential light sensitivity of marine nitrifiers has led investigators to make important implications for nitrification in marine environments (e.g. formation of primary nitrite maxima; Olson 1981). However, the physiology behind the photoinactivation of ammonium and nitrite oxidizing bacteria needs characterization, as a prerequisite to making any prediction on the process in which they are involved. This paper approaches this problem by spectroscopic analysis of the usual pigments (i.e. cytochromes) and of the light absorption characteristics of whole cell suspensions of nitrifiers. These observations were carried out to elucidate possible chromophore(s) present and to examine their relationship to the observed light response. Such measurements might provide an experimental basis to explore the photobiological control mechanisms of photoinhibition.

MATERIALS AND METHODS

Microorganisms and growth conditions. The marine ammonium oxidizing bacteria *Nitrosomonas cryotolerans* (ATCC 49181; Jones et al. 1988) and *Nitrosococcus oceanus* (ATCC 19707) along with the marine nitrite oxidizing bacteria *Nitrococcus mobilis* (ATCC 25380) and *Nitrobacter* sp. (Nb 297, provided by S. W. Watson) were grown in continuous cultures and harvested as described previously (Guerrero & Jones 1996a). For the experiments that required batch cultures, the same culture media was used with the addition of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) as a buffer. The flask cultures

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were kept in a rotatory shaker and incubated at 25°C.

Preparation of crude extracts. Cells were harvested by centrifugation from chemostat cultures, washed twice in 0.2 µm filtered sea water (FSW), and resuspended in 1 ml of phosphate buffer (pH 7.5, 50 mM). The cell suspension was placed in a 1.5 ml polyethylene microfuge tube and sonicated on ice for 3 min (Tekmar Sonic Disruptor, 15 W). The cellular debris was separated by microcentrifugation at 15000 × *g* for 5 min.

Absorbance studies. ***Absorbance scans:*** Absorption spectra of whole cell suspensions or cell-free extracts were measured at room temperature with a single-beam spectrophotometer (HP 8452A diode array) on line with a PC equipped with MS-DOS UV/VIS software, Version 1.0. Baseline measurements (phosphate buffer or FSW) were substracted automatically from sample scans. Absolute spectra are presented in either the reduced or oxidized state. Reduced scans were obtained by adding a few crystals of $Na_2S_2O_4$ to the cuvette that held the standard cell suspension. Oxidized scans were attained by vigorous aeration of the respective cuvette. Wavelength range for the scans was recorded and stored at 2 nm intervals.

Absorptivity changes: Absorbance changes *in vivo* and *in vitro*, were detected by absorption spectra of samples and controls before and after irradiation. For these experiments whole cells or extracts were resuspended in 0.05 M phosphate buffer (pH 7.5), instead of FSW. Monochromatic irradiation of the samples was carried out as described by Guerrero & Jones (1996a). Action spectra for the absorbance change induced by different wavelengths of light of equal fluence (25 W m^{-2}) were determined by measuring absorbance scans in triplicates just before irradiation (T_0) and 30 min after (T_{30}). A portion of the cell suspension was saved for protein determinations (Lowry et al. 1951), and results were normalized to ΔAbsorbance per mg of cell protein.

Cytochrome spectra. ***Whole cells:*** Reduced minus oxidized difference spectra were determined as follows: 250 ml of chemostat culture was harvested by centrifugation, washed twice in FSW and resuspended in phosphate buffer (pH 7.8) to an OD_{660} (optical density at 660 nm) of at least 1.2 Absorbance. This optically thick cell suspension was used to run a differential scan with a split beam spectrophotometer (Perkin Elmer Lambda 4B). $Na_2S_2O_3$ crystals were used to reduce one cuvette, while vigorous aeration before scanning was used to keep the other cuvette oxidized. The oxidizing effect of aeration was similar to adding 10 µl of a 30% H_2O_2 solution. Published extinction coefficients were used to estimate the amount of cytochrome per mg of cell protein (Smith 1977).

Cell-free extracts: For cytochrome determinations on crude preparations of cell membranes and cell-free supernatant fractions, the cells were ruptured by sonication. After sonication, the cells that were not lysed were separated by microcentrifugation (15000 × *g* for 15 min; Miller & Wood 1982). The sediment was taken as a crude preparation of cell membrane and the cell-free supernatant as the soluble (periplasmic + cytoplasmic) fraction. The rest of the procedure was as described above for whole cell suspensions.

Analytical methods. Total protein was determined within 48 h by Lowry's (1951) method using bovine serum albumin as standard. Samples were collected in triplicates, and the data points correspond to the mean of the triplicates. Correlation analyses (r) and Student *t*-tests were used to determine relationships between variables and statistical significance, respectively.

RESULTS

Absorption spectra of cells

In vivo absorption characteristics can give an indication of the possible chromophore(s) present in the cells. Like other cells, nitrifying bacteria exhibited at least a biphasic absorption, with a first maxima at 280 nm that fits the absorption spectrum of proteins and nucleic

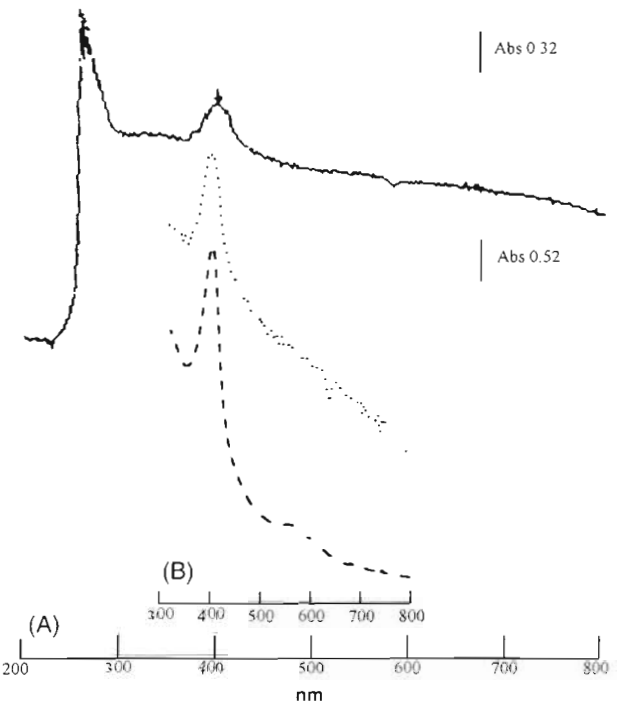

Fig. 1. Absorbance scans of *Nitrosomonas cryotolerans* as a prototype of nitrifiers' absorbance. (A) Wavelength range: 200 to 800 nm; (——) whole cells. (B) Wavelength range: 300 to 800 nm; (· · ·) whole cells, (– – –) crude-extract. Protein concentration: 0.5 mg ml^{-1}. Abs: absorbance

acids and a second at 408 to 410 nm (Fig. 1). A great deal of scattering was found when working with whole cell suspensions, which did not allow for 'smooth' resolution of the scans. It was possible to avoid scattering noise by using cell-free extracts instead of whole cells. This approach did not alter the maxima readings (Fig. 1), since the maximum absorption peak still showed up at 408 nm using either a whole cell suspension or cell-free extracts.

Absorption scans with cell-free extracts were performed to determine the potential chromophore in all the species studied (Fig. 2A). For comparison purposes, all of the cell suspensions were adjusted to a standard protein concentration within the range of 420 to 580 µg protein ml^{-1}. Absorbance characteristics of the 4 different species tested were qualitatively similar, since they all showed an absorption maximum at 408 nm in the oxidized state. The difference between species was observed to be mainly quantitative with respect to the 408 nm peak (Table 1).

It was also of interest to see how the absorbance characteristics of the cells correspond with their respective action spectra. When absorbance scans and previously published action spectra (Guerrero & Jones 1996a) were superimposed, it was consistently observed that the degree of photoinhibition directly correlated with higher absorbances at 408 nm (Fig. 3). For example, *Nitrobacter* sp. appeared as the organism with least absorbance (Abs_{408} mg^{-1} cell protein: 0.088) and also least photosensitivity (ca 5%) as opposed to *Nitrosomas cryotolerans* which showed the highest absorbance (Abs_{408} mg^{-1} cell protein: 0.326) as well as the highest photosensitivity (up to 82%; Table 1). *Nitrosococcus oceanus* was the only organism that did not fit this pattern. Interestingly, *N. oceanus* is also the only member of the NH_4^+ oxidizers that has been classified in a phylogenetically different group (Woese et al. 1985).

The chromophore seemed to accumulate during the late exponential phase of growth of the culture. Cells from an early exponentially growing culture (3 d old batch-culture; Table 2) had approximately 10 to 70%

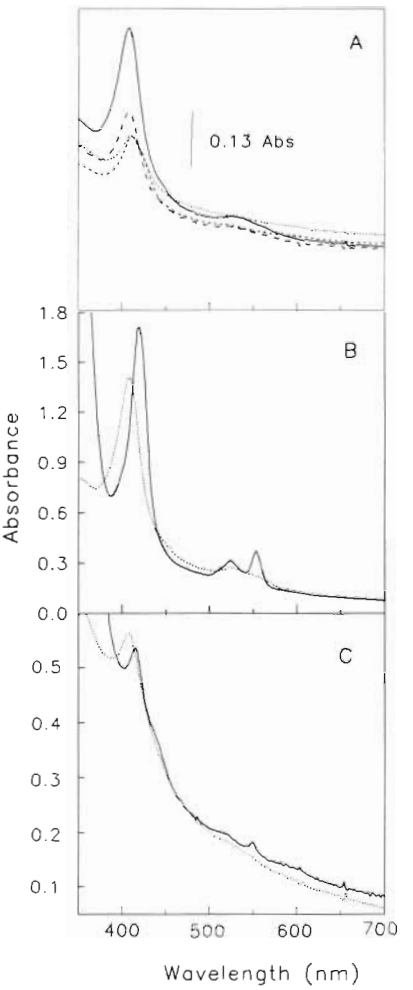

Fig. 2. (A) Absorption scans of cell-free extracts of nitrifying bacteria. (——) *Nitrosomonas cryotolerans*; (- - -) *Nitrosococcus oceanus*; (·····) *Nitrococcus mobilis*; (·· ··) *Nitrobacter* sp. Protein concentrations were 0.5, 0.58, 0.46, and 0.48 mg ml^{-1}, respectively. (B) Oxidized versus reduced absorbance scans of cell-free extracts of *N. cryotolerans*. (·····) Oxidized absorbance scan; (——) dithionite-reduced absorbance scan. Protein concentration: 0.5 mg ml^{-1}. (C) Oxidized versus reduced absorbance scans of cell-free extracts of *Nitrobacter* sp. Protein concentration: 0.46 mg ml^{-1} Abs: absorbance

Table 1. Pigment concentration per mg of cell protein. Abs_{408}: absorbance at 408 nm

Microorganism	Abs_{408}	Protein concentration (mg ml^{-1})	Absorbance mg^{-1} cell protein	Percent inhibition[a]
Nitrosomonas cryotolerans	0.163	0.5	0.326	82[b]
Nitrosococcus oceanus	0.073	0.58	0.126	75[b]
Nitrococcus mobilis	0.057	0.46	0.124	25[c]
Nitrobacter sp. (25W30N)	0.042	0.48	0.088	5[c]

[a]Light inhibition experiments were conducted in the same way as in Guerrero & Jones (1996a)
[b]% inhibition of NH_4^+ oxidizing activity at 400 nm (25 W m^{-2}) after a 2 h irradiation
[c]% inhibition of NO_2^- oxidizing activity at 400 nm (25 W m^{-2}) after a 4 h irradiation

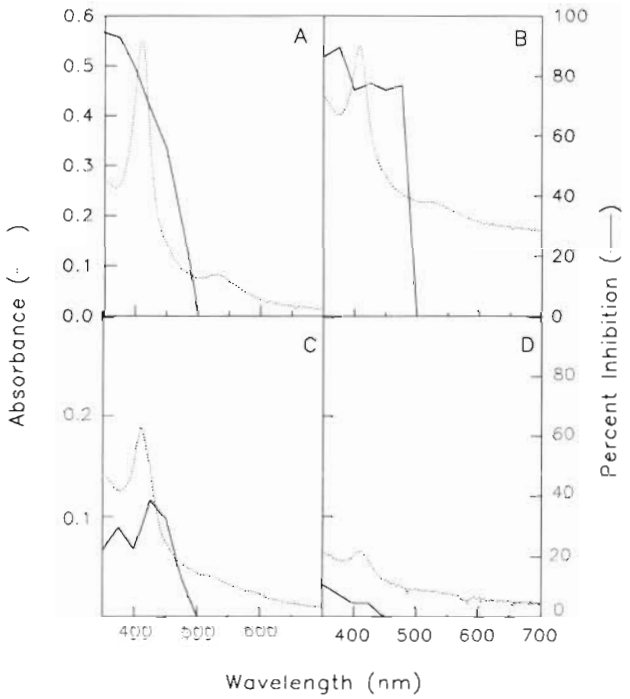

Fig. 3. Action spectrum versus absorbance scan. (A) *Nitrosomonas cryotolerans*, (B) *Nitrosococcus oceanus*, (C) *Nitrococcus mobilis*, (D) *Nitrobacter* sp. Protein concentrations as in Fig. 2A. (——) Percent inhibition; (· · · ·) absorbance scan of cell-free extract. Action spectra are from Guerrero & Jones (1996a)

of the pigment found in cells from the late exponential phase (8 d old batch-culture; Table 2). When batch-cultured cells were used for photoinhibition experiments, the greatest loss in activity was obtained with the older cultures as compared to the younger cultures ($r^2 = 0.84$).

Absorbance change

Light-induced absorbance changes were detected in the pigment found at 408 nm.

By measuring the extent of the absorbance change provoked by different wavelengths (375 to 500 nm) of equal light intensities, it was possible to construct the action spectrum for the light-induced absorbance change. In Fig. 4, absorbance changes are normalized to ΔAbs mg^{-1} cell protein. The action spectrum of *Nitrosomonas cryotolerans* shows a maximum at about 400 nm which closely corresponds to the absorption maximum (408 nm) of the pigment found in both whole cells and crude extracts (Fig. 4A). *Nitrobacter* sp. also showed significant changes in absorbance ($p < 0.05$) effected by the same wavelengths (Fig. 4B). Its action maximum was also found at 400 nm, yet the extent of the absorbance change was not as distinct as for *N. cryotolerans* or *Nitrosococcus oceanus*.

Cytochrome composition

Reduced minus oxidized difference spectra revealed a relatively uniform cytochrome composition for ammonium oxidizers and for nitrite oxidizers. It should be noted that electron transport chains are fairly versatile and vary according to changing conditions such as pH, temperature, pO_2, substrate, etc.; therefore, these results apply only to the growth conditions used in these experiments.

Dithionite-reduced minus oxidized difference spectra of *Nitrosomonas cryotolerans* and *Nitrosococcus oceanus* revealed 4 maxima at 421, 520, 550–552 along with a subtle peak at 603 nm (Fig. 5A, B). The relatively broad peak in the 550–552 nm region along with the absorption maxima at 421 and 520 nm are indicative of *c*-type cytochrome(s). The 603 nm absorption band corresponds to cytochrome *a* (cyt-*a*). The concentration of cyt-*c* as estimated by using published extinction coefficients (Table 3; Smith 1977) was in the range of 8.7 to 13 and 5.7 to 7.5 µmol mg^{-1} cell protein, respectively. Cyt-*a* was found in lower amounts, in the order of 1.7 to 1.9 µmol mg^{-1} cell protein.

Table 2. Culture dependent accumulation of 408 nm pigment. Abs_{408}: absorbance at 408 nm

Organism	Abs_{408}	Protein concentration (mg ml^{-1})	Absorbance mg^{-1} cell protein	Percent inhibition[a]
Nitrosomonas cryotolerans				
3 d (batch)	0.0057	0.045	0.126	50
8 d (batch)	0.0170	0.105	0.161	68
Chemostat	0.1292	0.348	0.67	30
Nitrosococcus oceanus				
3 d (batch)	0.0004	0.037	0.0108	59
8 d (batch)	0.0118	0.092	0.128	69
Chemostat	0.155	0.788	0.196	70

[a]% inhibition after 475 nm irradiation (2 h, 25 W m^{-2})

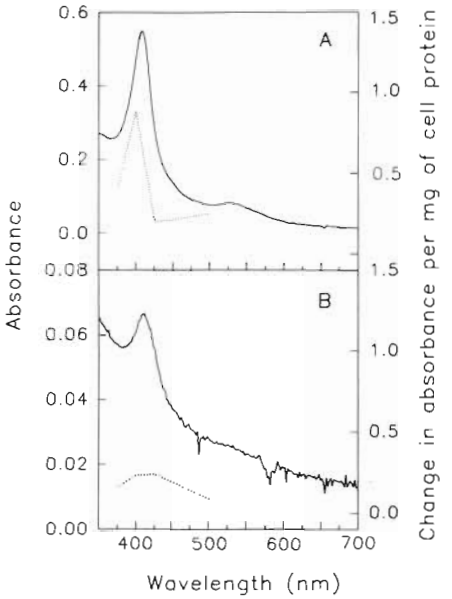

Fig. 4. Action spectrum of the light-induced absorbance change of the 408 nm chromophore versus absorption scans. (A) *Nitrosomonas cryotolerans*; 0.5 mg protein ml^{-1} (B) *Nitrobacter* sp.; 0.48 mg protein ml^{-1}. (——) Absorption spectrum; ( ) change in absorbance per mg of cell protein

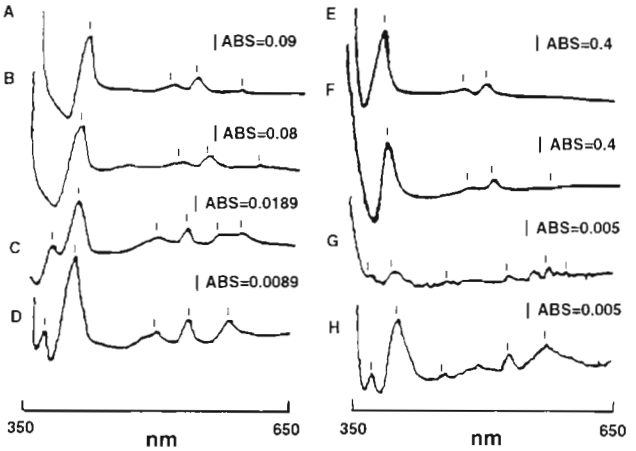

Fig. 5. Dithionite-reduced minus oxidized difference spectra of (A to D) whole cells and (E to H) soluble and particulate fractions. (A) *Nitrosomonas cryotolerans* (0.44 mg ml^{-1}), (B) *Nitrosococcus oceanus* (0.75 mg ml^{-1}); peaks were at 408, 520, 550 and 603 nm. (C) *Nitrococcus mobilis* (0.31 mg ml^{-1}), (D) *Nitrobacter* sp. (0.07 mg ml^{-1}); peaks were at 418, 440, 520, 550 and 590 nm. (E, F) *N. cryotolerans* soluble (E) and crude-membrane (F) fractions; protein concentration: 0.3 mg ml^{-1} (G, H) *Nitrobacter* sp. (25W30N) soluble (G) and crude-membrane (H) fractions; protein concentration: 0.026 mg ml^{-1}. ABS: absorbance

Nitrococcus mobilis and *Nitrobacter* sp. (25W30N) also shared a similar cytochrome constitution (Fig. 5C, D). Dithionite-reduced minus oxidized difference spectra showed absorption maxima at 418 and 440 nm in the soret region and at 520, 550 and 590 nm in the visible region of the spectrum. The absorption bands at 418, 520 and 550 nm correspond to cyt-*c* and the ones at 440 and 590 nm belong to cyt-*a* (DiSpirito et al. 1985).

Nitrite oxidizers exhibited quantitative differences among their cytochrome components. *Nitrobacter* sp. showed more of a cyt-*a* component (2.6 to 4.76 µmol mg^{-1} cell protein) than *Nitrococcus mobilis* (0.68 to 1.77 µmol mg^{-1} cell protein; Table 3). Yet, the cyt-*c* portion in *N. mobilis* was lower (1.8 to 2.4 µmol mg^{-1} cell protein) than in *Nitrobacter* sp. (4 to 6.2 µmol mg^{-1} cell protein). When ammonium oxidizers were fractionated, the precipitate (crude membrane) as well as the soluble (periplasmic + cytoplasmic) portion showed an analogous cytochrome make-up (Fig. 5E, F). In contrast, nitrite oxidizers appeared as having cytochromes preferentially located in the membrane fraction (Fig. 5G, H).

Table 3. Cytochrome composition and concentration (µmol mg^{-1} cell protein). Cyt-*a* reduced-oxidized $\Delta\epsilon$ mM: 20.5/heme; Cyt-*c* reduced-oxidized $\Delta\epsilon$ mM: 17.3

Microorganism	Cyt-*a*	Cyt-*c*
Nitrosomas cryotolerans	1.9	8.7–13
Nitrosococcus oceanus	<1.9	5.7–7.5
Nitrococcus mobilis	0.68–1.77	1.8–2.4
Nitrobacter sp.	2.6–4.76	4–6.2

The dithionite-reduced cell-free extracts of ammonium and nitrite oxidizers confirm the presence of a mostly *c*-type cytochrome in the supernatant of the crude extracts, because of its characteristic absorption maxima at 418, 520 and 550 nm. Without attempting to isolate cytochromes, but by preparing cell-free extracts of the nitrifying bacteria, it was possible to obtain cyt-*c*-like oxidized absorption spectra (Fig. 2B, C) similar to those found by DiSpirito et al. (1985). Thus, this technique provided an easy and dependable way to more precisely look into photoprocesses regarding cyt-*c* in these bacteria.

DISCUSSION

Absorption scans of fresh whole cells or cell-free extracts of nitrifying bacteria showed the presence of a broad peak at 408 nm which had a much higher absorbance than any other wavelength in the 300 to 500 nm range. The 408 nm absorption maximum was found in different proportions in all 4 species tested. Typically, there was a corresponding greater blue light sensitivity due to increasing pigment concentra-

tions (Table 1). One of the potential chromophores for this region of the spectrum are porphyrins. Porphyrins are universal in aerobic organisms inasmuch as cells have hemes, cytochrome and cytochrome oxidase. Porphyrins are also characterized by strong absorption maxima in the soret region (400 to 420 nm; Giese 1971, Jagger 1983); therefore, it was speculated that a porphyrin-containing pigment, such as a cytochrome, may act as the photoreceptor. Earlier work by Bock (1965) postulated that cytochrome-*c* specifically was affected by light, as it was 'photooxidized' in both *Nitrosomonas* sp. and *Nitrobacter* sp. after illumination. The light fluences used, however, were very high (>50 W m^{-2}) and this makes 'photobleaching' a more likely explanation (Müller-Neuglück & Engel 1961, Epel & Butler 1969). High light doses have been shown to provoke pigment photobleaching in other systems; *Zea mays*, *Neurospora crassa* (Brain et al. 1977), and *Prototheca zopfii* (Epel & Butler 1969). It is safe to assume that the fluences used in this work did not lead to photobleaching since dark recovery of nitrifying activity occurred previously (Guerrero & Jones 1996a, b) when using identical light doses.

Prior to this study soluble and membrane-bound cytochrome-*c* had been characterized among nitrifiers (Miller & Wood 1982, Tanaka et al. 1983, DiSpirito et al. 1985, Kirstein et al. 1986, Tsai & Tuovinen 1989, Hooper et al. 1991). The cell-free extract system used in this work showed properties very similar to the above cited purified oxidized or reduced cyt-*c* fraction (DiSpirito et al. 1985; Fig. 2B, C). When these cyt-*c* like fractions (cell-free extracts) were used to detect light-induced absorbance changes, the most distinct changes in absorbance were observed at approximately 400 nm for both *Nitrosomonas* sp. and *Nitrobacter* sp., the difference being that the extent of the absorbance change was always greater for ammonium oxidizers (Fig. 4A, B). Notably, changes in absorbance were more distinct in cell-free extracts than in whole cells, which may be a reflection of the loss of reducing agents normally found within intact cells as opposed to when the cells are broken. These results demonstrate that at low light intensities (i.e. <25 W m^{-2}), a ubiquitous cyt-*c*-like compound in the periplasm of nitrifying bacteria can become photooxidized and that this effect is reversible. *c*-Cytochromes of ammonium oxidizing bacteria are characteristically part of multiheme proteins, such as hydroxylamine oxidoreductase (HAO) and cyt-c_{554} (Hooper et al. 1991). Since *c*-cytochromes have both a catalytic (HAO) and electron-transfer role (cyt-c_{554}), growth of ammonium oxidizers in light may be affected by inhibition of cytochrome function and not by cytochrome destruction. Consequently, photoinactivation with blue light would depend on both: light dose and/or abundance of soluble cyt-*c* in the cells (Table 3).

Abundance of the chromophore (c-heme) corresponded to growth phase. Its accumulation is greater during the late exponential phase. Ecologically this might be significant, since cells from an early exponential phase of growth would be more light-tolerant than cells from subsequent stages. Therefore, conditions that favor exponential growth (waste-water treatment plants) are not expected to show major photoinhibition of nitrification. The opposite would be expected in oligotrophic waters such as the open ocean.

Under autotrophic growth conditions, ammonium oxidizers had a fairly uniform cytochrome composition with a broad peak for the cyt-*c* (550 to 554 nm) component and a barely detectable peak representing cyt-aa_3 (600 to 605 nm). Nitrite oxidizers, on the other hand showed 3 distinct components: cyt-*c*, cyt-a_3 (589 nm) and cyt-aa_3. The major difference found among the nitrite oxidizers was the ratio of cyt-*a* to cyt-*c* (greater for *Nitrobacter* sp. than for *Nitrococcus mobilis*). The variability in respiratory chains among soil *Nitrobacter* spp. has been recognized (Kirstein et al. 1986), especially with respect to different *b*-type cytochromes (558 to 564 nm). These *b*-type cytochromes have been reported from both autotrophically and heterotrophically grown cells, yet this study did not detect *b*-type cytochromes in marine nitrite oxidizers grown chemoautotrophically. These findings did confirm the presence of *a*-type cytochromes, which have been described in autotrophic and mixotrophic cells. With 1 exception (cyt-*b* in nitrite oxidizers), the cytochrome composition of both types of nitrifying bacteria in this study agrees with previous reports. What cannot be ascertained is whether this is the prevalent pigment composition in natural habitats. Cytochrome analyses involve high cell biomass, and this is the primary problem when working with nitrifying bacteria due to their very low maximum specific growth rate and low growth yields. This obliges the physiologists working on nitrifiers to keep cultures growing at near-maximum specific growth rates, which by itself limits extrapolation of laboratory results to field conditions. Yet, until more sensitive methods of detecting respiratory pigments are developed, these results can at least indicate which cytochromes when present are suggestive of photosensitivity in ammonium oxidizers, or of phototolerance in nitrite oxidizers.

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