Applicability of the MTT assay for measuring viability of cyanobacteria and algae, specifically for *Microcystis aeruginosa* (Chroococcales, Cyanobacteria)

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Several assay methods were screened for viability assessment in cyanobacteria using *Microcystis aeruginosa* FACHB 905. Compared with fluorescent diacetate (FDA), Evan's Blue and autofluorescence, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, which was based on the ability of viable cells to reduce MTT to formazan, was found to be reliable and was selected for further study. MTT concentration, incubation time and temperature were optimized for *M. aeruginosa*. Improvements to the sensitivity and reproducibility of the MTT assay included performing it in the dark to reduce the effects of formazan light sensitivity when extracted in DMSO. Another improvement involved collecting viability data by cell by counting rather than colourimetrically, which was concluded from the fact that oxidoreductase activity, responsible for MTT reduction, would elevate or decrease under stress conditions. Half-life of oxidoreductase in dead cell was calculated to be 3 h. The MTT assay was also found to be applicable to other cyanobacteria and diatoms, including field samples, but not for algae belonging to Chlorophyta, Euglenophyta, Pyrrophyta or Chrysophyta. Based on the above results, we proposed an optimized procedure for the MTT method on *Microcystis* strains. The use of this assay may be of importance to better understand the dynamics of bloom and the fate of *Microcystis* under natural or disturbed conditions.

Key Words: Algae, Oxidoreductase, *Microcystis*, MTT, Viability assessment

INTRODUCTION

Water blooms have attracted much attention in recent years. However, in contrast to the intense efforts to elucidate the conditions, mechanisms and strategies that control phytoplankton cell growth, relatively little attention has been focused on phytoplankton death (Bidle & Falkowski 2004). Loss via cell death has been poorly understood primarily because of the lack of a direct means of measuring viability (Lee & Rhee 1997). In addition, questions about how many cells had overwintered successfully and would resuscitate to form blooms as seeds, whether an algaecide was effective, and when a bloom would decline remain unanswered. To address these questions, a reliable viability assessment method for cyanobacteria and algae is necessary.

The classical viability definition is based on reproducibility. Cell viability could also be reflected by changes in morphology, motility or the membrane or physiological state, including production of enzymes for healthy metabolism, such as esterase and oxidoreductase, the ability to pump out or exclude certain dyes or levels of macromolecules important for life, such as ATP, proteins and nucleic acids (reviewed by Breeuwer & Abee 2000; Keer & Birch 2003).

In an effort to define a reliable viability assessment method for cyanobacteria and algae, Dorsey *et al.* (1989) established that a relationship existed between fluorescent diacetate (FDA) conversion rates and photosynthetic capacity. Pouneva (1997) recommended chlorophyll autofluorescence as the most convenient method for viability assessment. Markelova *et al.* (2000) suggested tetrazolium compounds could be useful for cyanobacterial viability assessments. Brussaard *et al.* (2001) and Agusti & Sanchez (2002) developed methods based on membrane permeability and applied them for viral infection and field samples, respectively.

Tetrazolium salts have been used extensively in determining the viability of cells, from bacterial to mammalian (Kairo *et al.* 1999), including spores (Stentelaire *et al.* 2001). They have been widely used in anticancer drug research to investigate cytotoxic and cytostatic effects on cancer cell lines and tumour cells (Berridge *et al.* 2005). The reduction of tetrazolium salts from colourless or weakly coloured, aqueous solutions to brightly coloured formazan has been the basis of their use as vital dyes in biochemical applications. Lately, experimental data have suggested that 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and other positively charged tetrazolium salts are primarily reduced not by the traditionally accepted succinate dehydrogenase but by oxidoreductase enzymes, the majority of which utilize the reduced pyridine nucleotide NADH (Berridge *et al.* 2005).

The application of the tetrazolium salts method in cyanobacteria or algae is seldom reported. In the present study, applicability of the MTT assay for this class of microorganism was explored for viability assessment, particularly for the toxin-producing species *Microcystis*

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aeruginosa. Besides optimization of the MTT assay procedure in *M. aeruginosa*, the half-life of oxidoreductase in dead *M. aeruginosa* cells and response of MTT reduction to stresses were studied. Furthermore, the applicability of the MTT assay to other cyanobacteria and algae was investigated.

MATERIAL AND METHODS

Culture conditions

Microcystis aeruginosa FACHB 905 was cultured in 1/2 BG-11 medium at 25°C under an illumination of 20–25 μmol m^{-2} s^{-1} (LI-COR LI-185B, USA) with a cycled photoperiod of 12 h light : 12 h dark. Cultures grown for 7–12 d, after inoculation into 250-ml flasks, were assumed to be active growing. To obtain heat-shocked dead cells, the cultures were boiled for 10 min.

Staining and viability calculation

During methods screening, FDA, Evan's Blue, autofluorescence and the MTT methods were compared using active growing and heat-killed *M. aeruginosa*. The commonly used algaecide $CuSO_4$ was also tested at a concentration of 0.15 mg l^{-1} treated culture. MTT (Amresco, USA) was prepared in PBS (pH 6.8) at a concentration of 0.5 mg ml^{-1} with 0.1 mol l^{-1} sodium succinate. Evan's Blue (Sigma, USA) solution was prepared at 4% in glycerol, and FDA (Sigma) was prepared in acetone at 5 mg ml^{-1}. The working concentrations of these reagents were 0.14 mg ml^{-1}, 0.25%, and 0.03 mg ml^{-1}, respectively. Stock solutions of MTT and Evan's Blue were stored at 4°C, and FDA was stored at −20°C. A 200-μl sample of culture was used for MTT, Evan's Blue or FDA staining. Samples were washed and suspended in 200 μl BG-11 medium before staining. After incubation, the dyes were removed with centrifugation at 8228 × *g* for 3 min. The pellets were resuspended in 200 μl distilled water.

MTT and Evan's blue staining were performed at 25 ± 1°C in an illuminating incubator for 2 h and 1 h, respectively, and 8 μl of each suspension were examined with a haemacytometer using light microscopy (Olympus CX41, Japan). At least 300 cells were observed in each.

FDA staining was carried out at 25 ± 1°C for 5 min in the dark and was detected by flow cytometry (Beckman Coulter Epic Altra, USA) or using a fluorescence microscope (Olympus BX41, Japan) equipped with a GFP filter set (exciter 395/40 nm, emitter 510/40 nm) (Chroma Technology Corp., USA). Cell concentrations were diluted to 10^6 cells ml^{-1} before detection using flow cytometry. The power output was 15 mW, and a wavelength of 488 nm was used to excite the fluorescent probes. Green fluorescence was measured through a 535 ± 10 nm bandpass filter (Jochem 1999), and at least 1×10^5 cells were collected. A value of 2 relative units was assigned based on the value of the heat-killed culture to separate live cells from dead ones. Cells with fluorescence readings above that value were considered FDA positive. Autofluorescence was visualized using a separate filter set (exciter 510/50 nm, emitter 570/90 nm).

Conversion of FDA to fluorescein relies on esterase activity in live cells, and conversion of MTT to formazan relies on oxidoreductase activity; therefore, for these assays, viability is equivalent to the ratio of positive cells to total cell numbers. Evan's Blue is membrane impermeable. Compromised membranes are implied in cells coloured with Evan's Blue; thus, the ratio of Evan's Blue negative cells to total cell numbers reflects culture viability.

Optimization of the MTT colourimetric assay for *M. aeruginosa*

In our initial MTT colourimetric assay, 100 μl MTT stock solution (0.14 mg ml^{-1} MTT) were added to a 250-μl sample, and the sample was incubated at 25 ± 1°C. After incubation, supernatant was removed, and 1 ml DMSO was added to dissolve the formazan crystals fully. The absorbance was measured after centrifugation at a wavelength of 556 nm with a spectrophotometer (UNICO UV2000, China). The staining intensity was expressed with the A_{556} when 1.87×10^6 cells were treated.

In our optimization experiments, the MTT assay was performed at different temperatures (10, 25 and 35°C), different MTT concentrations (0.019, 0.037, 0.068, 0.121 and 0.188 mg ml^{-1}) and with different incubation time (15, 30, 45, 60, 90, 120, 180 and 240 min). Finally, the relationship between the staining intensity and the percentage of viable cells (mixing active growing cells and heat-killed cells) was studied using the optimized procedure.

Determination of oxidoreductase half-life in dead *M. aeruginosa*

Microcystis aeruginosa exposed to 0.25 mg l^{-1} $CuSO_4$ for 12 h in a 4°C refrigerator were unable to survive in fresh 1/2 BG11 medium even under optimal conditions. Therefore, the exposed sample was assumed to be dead though more than half the cells tested MTT positive. A_{556} was determined after the exposed culture was transferred into fresh 1/2 BG11 medium in a 25°C incubator with 25 μmol photon (PAR) m^{-1} s^{-1} photon irradiance. The declining absorbance curve in dead *M. aeruginosa* was simulated with the formula $[Et] = y_0 + [Ei]\ e^{-kdt}$, where $[Et]$ is the enzyme activity at time t, $[Ei]$ is the initial enzyme activity, k_d is the rate of activity decay and t is time. $T_{1/2}$ was equal to $0.693/k_d$.

Response of oxidoreductase to stresses

The responses of oxidoreductase activity to temperature, nitrogen or phosphorus limitation and dark or formaldehyde exposure were studied. Nitrogen- or phosphorus-limited cells were obtained by inoculating samples of culture into corresponding nutrient-free media. In the nitrogen-depleted medium, $NaNO_3$ was omitted, and $FeC_6H_5O_7$ was used in place of $Fe(NH_4)_3(C_6H_5O_7)_2$. In the phosphorus-free medium, K_2HPO_4 was omitted. Dark conditions were obtained by wrapping the flasks with silver paper. The formaldehyde concentration was 0.0015%.

Table 1. Application of MTT in other algae. "+" indicates that the species could be stained with MTT; "–"indicates that the species could not be stained with MTT.

Species	Strain	Staining
Cyanophyta		
Anabaena sp.	PCC7120	+
Gloeocapsa alpicola	FACHB400	+
Merismopedia sp.	FACHB286	+
Oscillatoria tenuis	FACHB247	+
Phormidium mucicola	FACHB723	+
Synechocystis sp.	PCC6803	+
Chlorophyta		
Chlamydomonas reinhardtii	FACHB479	–
Chlorella pyrenoidosa	FACHB9	–
Dunaliella parva	FACHB815	–
Haematococcus pluvialis	FACHB712	–
Pediastrum sp.	FACHB721	–
Scenedesmus obliqnus	FACHB416	–
Selenastrum capricornutum	FACHB271	–
Bacillariophyta		
Cyclotella meneghiniana	FACHB739	+
Fragilaria sp.	FACHB218	+
Melosira granulate var. *angustissima*	NIES333	+
Navicula incerta	FACHB371	+
Nitzschia palea	FACHB204	+
Others		
Euglena gracilis	FACHB848	–
Euglena spirogyra	FACHB922	–
Glenodinium sp.	FACHB328	–
Porphyridium purpareum	FACHB806	–
Prymnesium parvum	FACHB923	–

The MTT test applied to field samples and other cyanobacteria and algae

Tests were performed to investigate whether other cyanobacteria and algae would present obvious differences after MTT incubation under light microscopy. Field samples were collected from Dianchi (Yunnan, China), a typical heavy eutrophicated lake. Twenty-three pure cultures of cyanobacteria and algae, including six Cyanobacteria, seven Chlorophyta, five Bacillariophyta, two Euglenophyta, one Chrysophyta, one Pyrrophyta and one Rhodophyta were also tested (Table 1). All of them were obtained from FACHB (Freshwater Algae Culture Collections, Institute of Hydrobiology, Chinese Academy of Sciences). The assay conditions were the same as the optimized MTT assay conditions described in the Results section with the exception that the incubation time was 2 h to make the tested strain reacted with MTT fully. After incubation with MTT, the culture was observed under light microscopy to make a judgment of whether formazan aggregated obviously in the cell.

Data analysis

All experiments were performed in three replicates. Data in this study are presented as means ± standard deviations (s) and analyzed using Microcal Origin Software (Version 6.0, Microcal Software Inc.) Significant analyses were performed when investigating response of oxidoreductase to stresses. Differences by analysis of variance between treated samples and control were considered to be significant at $P < 0.05$.

RESULTS

Screening of viability assessment methods using *M. aeruginosa*

For active growing cells, FDA, Evan's Blue, autofluorescence and MTT methods gave similar estimates of viability, the result from FDA being only slightly lower (Fig. 1). Observations under fluorescence microscopy also demonstrated that some cells with strong autofluorescence presented less FDA green fluorescence. The same result

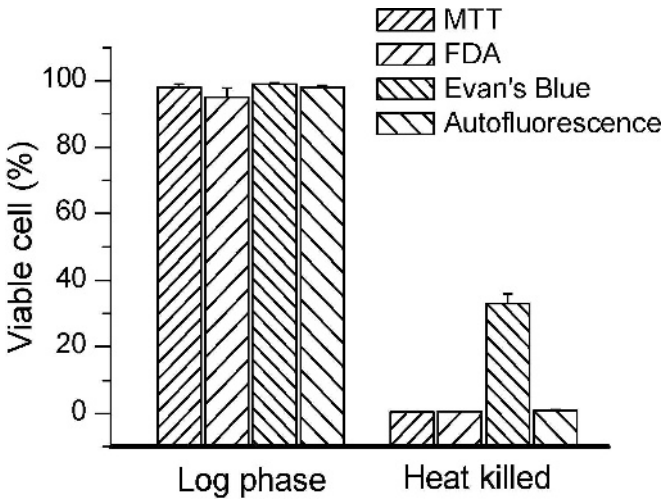

Fig. 1. Viability assessment with FDA, Evan's Blue, autofluorescence and MTT for active growing cell and heat-killed sample.

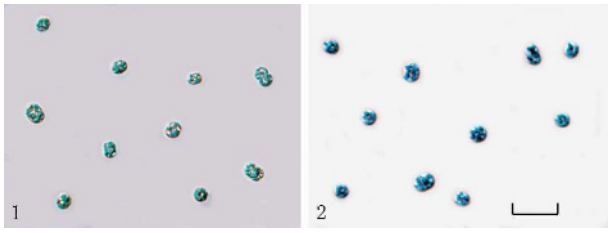

Fig. 2. Active growing *M. aeruginosa* under light microscopy (1) without staining. (2) Stained with MTT. Bar = 10 μm.

was obtained with flow cytometry. In contrast, the MTT reduction product, blue formazan, was clearly discernible in viable cells (Fig. 2).

For the heat-treated *M. aeruginosa*, Evan's Blue gave an obviously higher estimation of viability. All the other methods suggested that most of the cells were dead. However, 32% of the sample was Evan's Blue negative (Fig. 1), suggesting those cells had intact membranes and should be viable. Even when the concentration of Evan's Blue was elevated to 0.5% or if staining was performed after 1 d, 20% of cells still tested negative.

Evan's Blue also overestimated viability in the $CuSO_4$ (0.15 mg/L)-treated sample. After 8 h of $CuSO_4$ treatment, only 66% of the cells were viable according to the FDA and MTT methods. The same percentage of viable cells was recorded at 24 h. Evan's Blue gave an estimation of 87% at 8 h and decreased to about 70% 24 h later. Autofluorescence estimated lower viability than Evan's Blue but higher than FDA and MTT (Fig. 3).

Optimization of the MTT colourimetric assay for *M. aeruginosa*

SELECTION OF AN EXTRACTION SOLVENT FOR MTT FORMAZAN: To quantify MTT staining intensity, it was necessary to establish a standard colourimetric assay. Formazan was much more stable dissolved in isopropanol than in DMSO, but extraction with isopropanol left blue crystals, even after mixing for 10 min. In contrast, the formazan was almost completely dissolved in DMSO within 2 min. However, we found that formazan dissolved in DMSO was light sensitive. Under illumination of 35 μmol m^{-2} s^{-1} at 35°C, formazan had a half-life of 8 min, and the A_{556} decreased from 0.820 to 0.156 (Fig. 4). However, under dark conditions, the A_{556} did not decrease at all for at least an hour. We selected DMSO as the extraction solvent based on the rapid extraction ability and the stable properties in the absence of light.

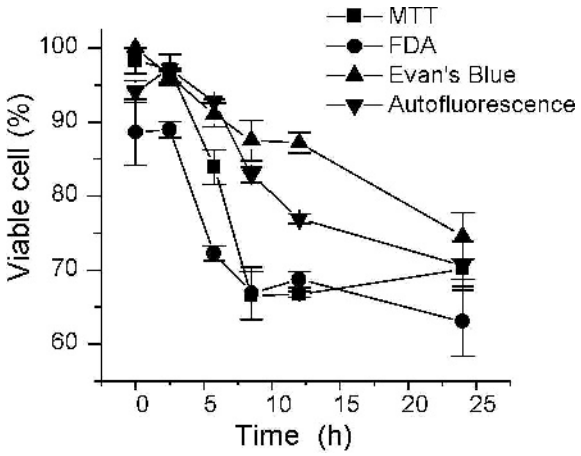

Fig. 3. Decrease of viability in *M. aeruginosa* after 0.15 mg l^{-1} $CuSO_4$ exposure evaluated with FDA, Evan's Blue, autofluorescence and MTT.

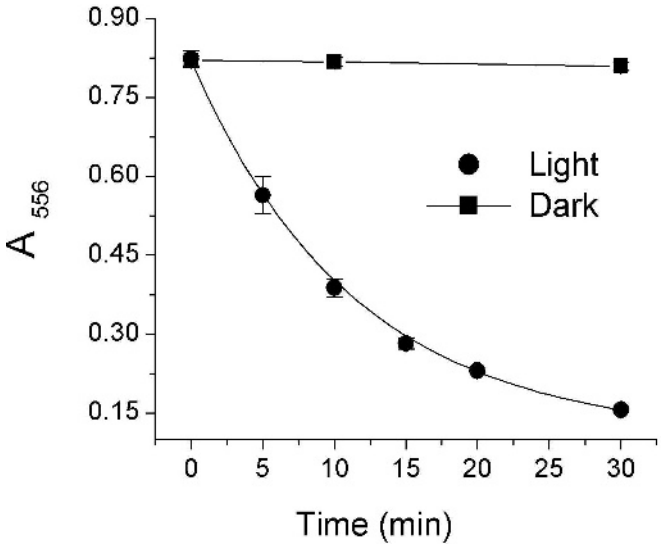

Fig. 4. Decline of DMSO-dissolved formazan. The extraction process was performed under light or dark.

OPTIMIZATION OF INCUBATION TEMPERATURE, MTT CONCENTRATION AND INCUBATION TIME: The MTT reduction reaction was dependent on incubation temperature. The mixture incubated at 35 ± 1°C showed the highest staining intensity, and the lowest was obtained at 10 ± 1°C (Fig. 5–1). The staining intensity at 35 ± 1°C was about twice that measured at 25 ± 1°C.

The staining intensity increased proportionally with concentration in the range of 0–0.07 mg/ml^{-1} (Fig. 5–2). By plotting these data according to the Lineweaver-Burk representation ($y = 1.38\times/(0.027 + x)$, $R^2 = 0.98$), a Michaelis-Menten constant (Km) of 0.027 mg ml^{-1} was determined. A concentration of 0.1 mg ml^{-1} MTT was adopted for subsequent experiments.

As shown in Fig. 5–3, the staining intensity for a suspension of 7.5×10^6 cells ml^{-1} of *M. aeruginosa* was linear for up to 2 h incubation ($R^2 = 0.9992$). Considering the optimum absorbance range for linearity (0.1–1) in most spectrophotometers, 1 h was a long enough duration for the assay, and the deposited formazan was also enough to detect by light microscopy.

In the finalized assay procedure, 250-μl samples were combined with 60 μl MTT stock solution and incubated at 35 ± 1°C for 1 h. According to our results, the relationship between A_{556} and viable *M. aeruginosa* was linear from 2.5×10^6 to 1.5×10^7 cells ml^{-1} (Fig. 5–4).

Response of oxidoreductase to stresses

Under light microscopy, more formazan was observed in most of the cells grown at 10°C than in cells cultured at

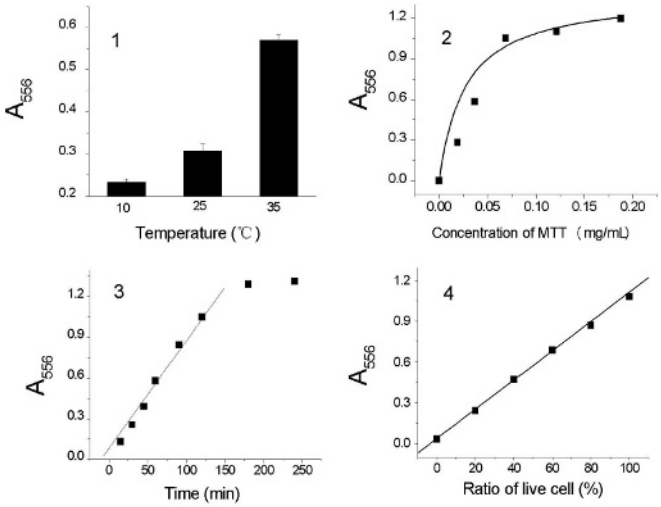

Fig. 5. Optimization of MTT assay with *M. aeruginosa*. (1) Effect of incubation temperature on staining intensity. 250 µl 7.5×10^6 cells ml^{-1} incubated for 1 h. (2) Relationship between staining intensity and MTT concentration. 250 µl 7.5×10^6 cells ml^{-1} incubated for 2 h. (3) Dependence of staining intensity on incubation time. 250 µl 7.5×10^6 cells ml^{-1} was used. (4) Linear regression between staining intensity and the percentage of viable cell. 250 µl 1.5×10^7 cells ml^{-1} incubated for 1 h. All the reactions were incubated at 35 ± 1°C with the exception of temperature test.

25°C. Based on this phenomenon, we compared the A_{556} values for cultures grown at different temperatures.

Our results showed that cells cultured at 10°C, with a lower growth rate (Fig. 6–1), produced over 30% more formazan than those grown at 25°C, while cells at 35°C, having the highest growth rate, produced the least formazan (Fig. 6–2). MTT-reducing activity was the lowest when cells were incubated at 10°C (Fig. 5–1). We proposed that cells grown at 10°C express more oxidoreductase to offset their weakened ability to transport electrons at lower temperatures.

Response of oxidoreductase was also studied in cells suffering from other stresses (Fig. 7). Under dark conditions, more than 95% of the cells were MTT positive, but A_{556} decreased significantly ($P < 0.01$) in the first 12 h and remained low. A_{556} also decreased immediately when cultures were exposed to 0.0015% formaldehyde ($P < 0.05$). No significant increase was observed before the decrease occurred in nitrogen-depleted cultures, but A_{556} increased significantly after 28 h in phosphorus-depleted medium, suggesting that the cells might increase oxidoreductase activity to survive the phosphorus limitation ($P < 0.05$).

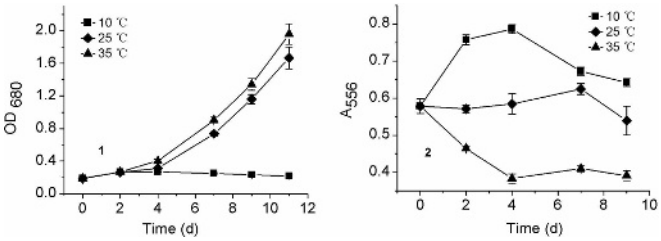

Fig. 6. (1) Growth curve and (2) staining intensity in *M. aeruginosa* cultured at 10°C, 25°C and 35°C.

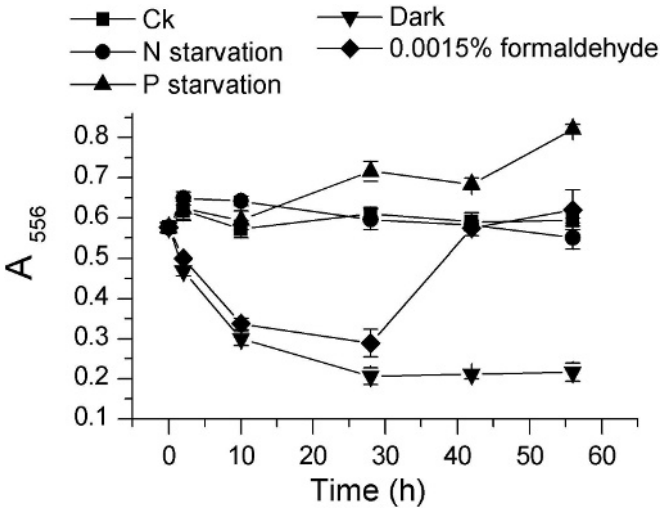

Fig. 7. Response of oxidoreductase to stress condition.

Half-life of oxidoreductase in dead *M. aeruginosa*

The A_{556} of *M. aeruginosa* exposed to 0.25 mg/L $CuSO_4$ for 12 h at 4°C decreased from 0.42 to 0.04 in less than 10 h (Fig. 8). The formula $[Et] = -0.03105 + [Ei]e^{-0.232t}$ ($R^2 = 0.9826$) fit the curve well. The rate of decay (k_d) and the half-life ($t_{1/2}$) were 0.232 h^{-1} and 3 h, respectively. The 3-h half-life ensured a low deviation from the true value due to false MTT positive results.

Application of the MTT assay to field *Microcystis* samples, other cyanobacteria and algae

MTT staining could be extended to field *Microcystis* samples. Field *Microcystis*, including *M. aeruginosa, M. viridis* and *M. wesenbergii*, could all be stained with MTT. Most of the colonies were definitively MTT positive under microscopic inspection. However, portions of the colonies were hard to clearly determine because of interference from gas vesicles. According to our observations of 23 strains of cyanobacteria and algae, the MTT method could be applied to all the tested Cyanobacteria, Bacillariophyta and Rhodophyta but was

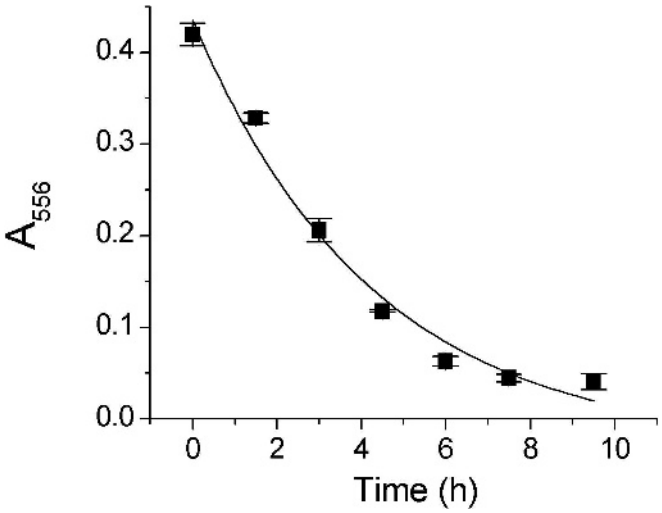

Fig. 8. Decline of oxidoreductase activity in dead *M. aeruginosa*.

not ideal for algae belonging to Chlorophyta, Euglenophyta, Chrysophyta and Pyrrophyta (Table 1).

DISCUSSION

The MTT assay was previously suggested to be a sensitive and reliable method for measuring cell viability. We compared this assay with FDA, Evan's Blue and autofluorescence and found the MTT assay to be a relatively reliable and easily performed viability assessment method for *M. aeruginosa*.

The formazan extraction process was crucial to the accuracy and reproducibility of the MTT colourimetric method. DMSO had been suggested as the best solvent for dissolving formazan (Twentyman & Luscombe 1987), but the effect of light on the DMSO-dissolved formazan was seldom considered, possibly because many results were read out quickly with an ELISA reader. DMSO-dissolved formazan had a half-life of 8 min under 35 $\mu mol\ m^{-2}\ s^{-1}$ light intensity at 35°C. This rapid decline could be expected to lead to an underestimation of staining intensity. Protection from light should be ensured during the extraction process.

The *M. aeruginosa* cells appeared to increase their oxidoreductase activity in response to stressful environments. This has also been reported for other cell cultures (Sieuwerts *et al.* 1995; Zhang & Cox 1996; Bernhard *et al.* 2003) but has never been reported in cyanobacteria or algae. Capasso *et al.* (2003) compared viability of nitrogen-limited, 0.0015% v/v formaldehyde-treated and 0.5% v/v acetic acid–treated *Dunaliella viridis* with that of active growing cultures, employing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenil)-2H-tetrazolium (MTS), another tetrazolium salt. As a result, reduction of tetrazolium MTS to formazan proceeded at a much lower rate in stressed cultures than in healthy cultures. According to our results, tetrazolium MTT reducing ability decreased after 0.0015% v/v formaldehyde exposure but would increase at low temperatures and upon exposure to phosphorus-depleted conditions.

The MTT method is often performed colourimetrically, and viability is expressed by the ratio of A_{556} in test cultures to the value in healthy cultures (Gaboriau *et al.* 1997). It was, indeed, alluring to perform viability testing using the colourimetric method, especially for material such as a field colony of *Microcystis*. For our assay, we had to break apart the cells into single cells before viability estimation by cell counting, but this step is not necessary if estimating colourimetrically. However, the elevated oxidoreductase hinders this method. If oxidoreductase expression was higher under stress (low temperature or phosphorus starvation) and even part of the cells became MTT negative, the A_{556} still indicated higher values than in the healthy culture and gave a much higher estimation of cell viability. In the case of decreased oxidoreductase activity, such as under dark conditions and at high temperatures (35°C), A_{556} decreased considerably in 2 d, but more than 95% of the cells were still MTT positive. That is, they were viable and would divide and propagate after the stress was withdrawn. In light of this consideration, we strongly recommend that viability based on MTT reduction be calculated by cell counting.

The elevated oxidoreductase levels would also lead us to believe that cells rich in formazan were in a healthy state when observed by light microscopy. No doubt that they were viable, but in fact they were under stress. Kept under stress conditions, they appeared to enter death phase and become MTT negative, as more MTT negative cells were observed in 10°C cultures than in 25°C culture (data not shown). Either way, oxidoreductase in the dead *M. aeruginosa* at 25°C had a half-life of about 3 h, which ensured that the number of false positives was moderately low.

FDA was effective for *Thalassiosira, Dunaliella, Emiliania* and *Chlorella* spp. but not for *Synechococcus, Phaeodactylum* or *Prochlorococcus* spp. (Agusti & Sanchez 2002). Methods based on membrane permeability like Evan's Blue failed to differentiate dead cells under some stresses such as CO_2 limitation (Vardi *et al.* 1999) or nitrogen starvation (Sauer *et al.* 2001) or, as in this study, exposure to heat and $CuSO_4$. MTT also had its limitations as an assay. First, MTT was restricted as to the range of species it was suitable for testing. It was not applicable for *Chlorophyta, Euglenophyta, Chrysophyta* and *Pyrrophyta* spp. In addition, when cultures were grown above 30°C, less formazan was aggregated. It would be exhausting to differentiate the dead cells under light microscopy. Finally, for some field samples, gas vesicles distributed widely in single cells interfered with our observations. Though phycoerythrin was suspected to disturb the reaction, the obvious difference under light microscopy between *Porphyridium purpureum*, one strain of Rhodophyta rich in phycoerythrin, and its MTT treated samples excluded our suspicion. Regardless of these shortcomings, the MTT method was inexpensive, simple and relatively reliable compared to other assays. It was also rapid to perform and would be an effective tool for cyanobacteria and algae viability assessment when applied for species such as Bacillariophyta and Rhodophyta.

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