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Coomassie Blue G250 for Visualization of Active Bacteria from Lake Environment and Culture

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Abstract

Bacteria play a fundamental role in the cycling of nutrients in aquatic environments. A precise distinction between active and inactive bacteria is crucial for the description of this process. We have evaluated the usefulness of Coomassie Blue G250 for fluorescent staining of protein containing potentially highly active bacteria. We found that the G250 solution has excitation and emission properties appropriate for direct epifluorescence microscopy observations. It enables fast and effective fluorescent visualization of living, protein-rich bacteria, both in freshwater environment and culture. Our results revealed that the number of G250-stained bacteria from eutrophic lake was positively correlated with other standard bacterial activity markers, like number of bacteria containing 16S rRNA, bacterial secondary production or maximal potential leucine-aminopeptidase activity. In case of the *E. coli* culture, the percentage of bacteria visualized with G250 was similar to that of bacteria which accumulated tetracycline. Compared to other common methods utilizing fluorogenic substances for bacteria staining, the approach we evaluated is inexpensive and less hazardous (for example mutagenic) to the environment and researchers. It can be regarded as an additional or alternative method for protein rich, active bacteria staining.

K e y w o r d s: active bacteria, aquatic bacteria, aquatic environment, bacterial culture, Coomassie Blue G250, fluorescence

Introduction

Coomassie Blue G250 is commonly used for the detection and visualization of proteins. It is the main chemical compound in the classical colorimetric Bradford assay (Bradford, 1976). The binding ability of G250 to proteins has been used for years for quantification of the concentration of proteins separated on polyacrylamide gels (Neumann *et al.*, 1994) or for the observation of protein rich particles under light microscope (CSP – Coomassie Staining Particles, Long and Azam, 1996). For protein detection not only absorbance of light by G250, but also its fluorogenic properties were used. For example Luo *et al.* (2006) proposed to use infrared fluorescence emission (excitation at 550 nm, emission at 720 nm) of G250 for protein detection after electrophoresis or western blotting.

Because of the high affinity of G250 to proteins, it might be, theoretically, a promising factor for the staining of bacterial cells containing proteins. Coomassie blue G250 should be particularly effective in staining live, active microorganisms with a high concentration of proteins such as ribosomal proteins of translationally active cells, structural proteins, enzymes of various metabolic pathways, and proteins placed in the cell membrane, outer membrane, cell wall and periplasmic space. Conversely, the debris of dead microorganisms, like for example empty bacteria envelopes, "cell ghosts", detected first by Zweifel and Hagström (1995), should be stained ineffectively because of their low protein content – the effect of fast protein degradation. Degradation rate in the case of dead bacterial cells exceeds 10% of intracellular proteins per hour (Gottesman and Maurizi, 1992).

There are several methods for the detection of active microorganisms. The most important of them are based on: the intracellular reduction of CTC (5-Cyano-2,3-ditolyl tetrazolium chloride), the detection of membrane integrity by LIVE&DEAD test, DNA staining by DAPI followed by propanol washing and staining by anionic or cationic membrane-specific dyes (Rodriguez *et al.*, 1992; Suller and Lloyd, 1999; Luna *et al.*, 2002; Zweifel and Hagström, 1995). However, the precise distinction of living, active bacteria from inactive ones is still an important subject of research. In addition, a majority of widespread methods of microorganism staining involve

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the use of chemical compound binding to nucleic acids, and are thus highly carcinogenic, posing a threat to researchers and the environment.

According to our knowledge, G250 is not commonly used for the direct visualization of the planktonic microorganisms. The reason for this could be that the absorbance of Coomassie Blue is inadequate for a quality visualization of small microorganisms, however it can be used for showing large protein-rich biofilms structure (Larimer *et al.*, 2016). The observed infrared light emission by Coomassie Blue (Luo *et al.*, 2006; Carlsson *et al.*, 2012) is not useful for visual observation and inconvenient for digital camera detection. However, our findings suggest that the fluorescence exhibited by this dye in the visible range of light spectrum could be promising for this purpose.

The main goal of this article was to evaluate the usefulness of G250 for the fluorescence staining of protein rich, potentially highly active bacteria. In particular: 1) characterization n of the fluorescence spectrum of Coomassie Brilliant Blue G250 in the visual light spectrum emission range; 2) testing the bacteria staining procedure on the *Escherichia coli* culture and natural aquatic microorganisms community; 3) comparing the effectiveness of bacteria staining by G250 with other methods commonly used for the evaluation of bacteria abundance and activity.

Experimental

Material and Methods

Study area and sampling. Environmental samples were taken from the pelagial zone of the deepest part of eutrophic Lake Mikołajskie (26 m max. depth, N 53°47'31.2", E 21°34'56.3") during two years of research in July, September 2010 and August 2011. The water was collected from six depth layers: 1) 0.5–2 m, 2) 3–5 m, 3) 7–9 m, 4) 11–13 m, 5) 16–18 m, and 6) 21–23 m. Within each layer, three one-liter subsamples were taken every 0.5 m and mixed together. Samples were subjected to further laboratory analyses within 2 h. To avoid the problem of underestimation of the number of attached bacteria, all the lake water samples were filtered through 3 µm polycarbonate filter (Millipore) to obtain sample fractions containing mostly free-living bacteria.

For *E. coli* staining with G250 and tetracycline, 3 days old LB broth culture of *E. coli* K12 strain, sensitive to tetracycline was used (incubation temp. 36°C, shaking).

The characteristics of CB G250 fluorescence spectrum. The spectrum of G250 was scanned in the 0.05% solution of G250 in sterile 0.2 µm filtered 0.1 × PBS in temp 24°C using the BioTek Synergy H1 Hybrid Reader (BioTek corp.). To confirm the results, we used Shimadzu Rf-1501 Spectrofluorometer (step 5 nm, delay 100 ms).

For testing the influence of Coomassie Blue G250 (Sigma) concentration on fluorescence emission intenity, the concentrations 0, 0.001, 0.003, 0.00625, 0.0125, 0.025, 0.05, 0.08, and 0.1% of G250 in 0.1 × PBS were measured using the maximum absorption 340 nm and the maximum emission 390 nm of G250. The measurement was done on Shimadzu Rf-1501 Spectrofluorometer.

For detecting the potential influence of proteins (albumin) on emission intensity of G250, solutions with increasing concentrations of 0, 10, 20, 40, 60, and 100 mg/l BSA (bovine serum albumin, BSA, Sigma-Aldrich) in double-distilled water were prepared. The G250 in PBS was added to each concentration (fin. conc. in BSA solution 0.05% G250 and 0.1% PBS). After 30 min of incubation in 24°C, sample fluorescence was measured (Ex. 340, Em. 390 nm). The spectrum of G250 (fin conc. 0.05%) in 40 mg/l BSA solution was scanned using the BioTek Synergy H1 Hybrid Reader (BioTek corp., step 5–10 nm, delay 100 ms).

G250 staining procedure. Half percent Coomassie Blue G250 (Sigma) stock solution in sterile, 0.2 µm filtered 1 × PBS (Sigma, in ddwater) buffer was prepared at temp. 20–24°C. A stock solution can be stored in 4–8°C, in darkness for several days. Before staining the samples, the 0.5% G250 stock solution was heated to the temperature of 20–24°C and filtered through a 0.2 µm polycarbonate filter (Millipore).

For staining, 1 ml of G250 stock solution was added to 9 ml of the sample (free-living bacteria fraction < 3 µm), mixed and incubated for 30 min in the dark, at a temperature of 20–24°C. After staining, 1 to 10 ml of stained samples (depending on the expected microorganism count number) were suspended on the surface of a 0.2 µm polycarbonate filter (Millipore), filter was washed 3 times with 5 ml 0.1 × PBS buffer and dried at 20°C for 1 h. After drying, the filter was mounted on a microscopic slide with one drop of epifluorescence Nikon immersion oil. For bacteria counting we used a computer image analyzing system composed of a Nikon epifluorescence E450 microscope, Nikon Digital Camera DXM 1200F and NIS elements software (Nikon). The bacteria were counted from digital images of 10–30 random fields for each membrane filter (from 50 to 100 bacteria per field, 1000–3000 bacteria cells per each membrane filter, picture area: 5510 μm^2). We recommend the use of UV2A microscope filter (UV2A, Ex. 330–380 nm, DM. 400 nm, Em. 420-α nm) for stained bacteria observation. In case of intense background fluorescence in UV2A filter, the B-2A filter (Ex. 450–490 nm, DM. 505 nm, Em. 520–∞ nm) could be used instead. Both filters are appropriate for the G250 fluorescence spectra.

The number of bacteria per 1 ml of the sample was calculated from the following equation: $BN = [(N \times F_{area})/(M_{area} \times V)] \times 1.1$, where N the number of bacteria visible under epifluorescent microscope; F_{area} the area of filter with suspended bacteria; M_{area} the area of photographed filter surface; V the volume of filtered samples; 1.1 the G250 solution dilution coefficient.

Comparison of tetracycline and G250 staining of *E. coli* bacteria from culture. For the comparison of the staining results obtained using tetracycline and G250, three days old LB broth culture of tetracycline-sensitive *E. coli* K12 strain was used (in three repetitions).

E. coli culture for G250 staining was diluted 500 times in double 0.2 μm filtrated, sterile 0.1 × PBS buffer (pH 7.4), and then immediately stained according to the standard G250 staining procedure described above. Nikon UV2A filter (UV2A, Ex. 330–380 nm, DM. 400 nm, Em. 420-α nm) was used for observation. *E. coli* cells stained with G250 were observed as vivid blue cells among dark ones; the percentage of bright cells was calculated.

The test of tetracycline incorporation into *E. coli* cells was made according to Ammor *et al.* (2006), with a few modifications. Instead of the fluorometric measurement of incorporated tetracycline in bacteria suspension, the direct observation of *E. coli* cells was used. To 9 ml of the 500-times diluted samples of *E. coli* culture, we added 1 ml of tetracycline solution in deionized sterile water to obtain a final tetracycline concentration of 100 μg ml^{-1}. The samples were incubated for 2 h in 24°C. Subsequently, the bacteria were collected on 0.2 μm policarbonate filters (Millipore) and dried. After drying, the filter was mounted on a microscopic slide with one drop of epifluorescence Nikon immersion oil. Because tetracycline has a maximum excitation of 390 nm and emission of around 520 nm (Glazier and Horvath, 1995), tetracycline stained bacteria were visualized and photographed using the same filter as the G250 stained bacteria (Ex. 330–380 nm, DM. 400 nm, Em. 420–∞ nm, magnif. 1000×). *E. coli* with accumulated tetracycline were observed as bright green cells among dark ones; the percentage of bright cells was calculated.

Standard procedures for environmental sample analysis. The number of DAPI stainable free-living bacteria (DAPI BN) was determined by the direct counting of cells collected on 0.2 μm, black polycarbonate membrane filters (Millipore) under epifluorescence microscope (Porter and Feig, 1980). DAPI (4,6-diamidino-2-phenylindole) in final concentration 1 μg ml^{-1} was used (for 10 min., at 24°C). For bacteria counting, we used a computer image analyzing system composed of a Nikon epifluorescence E450 microscope, Nikon Digital Camera DXM 1200F and NIS elements software (Nikon). The bacteria were counted from digital images of 10 to 30 random fields for each membrane filter (from 50 to 100 bacteria per field, 1000 to 3000 bacteria cells per each membrane filter, picture area: 5510 μm^2, UV-2A Nikon fluorescence filter – Ex. 330–380 nm, DM. 400 nm, Em. 420-α nm).

Detection of microorganisms belonging to Domain Bacteria by fluorescence *in situ* hybridization (FISH) was carried out using the EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') CY3 labeled oligonucleotides developed by Amann *et al.* (1997). The bacteria were permeabilized (2% paraformaldehyde, 4°C, 18 h), retained on 0.2 μm polycarbonate filters, hybridized according to Knoll *et al.* (2001), and counterstained with DAPI. Fluorescently labeled cells were counted using CY3 Nikon filter and Nikon epifluorescence E450 microscope, as described above. The percentage of hybridized cells was used for the calculation of FISH stained bacteria abundance.

Secondary production of free-living bacteria was determined by means of thymidine (TdR, 90–97.5 Ci nmol^{-1}; NEN Du Pont) incorporation (Chróst and Rai, 1993).

To determine the percentage of free-living bacteria with intact cell membranes, LIVE/DEAD® BacLight™ Bacterial Viability Kit, Invitrogen Molecular Probes was used according to Invitrogen, Molecular Probes standard procedure (Luna *et al.*, 2002). The bacteria were stained in unpreserved samples within a few hours after the samples were taken. After staining, the bacteria were collected on 0.2 μm black polycarbonate membrane filters (Millipore) and dried. After drying, the filters were mounted in BacLight™ mounting oil on microscopic slides. For LIVE/DEAD® BacLight™ stained bacteria visualization we used the B2A Nikon filter (Ex. 450–490 nm, DM. 500 nm, Em. > 515 nm). The green (MEM+) and red (MEM-) colored bacteria (with integral and damaged membranes, respectively) were counted from digital images of 10–30 random fields for each membrane filter, as with DAPI staining. We calculated the percentage of MEM+ bacteria in the total, DAPI visible bacteria number.

Maximal potential leucine-aminopeptidase activity (V_{max} AMP) was measured fluorometrically (Chróst, 1990; Kiersztyn *et al.*, 2012). The plot of the reaction velocity (v) against substrate concentration [S] displayed a rectangular hyperbolic relationship, described by the equation $v = V_{max} \times [S] / (K_m + [S])$. Nonlinear regression analysis was applied to calculate the kinetic parameters of enzymatic reactions using Origin 8.6 software (OriginLab Corporation, Northampton, USA).

Dissolved Organic Carbon (DOC) concentration was measured as C-CO_2 concentration after complete burning in O_2 atmosphere using Shimadzu TOC 5050A analyzer (Tupas *et al.*, 1994).

The statistical analyses were performed using Origin 8.6 (OriginLab Corporation, USA) and Statistica (StatSoft, Poland). Pearson correlation matrices,

multidimensional scaling (based on the correlation matrix of analyzed parameters), one-way ANOVA test and Mann-Whitney U test were used for data analysis.

Results

Analysis of the fluorescence spectrum of G250. In the visible range of light spectrum, 0.05% G250 in 0.1 PBS (pH 7.4) exhibited two excitation-emission maximum pairs: the first at Ex. 340 nm, Em. 390 nm and the second at Ex. 440 nm, Em. 490 nm. The normalized spectra of the relative fluorescence intensities of G250 are shown in Fig. 1A and Fig. 1B. The emission of fluorescence at 490 nm was less intensive than at 390 nm. In both emission-excitation pairs it was possible to observe G250-labeled microorganisms, under epifluorescence microscope equipped with a set of standard Nikon filters UV-2A (Ex.: 330–380; DM: 400; Em.: 420 – ∞) or B-2A (Ex.: 450–490 nm, DM: 505, Em.: 520 – ∞). The numbers of bacteria observed using the two different wavelengths were positively, significantly correlated ($r = 0.95$, $p = 0.004$) and stayed in the same range. The mean values of G250-stained bacteria number at all depths were $2.6 \pm 0.8 \times 10^5$ cell ml^{-1} and $3.9 \pm 1.2 \times 10^5$ cell ml^{-1} for UV-2A and B-2A filters, respectively, and were not significantly different from each other at the statistical probability level of $p = 0.05$ (using the one-way ANOVA test). Some typical microscopy images of bacteria from lake Mikołajskie stained with Coomassie G250 and the DAPI-stained bacteria are shown in Fig. 2.

Relationship of G250 concentration and G250 fluorescence. The relationship between the concentration and fluorescence emission intensity of G250 dye at Ex. 340 nm and Em. 390 nm is presented in Fig. 3. The highest fluorescence intensity was observed when Coomassie Blue G250 concentration was about 0.05%. This concentration was chosen as the final optimal concentration of G250 for the microorganism staining procedure.

Influence of BSA on G250 fluorescence. We did not observe significant linear correlation between relative fluorescence of G250 and albumin concentration ($r^2 = 0.34$, $p = 0.13$). In a solution containing BSA and G250 we observed an additional peak of albumin fluorescence at Ex. 280 and Em. 340. These values are distant from the maximum excitation / emission wavelength for G250.

Comparison of *E. coli* staining with tetracycline and G250. Sample microscopic images of *E. coli* cells observed after tetracycline treatment (A), stained with G250 (B) and unstained (C) are shown in Fig. 4. The

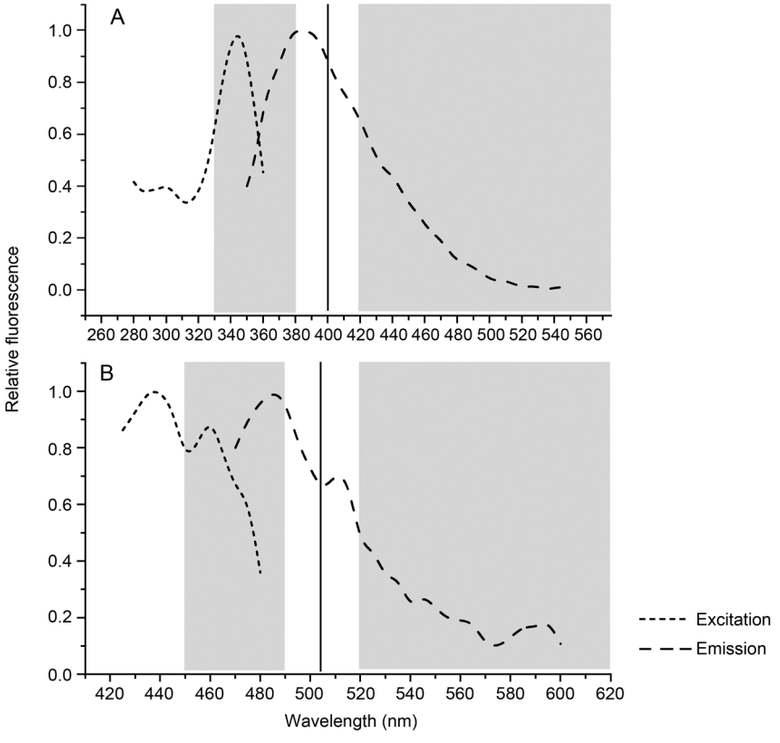

Fig. 1. The normalized, relative fluorescence spectrum of 0.05% Coomassie Blue G250 in 0.1 × PBS buffer excited by A) UV light and B) blue light. The grey boxes represent the excitation and emission parameters of epifluorescence filters used for microscopic observation of G250-stained microorganisms: Nikon UV2A, Ex. 330–380 nm, DM. 400 nm, Em. 420-α nm and Nikon B-2A, Ex. 450–490 nm, DM. 505 nm, Em. 520–∞ nm respectively. The vertical line shows the wavelength barrier of dichromatic mirror.

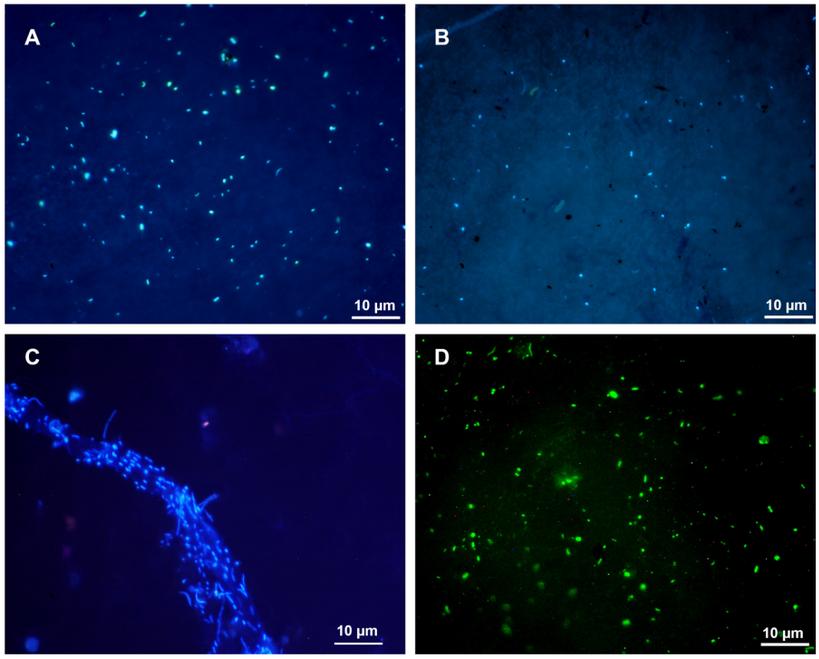

Fig. 2. Sample microscopic images of bacteria from Lake Mikołajskie: A) DAPI-stained bacteria (UV2A Nikon filter), B) G250-stained bacteria (UV2A Nikon filter), C) G250-stained bacteria (UV2A Nikon filter), D) G250-stained bacteria (B-2A Nikon filter). Mag. 1000×, Depth: 0.5–2 m, Lake Mikołajskie, August 2011. Image C) shows attached bacteria from unfiltered sample. The remaining images depict microorganisms from the 0.2–3.0 µm fraction.

staining of *E. coli* with G250 or tetracycline yielded similar images of vivid-bright cells of bacteria among dark ones (Fig. 4A, B). The mean percentages of bright cells among total visible cells were similar for G250 and tetracycline staining (40.2 ± 2.5% and 41.4 ± 3.2%, respectively; Mann-Whitney U test $p = 0.71$). For the unstained sample, an image of dark, hardly visible *E. coli* cells was obtained (Fig. 4C).

Comparison of the number of aquatic bacteria visualized by G250 staining with other indicators of potential bacteria activity. To evaluate the usefulness of G250 for aquatic, active bacteria detection, we compared the number of Coomassie Blue G250 stained bacteria (CB BN) with parameters characterizing the abundance and vitality-activity of microorganism communities like: DAPI stained cell abundance (DAPI BN), secondary bacterial production (Bacterial Prod.), the number of cells belonging to the domain Bacteria containing rRNA visualized using FISH staining procedure (FISH BN), maximal potential activity of aminopeptidase (V_{max}AMP), the live cell number (cells with intact cell membrane, MEM + BN), and DOC concentration. The matrix of linear correlation coefficients between these parameters is shown in Table I. We found

Table I

Matrix of Pearson correlation coefficients between the following parameters: DAPI BN – DAPI-stained cell abundance; Bacterial Prod. – secondary bacterial production; FISH BN – the number of Bacteria containing rRNA visualized using FISH staining procedure; VmaxAMP – maximal potential activity of Leucine aminopeptidase; MEM + BN – bacteria cells with intact cell membrane; CB BN – number of G250-stained bacteria; DOC con. – concentration of dissolved organic carbon. Stars indicate that the correlation is significant at the level of $p < 0.05$.

	DAPI BN	CB BN	Bacterial Prod.	Vmax AMP	MEM + BN	DOC conc.	FISH BN
DAPI BN	–						
CB BN	–0.62*	–					
Bacterial Prod.	–0.31	0.79*	–				
Vmax AMP	0.36	0.71*	0.82*	–			
MEM+ BN	–0.43	–0.36	–0.27	–0.14	–		
DOC con.	–0.26	0.69*	0.60*	0.57	0.30	–	
FISH BN	0.25	0.84*	0.52	0.56	–0.48	0.59	–

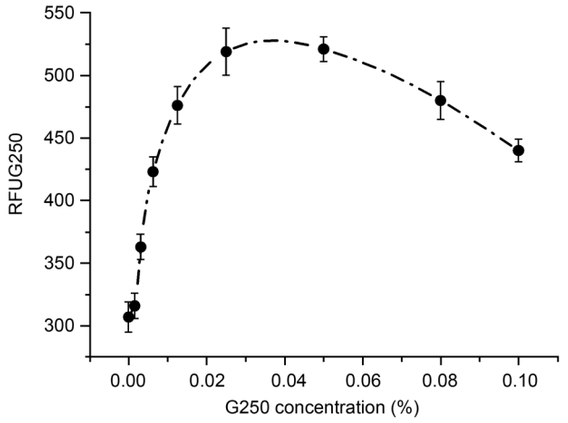

Fig. 3. Relationship between Coomassie Blue G250 concentration (in 0.1 × PBS, pH 7.4) and relative fluorescence emission intensity at 390 nm after excitation at 340 nm.

positive and significant correlations between CB BN and three bacterial activity indicators. These correlations were especially strong for secondary bacterial production ($r = 0.79$, $p < 0.001$) or FISH BN ($r = 0.84$, $p < 0.001$). We found no correlation of CB BN with MEM + bacteria number and a negative, significant correlation with DAPI BN ($r = -0.69$, $p = 0.007$). Fig. 5, reports the DAPI stainable bacteria number and Coomassie stainable bacteria number, together with bacterial production, at various depths during two years of research. The decrease in CB BN (but not DAPI BN) in August 2011 corresponded with a considerable decrease in secondary bacterial production. This suggests that only productive, live bacteria may be susceptible to effective staining with G250. Generally, the percentage of G250 bacteria in DAPI BN ranged from 1.9% to 82.7%, with a mean value of 36.8% and a median of 40.9%.

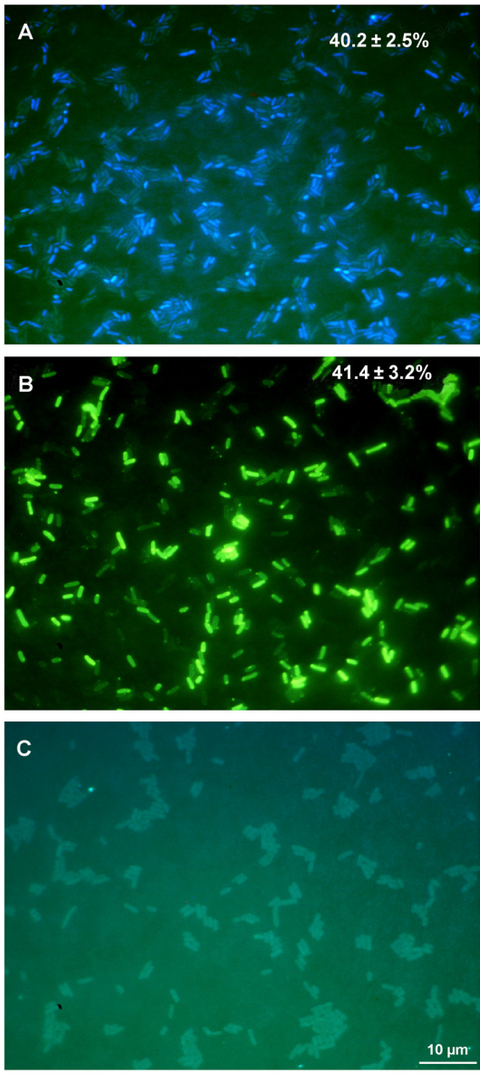

Fig. 4. Microscopic image of *E. coli* from three days' culture in LB medium: A) G250-stained, B) with tetracycline accumulated, and C) not stained. The percentages on image (A) and (B) indicate the fraction of bright cells in the total dark and bright cell numbers.

Discussion

This article evaluates the use of a well-known dye Coomassie Blue G250 for fluorescent visualization of active bacteria. To obtain the anionic form of G250 in our samples, we buffered the lake water samples with 0.1 × PBS (pH 7.4). The anionic form of G250 binds predominantly to arginine, lysine and histidine by electrostatic attraction, creating a complex, ensuring effective staining of proteins (Georgiou *et al.*, 2008). Given the well-known problem of fluorescence self-quenching (Penzkofer and Lu, 1986), we established experimentally the optimal concentration of G250 at 0.05% in PBS solution. Though the latter provided the most intense fluorescence at 340 nm excitation and 390 nm emission, concentrations lower and higher than 0.05% still allowed effective emission detection. We did not observe significant changes in fluorescence in the presence of, even high, concentrations of proteins (*i.e.*, BSA). We observed only slight emission light intensification (at 390 nm) with increasing BSA concentration, and additional BSA excitation-emission peaks (max. emission at 340 nm). It is interesting to note that the BSA emission peak is in the range of the G250 excitation maximum. This may be explained by the absorbance of BSA emission light by G250 (Katrahalli *et al.*, 2010), causing a slight increase in G250 fluorescence, especially under high BSA concentrations.

The G250 staining mechanism is different from that of the most common dyes used for bacteria visualization, like SYBR-I, SYBR-II, or DAPI, which is based on the conversion of the dye to its fluorescent form after

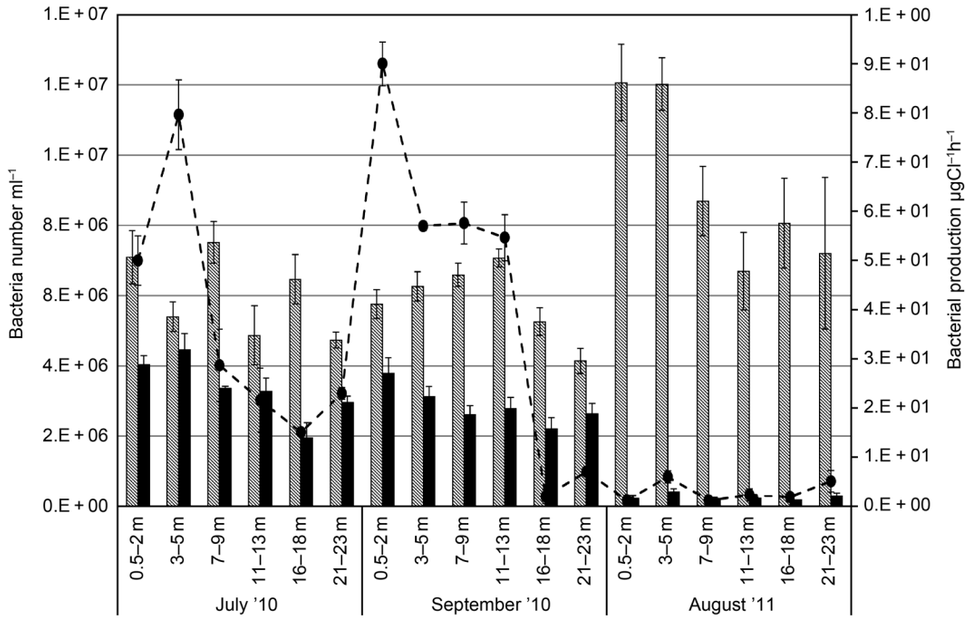

Fig. 5. The comparison of DAPI-stained bacteria number (dashed bars, left axis), G250-stained bacteria number (black bars, left axis) and bacterial secondary production (black dots, right axis) from different depths of lake Mikołajskie in July, September 2010 and August 2011.

interaction with DNA (Lebaron *et al.*, 1998). In the case of Coomassie blue G250, fluorescence intensity does not change after protein binding, and the visualization of protein rich bacteria is possible due to the contrast between the concentrated Coomassie bound to microorganisms and the background fluorescence. This background fluorescence is primarily a result of G250 binding to dissolved proteins remaining on the filter surface after sample filtration. A practical consequence of this fact is that the filter must be profusely washed prior to observation to provide high quality pictures.

Cellular proteins constitute from 15% to nearly 50% of the volume of bacteria, and more than 60% of bacterial dry weight (Simon and Azam, 1989). The protein synthesis rate calculated on the basis of H^3-leucin incorporation has been used for years as an indicator of live bacterial production (Bastviken and Tranvik, 2001) and bacterial incorporation of amino acids has been used for active bacteria detection by autoradiography (Simon, 1988). In dead bacterial cells, the protein content decreases rapidly after inhibition of protein synthesis due to the fast internal protein hydrolysis rate (more than 10% of internal proteins per hour) (Gottesman and Maurizi, 1992). This led us to consider protein richness as an indicator of bacterial cell activity and physiological condition. We tested the ability of G250 to selectively stain potentially live bacterial cells by comparing the staining results obtained by adding G250 and tetracycline to diluted samples of *E. coli* culture. We chose tetracycline for two reasons. First, it is a bacteriostatic and does not lead to fast disruption of affected bacteria (cells may still be visualized) – it only inhibits protein synthesis by binding reversibly to the 30S ribosomal subunit at a position that blocks the binding of the aminoacyl-tRNA to the acceptor site on the mRNA-ribosome complex. Second, tetracycline is fluorescent with an excitation maximum at 490 nm and emission maximum at 520 nm (Glazier and Horvath, 1995), enabling the observation of cells with accumulated tetracycline and measuring the accumulation rate of tetracycline in bacterial cells by detecting cell fluorescence (Ammor *et al.*, 2006). Only active, energy-dependent transport of tetracycline followed by binding to active ribosomes leads to intracellular accumulation of tetracycline at concentrations greater than those in the medium (Franklin and Snow, 2005). We observed similar percentages of tetracycline visualized and G250 stained *E. coli* cells in the total number of *E. coli* cells from culture, which suggests that G250 stains mainly active cells with a high protein synthesis rate.

The assumption that G250 primarily stains live bacteria is supported by environmental analysis. The analysis was conducted on the free-living bacterial fraction (< 3 µm lake water filtrate through 3.0 µm filter) to minimize potential measurement error arising from the strong heterogeneity of samples containing bacteria living in deep biofilm layers (Griebler *et al.*, 2001). Bacteria living in deep biofilm compartments require special treatment prior to their counting like, for example, gentle sonication to release them from

the biofilm matrix. Such treatment might influence the measurement of some parameters used as activity indicators and had a negative influence on the vitality of the cells (Böllmann *et al.*, 2016). For example it could lead to protease release from damaged cells or change cell membrane integrity, influencing L&D test results. This does not mean that attached bacteria may not be visualized using G250. Without preliminary filtration it is possible to obtain images of well visible, G250-stained attached bacteria, as shown in Fig. 2C.

In contrast to the large *E. coli* cells from culture, which were visible (though barely) under epifluorescence microscope even without staining, the small (usually 0.2–1.0 μm of diameter, Simon, 1988) bacteria living in natural lake water, were visible and could be counted only after staining. We compared the number of G250-stainable bacteria with several parameters describing bacterial community abundance and activity (Table I), finding a positive correlation between G250-stained bacteria number and number of bacteria obtained using FISH. The FISH method allows visualization only of cells containing a large number of ribosomes. Such cells may generally be treated as translationally active, live cells (Karner *et al.*, 1997; Smith and del Giorgio, 2003; Freese *et al.*, 2006). The strong linear correlation between the number of FISH-detected and G250-detected bacteria supports the thesis that the G250-staining procedure visualizes bacteria which are, at the moment of staining or soon before it, in good vital condition, as suggested by high ribosome concentration. This thesis is also consistent with some of our other observations. First, the strong, significant correlation between G250-stained bacteria number and secondary production based on H^3-thymidine incorporation rate, which is used as an indicator of bacteria divisions (Fuhrman and Azam, 1982; Bengtsson *et al.*, 2012). Second, the fact that the rapid decrease in bacterial secondary production in August 2011 (Fig. 5) was accompanied by a parallel decrease in G250-stained bacterial abundance. Third, the significant positive correlation between the maximal potential activity of aminopeptidase and G250-stained bacteria number. Extracellular aminopeptidase is predominantly produced by bacteria, and is located in the periplasmic space or built into bacterial walls (Chróst, 1990; Kiersztyn *et al.*, 2012). Fourth, the strong correlation between G250-stained bacteria number and DOC concentration. DOC is the main source of carbon for free-living bacteria and an important factor of the bottom-up mechanism controlling bacteria activity in lake waters. All these observations lead us to conclude that G250 may reasonably be used for the staining of active bacteria.

Our conclusion also explains the fact that we did not find any positive correlation between the number of DAPI stained bacteria, live bacteria according to L&D test and G250. DAPI has been shown to stain not only active bacteria cells, but also empty bacterial envelopes (ghost cells) or micro-particles with DNA adsorbed (Zweifel and Hagström, 1995). On the basis of many activity markers, it has been found that in various aquatic environments the percentage of live cells in the total DAPI-stained cell count varies from a few percent up to 90%, depending on sampling period and environmental conditions (Haglund *et al.*, 2003; Warkentin *et al.*, 2007).

The lack of correlation between the number of live bacteria visualized by L&D test and that of G250 stained bacteria may be due to the specific properties of L&D test. This test is based on the assessment of cell membrane integrity and contains two dyes: red emission fluorescent propidium iodide which cannot penetrate the intact bacterial cell membrane and green emission SYTO® 9 which can penetrate the cell membrane easily. Both of them require nucleoid presence inside the cell for effective staining. Staining by propidium iodide is likely to cause an underestimation of the actual number of dead cells due to the fact that, apart from the predation of protozoa on bacteria, another important cause of bacterial mortality is viral lysis, resulting in DNA fragmentation (Shibata *et al.*, 1997). This problem is compounded by the fact that the presence of propidium iodide in a cell (suggesting that the cell is dead) might be an effect of small cell membrane damage during sample preparation. The lack of significant correlation between MEM+ cell abundance and bacterial production or aminopeptidase activity also point to the uncertainty of the L&D method for live bacteria detection in natural lake samples.

We conclude that the staining of bacterial cells using G250 offers a reasonably accurate assessment of the number of active bacteria in culture and freshwater environment. It may thus be regarded as a viable alternative or supplement to other microscopic methods of direct active bacteria visualization. G250 is inappropriate for staining bacteria with low activity and in dormant state. Its usefulness lies in its ability to visualize highly active bacterial cells – the fraction of the bacterial community mainly responsible for the global bio-geochemical processes in the natural environment.

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