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Application of Fluorescence Markers for the Diagnosis of Bacterial Abundance and Viability in Aquatic Ecosystem

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Abstract Application of modern microbial diagnostics based on fluorosensors is very crucial to investigate bacteria from natural samples (aquatic as well as transparent biofilms on material surfaces). This method allows researcher to fast recognize the existence of the invisibly small bacteria, to differentiate between live and dead cells as well as active and inactive cells *in situ*. This paper wanted to elaborate and discuss several perspectives on the usage of different types of fluorescent markers, e.g. redox sensitive, nucleic acid stains with different membrane permeability to natural bacterial assemblages as well as bacterial strains isolated from aquatic samples as a tool to differentiate living, active from dead or inactive bacteria.

Keywords Fluorosensors, Aquatic Bacteria, Bacterioplankton, Bacterial Viability

1. Introduction

Bacteria play an important role as decomposer for organic matter in aquatic environment. This process is a key process in aquatic microbial food webs as well as its application in the water bioremediation process[e.g. 4, 20]. Modern microbial diagnostics are very important to recognize the existence of the invisibly small bacteria and the transparent biofilms on material surfaces as early as possible. Classical microbiological detection methods often include an isolation and cultivation steps that is not only time consuming (up to several days), but also missing most individuals or even species. Only up to 6% of all bacteria from water samples were found to grow colonies on agar plates, most often they don't exceed 1% of total number[35]. Molecular genetic methods, esp. genomic profiling protocols, were introduced into environmental research, esp. into marine microbiology, several years ago with great success[29]. Fluorescent gene probes identify bacterial species without isolation[1]. However, if they detect DNA, they will also find dead cells and if they are designed to hybridize with RNA they will not distinguish active and inactive individuals. The detection of small marine bacteria with a low ribosome content using CARD-FISH (catalyzed reporter decomposition – fluorescence in situ hybridization) was of great advantage[33] since many "wild" cells are rather small compared to cultured ones. The novel microbial diagnostic strategy based on molecular fluorosensors is especially advantageous in applied environmental microbiology, because microorganisms do not need to be isolated and they can be investigated on non-transparent surfaces with minor to none material damage. The specificity and universal applicability of fluorosensors for heterotrophic and autotrophic microorganism is a good advantage to develop it as a new microbial diagnostic kit in applied environmental microbiology.

A bacterioplankton community constitutes bacterial cells with different physiological states, viable as well as non-viable, defect, cells. As viable cells may be active or inactive with respect to many different activities, e.g. substrate uptake, respiration, hydrolysis, and cell deviation. The enumeration of bacterioplankton composition i.e. between total bacterial number and their active and/or viable cells is a very important step to understand bacterial role in the decomposition of organic matter because only viable bacterial cells mediate organic matter turnover. Therefore, it is necessary to choose the method to visualize active cells according to the aim of the investigation i.e. matter breakdown, turnover, degradation, or biomass production. This present paper discusses the methodological considerations of different types of fluorescent markers application, e.g. redox sensitive, nucleic acid stains with different membrane permeability to natural bacterial assemblages as well as bacterial strains isolated from aquatic samples as a tool to differentiate living, active from dead or inactive bacteria.

2. Bacterial Abundance and Biomass

Bacteria can be enumerated using several methods, such

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as traditional agar plating (colony forming units), direct cell counting without staining, optical density (OD) measurement and by protein content or the concentration of cell wand constituents. Most aquatic heterotrophic bacteria are too small to be observed by light microscopy. OD measurements are hampered by dissolved matter, particles changing and varying size as well as surface properties of the bacteria themselves. Epifluorescent direct counting is the best method available for the enumeration of total bacteria in environmental samples[19]. Bacterial abundance from plankton samples as well as bacterial isolates can be estimated after staining with 4',6 diamidino-2-phenylindole dihydrochloride (DAPI, Roth) DAPI, which is together with acridinorange, the most often used bacterial stain[e.g. 43, 45]. Several weaknesses of the DAPI staining results have been reported, such as an underestimation of bacterial numbers under starvation condition[28] and the inclusion of virus particles[7] caused by the high quantum yield of new stains like SYBR Gold and Green[18]. This weaknesses can be overcome by combining DAPI stain with SYBR. Counterstaining DAPI with SYBR Green has been proven to clearly distinguish between bacteria and nonspecifically stained bacteria-like particles[16]. Still, it has been widely used to enumerate bacterial abundances in different aquatic environments over the past decades and is, therefore, referred to as the reference parameter for bacterial abundance and biomass as well.

Several preservation techniques have been applied to bacterial samples. Aldehydes and their derivatives are the most commonly used preservatives. They act in cell membranes by cross-linking proteins, thus improving bacterial cell rigidity. Formaldehyde (FMA) and glutaraldehyde (GTA) are the most common aldehydes applied in aquatic samples[22]. Final concentrations of FMA in samples must not exceed 0.5% because 1% and 2% of FMA already resulted 20% and 80% reductions in fluorescence, respectively[26]. GTA is most frequently used at a concentration of 1%[22], although Clarke and Joint[9] found no change in cell numbers for up to 1 month with storage in 2.5% (vol/vol) electron microscope-grade GTA. GTA could be used at final concentration of more than 2% because with 5% fixative, an approximately 30% reduction in fluorescence was observed[26]. Application of the commercially available liquid forms of FMA, i.e. formalin, which is combined with methanol as the fixative, was preferable to use for the enumeration of bacterial isolates[40]. GTA used to fix bacterioplankton because fixing samples using FDA destroyed pigment fluorescence and thus total bacterial number can be overestimated caused by confusion of cyanobacteria with bacteria.

3. Bacteria Cell Viability and Activity in Aquatic Ecosystem

Membrane integrity is an important prerequisite for activity[15]. Viability markers have been used extensively in medical studies. Impermeant nucleic acid stains such as SYTOX[37] or TO-PRO[31] were applied to bacterial isolates, especially to test antibiotic susceptibility or other stress factors. Propidium Iodide (PI) has already been applied to natural bacterial assemblages[5, 46]. The following markers were applied intensively this last decade for the diagnosis of natural bacterial assemblages from samples especially for the bacterial structure enumeration (live/dead and active and/or inactive bacteria) in aquatic ecosystem.

Intracellular esterase activity

Bacteria with intracellular esterase activity can be detected using 5-chloromethylfluorescein diacetate (CellTrackerTM Green CMFDA, Molecular Probes Europe) as an artificial substrate for esterase. CMFDA starts to fluoresce after enzymatic hydrolysis and is bound to the intracellular protein pool with its chloromethyl-group[18]. This reaction formed a covalently bound fluorescent molecule staining the entire cells yellow-green. Using 1000-fold magnification of an epifluorescence microscope, active bacteria can be enumerated. These active bacteria were considered as a viability parameter for the physiological state of bacteria[38].

CellTracker™ Green CMFDA (Molecular Probes) can be used to detect the intracellular esterase activity of bacterial communities. Intracellular esterase activities have been analysed using various fluorescein esters, e.g. carboxyfluorescein diacetate (cFDA)[13, 34], Chemchrome [10] and fluorescein diacetate (FDA)[e.g. 2, 14]. However, this activity was followed mainly by flow cytometry, which is restricted to the investigation of large and homogenously distributed cells or bacterial cultures. CMFDA was applied to many medical questions, on human cells and tissues[e.g. 12, 23] or as a cell tracker dye for eukaryotic microbes, e.g. flagellates and yeast[6, 24]. CMFDA is even more preferable due to its high cell retention[13]. Unlike other fluorescein derivatives, chloromethylfluorescein is bound via the glutathion *S*-transferase to the cells protein pools[18]. The presence of large pools of free enzymes may yield false positive signals because of the hydrolysis of CMFDA outside the cells and subsequent uptake[38].

In contrast, the very low portion of stained log-phase cells points to another problem. The obstructed permeation of fluorescein diacetate derivatives into some bacterial species can drive to an incomplete cell loading resulting in false-negative results[8, 21]. Longer incubation times than several previous reports (5 hours or more) were recommended in the application of this method to ensure sufficient quantity of CMFDA esterase substrate in all bacterial cells. Other treatments, which improve cell loading (e.g. heat or acid), may improve dye loading but cause damage the cells.. Therefore, intracellular esterase is not a general viability marker. It is essential to combine results using CMFDA with other viability and or activity markers.

Cellular respiration

Respiring active bacteria can be counted based on

reduction of the fluorosensor 5-Cyano-2,3-ditolyltetrazoliu mchlorid (CTC, 19292-100 Polysciences Inc.)[36]. Active bacteria reduce monotetrazoliumredox (CTC) to the red fluorescing formazan derivative (CTF) given so that there are sufficient amounts of NADPH+ available, e.g. via the respiratory chain. This fluorescence appeared intracellular by the formation and deposition of red fluorescing crystals by epifluorescence microscope.

CTC (5-cyano-2,3-ditolyl tatrazolium chloride) has been used to evaluate the respiratory activity of bacterial populations from marine and freshwater sites[e.g. 17, 42]. CTC is reduced to fluorescing formazane crystals by cellular respiration and, thus, is a direct indicator for viability[42] and cell specific activity[11]. Since not all cells are big or active enough to form a visible formazan crystal, only the metabolically most active bacteria are considered to give a signal[e.g. 25, 32]. In nature, only those cells that are able to reduce detectable amount of CTC constitute the most active cells in bacterial communities and are responsible for the majority of bacterial carbon turnover[41]. Therefore, CTC reduction can be interpreted as an indicator for bacterial cell viability as well as activity. The ability of CTC to observe metabolically active anaerobic bacteria has also been reported. CTC can be used to capture the metabolic activity of anaerobic bacteria, however care must be taken to avoid abiotic reduction of CTC[3].

Membrane integrity

Membrane integrity can be checked to confirm bacterial viability. Membrane integrity can be visualized with a Live/Dead Kit (Live/Dead Bacterial Viability, Molecular Probes Europe). Under blue excitation intact cells, marked by SYTO® 9, appeared yellow green and permeable, propidium iodide stained cells fluoresced red[5] under an epifluorescence microscope. Bacteria with permeable, i.e. damaged, membranes do not respire anymore, may loose cellular contents and at the same time may be exposed to eventually harmful substances without their intact, protective membranes. They are therefore considered dead or dying[38].

The other application of fluorosensor based on membrane integrity is LIVE® Bac*Light*™ bacterial Gram stain kit to direct- and rapidly determine Gram characteristic of bacterial isolates based on differential nucleic acid staining of live Gram-positive and Gram-negative bacteria. This kit contains membrane permeant green-fluorescent SYTO® 9 and the red fluorescent hexidium iodide (HI) dyes. The SYTO® 9 stains labels both live Gram-positive and Gram-negative bacteria, whereas HI preferentially labels live Gram-positive bacteria. When a mix population of live Gram-positive and negative bacteria simultaneously stained with combination of both dyes, live Gram-positive bacteria fluoresce red-orange and the Gram-negative bacteria fluoresce green. Dead bacteria do not exhibit predictable pattern[18]. The LIVE/DEAD® Bac*Light*™ (LD) bacterial viability confirms the bacterial membrane integrity. SYTO® 9 stains generally all cells in a population, those with intact membrane and those with damaged membranes. In contrast, PI labels only dead bacterial cells with damaged membranes[39]. Although log phase bacterial cultures are usually applied during investigation, washing steps and centrifugation may influence the integrity of membranes (discussed below).

LIVE® Bac*Light*™ bacterial Gram stain kit is based on differential nucleic acid staining of intact Gram-negative and intact Gram-positive bacteria. Unlike the conventional Gram staining procedure, this kit allows a rapid classification using a single staining solution without fixatives and washing steps. The kit contains also SYTO® 9 and the red fluorescent hexidium iodide (HI). Both of these fluorescent dyes stain nucleic acids and differ in spectral characteristics. The SYTO® 9 dye is permeable to all cell membranes, including mammalian cells and bacteria. However, dyes should be able to across the bacterial cell wall that is actually preliminary barrier before bacterial cell membrane. HI is a moderately lipophilic phenanthridinium dye that is permeant to mammalian cells and almost all Gram-positive bacteria. Destabilization of bacterial lipopolysaccharides (LPS) by EDTA[30] may indicate that the LPS-rich outer membrane of Gram-negative bacteria plays a role in exclusion of HI from these organisms. Thus, HI inclusion can also indicate the outer membrane disruption of Gram negative bacteria as was verified by ethanol treatments[27]. Therefore, maintaining the original salinity of *in situ* conditions of the isolates from aquatic samples is also important to avoid destabilization of bacterial (outer) membranes. Therefore, it is recommended to add NaCl into the medium according to its original salinity. Only 2 to 5% of total bacterial cells were dead after centrifugation following washing bacterial cells with buffer containing NaCl according to its *in situ* salinity. Due to the fragile structure of the LPS layer[30] and the need of intact bacterial cell limit the application of this technique for uncultivated samples.

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