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False positives in Biolog EcoPlates™ and MT2 MicroPlates™ caused by calcium

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ABSTRACT

Biolog MicroPlates™ (e.g. EcoPlate™, MT2 MicroPlate™, GN MicroPlate™) are useful tools for characterizing microbial communities, providing community-level physiological profiles to terrestrial and aquatic ecologists. The more recently designed Biolog EcoPlates have been used frequently in aquatic ecology with success. This study, however, reveals one major problem when using EcoPlates to evaluate samples within an estuarine or seawater matrix. At concentrations greater than 100 parts per million, the cation calcium begins to interfere with the microplate chemistry, causing false positive readings. Experiments, in which multiple treatments of natural and artificial seawater were tested, as well as calcium-addition experiments, demonstrate that calcium inhibits complete dissolution of the minimal growth medium in wells. Future studies involving Biolog EcoPlates and MicroPlates should take this effect into account, and the dilution of samples is strongly recommended to diminish the "calcium effect."

1. Introduction

Biolog EcoPlates™ (Biolog Inc., Hayward, California) are used for community-level physiological profiling of a variety of environmental matrices. The 96-well microplate contains a triplicate set of 31 carbon sources and a control well. Each well contains a minimal growth medium and tetrazolium dye, which is reduced to formazan during bacterial respiration, resulting in an insoluble violet color. Control wells contain no added carbon substrate and account for background readings from the growth medium, redox dye, and bacterial respiration of any dissolved carbon compound contained in the environmental matrix. Initially used widely for soil microbial analysis (>83% of papers using Biolog methods published prior to June 2001), Biolog MicroPlates are now used with a variety of different inoculums (Choi and Dobbs, 1999; Preston-Mafham et al., 2002). Specifically, their use in estuarine and ocean sciences has increased in recent years (Lyons et al., 2010; Sala et al., 2010; Gilbert et al., 2012; Mouchet et al., 2012). Because of their growing use, ensuring the accuracy and validity of data gathered from EcoPlates is essential. There are a number of known problems with the method, most notably those of inoculum density and the inability of some bacteria and all fungi to reduce the tetrazolium dye, resulting in an incomplete picture of the microbial community (Preston-Mafham et al., 2002; Insam and Goberna, 2004; Weber and Legge, 2010). The current study indicates another significant problem, false positives, of which investigators must be aware to ensure accurate results and interpretation of data.

There have been previous observations (Samantha Bickel, Virginia Institute of Marine Science, personal communication) of high absorbance readings without color development. Noble and Gow (1998) also reported that "sea salts or solutions containing cations such as Ca^{2+} are not recommended because some divalent cations may cause precipitation of the redox agent." The authors know of no previous studies, however, that have systematically investigated whether divalent cations abundant in seawater may be associated with the phenomenon.

The present study was motivated by high absorbance readings, without commensurate color development, in EcoPlates used to test marine environmental samples. For the purposes of this paper, this phenomenon is henceforth described as "undissolved material" with reference to its appearance. To pinpoint the source of the false positives as well as elucidate the exact response of the microplates, a series of experiments was designed using seawater and freshwater treatments. All treatments were sterilized prior to inoculation to eliminate any effects of respiration caused by heterotrophic bacteria.

2. Materials and methods

2.1. Experiments with Biolog EcoPlates™

Eight treatments of natural (NSW) and artificial (ASW) seawater were prepared to test their reactions in Biolog EcoPlates and evaluate their usefulness as diluents. The treatments were: 1. autoclaved NSW, 30 parts per thousand (ppt), 2. sterile filtered NSW (30 ppt), 3. sterile

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filtered ASW (+Ca/+Mg), 4. autoclaved NSW (15 ppt), 5. sterile filtered ASW (+Ca/-Mg), 6. Phosphate Buffered Saline (PBS, isotonic), 7. sterile filtered ASW (−Ca/+Mg), and 8. sterile filtered ASW (−Ca/−Mg). Artificial seawater treatments were prepared using recipes from The Marine Biological Laboratory (MBL) (Cavanaugh, 1956). Calcium and magnesium concentrations for each treatment were either measured (Red Sea Aquariums Inc.; Millipore Corp.) or calculated (Table 1). Due to the conservative nature of calcium and magnesium ions in seawater, the recipes used are typical of natural seawater cation concentrations found globally. All treatments were measured for salinity using a handheld salinity refractometer (Fisher Scientific) and pH using a benchtop pH meter (Accumet B15Plus). Treatments were sterile-filtered using Stericups® (Millipore Corp.), with autoclaved treatments filtered using a 0.22 μm nitrocellulose filter (Millipore Corp.) before sterilization at 121 °C for 20 min. Aliquots (150 μl) of each treatment were inoculated into the control wells of EcoPlates inside a laminar flow hood (BioSafety Cabinet). Three to four replicates, i.e., one to two plates, of each treatment were prepared. Plates were read on a BioTek ELx808 microplate reader at a wavelength of 590 nm and absorbance was determined at the time of inoculation (time = 0 h) and every 24 h for up to seven days. Plates were dark incubated at 23 °C between readings. Microscopic observations of control wells were made after seven days.

In addition to NSW and ASW treatments, salts were added to fresh water collected from a pond near the University of Connecticut, Avery Point campus in Groton, CT. Salts (calcium chloride, magnesium chloride, and magnesium sulfate) were added in the amounts per volume for artificial seawater (taken from MBL seawater recipes, Cavanaugh, 1956). Three treatments were prepared: 1. pond water plus calcium (PW + Ca), 2. pond water plus magnesium (PW + Mg), and 3. pond water with no salt additions (PW). All treatments were sterile filtered using Stericups® (Millipore Corp.), and aliquots (150 μl) of each were inoculated into control wells of EcoPlates. Plates were processed, read, and incubated as described above.

2.2. Experiments with Biolog MT2 MicroPlates™

2.2.1. Treatments with and without cations

EcoPlate experiments were repeated in Biolog MT2 MicroPlates to obtain a larger sample size for statistical analysis. In each of 96 wells, MT2 MicroPlates provide a minimal growth medium and tetrazolium dye, but no carbon substrate. Each MT2 MicroPlate well, therefore, is the same as an EcoPlate control well, providing 96 replicate controls per plate instead of three. Eight treatments of NSW and ASW were prepared, as described in Section 2.1. Plates were processed, read, and incubated as previously described in Section 2.1. Twenty-four to forty-eight replicates (wells) of each treatment were inoculated. Salinity and pH of treatments were measured as indicated in Section 2.1. Microscopic observations of plate wells were made after seven days.

Table 1
Measured (no notation) and calculated values of calcium (Ca^{2+}) and magnesium (Mg^{2+}) for all seawater and pond water treatments. NSW = Natural Seawater, ASW = Artificial Seawater, PW = Pond Water, Auto = Autoclaved, Filt = Sterile Filtered, and PBS = Phosphate Buffered Saline. Salinity values are indicated where pertinent.

Treatment	Ca^{2+} (ppm)	Mg^{2+} (ppm)
Natural Seawater	412[a]	1280[a]
Pond Water	<30[b]	<20[b]
Auto NSW (30)	395–415	1185–1245[c]
Filt NSW (30)	405–435	1215–1305[c]
ASW (+Ca/+Mg)	400–420	1178[c]
PW + Ca	370[c]	–
Auto NSW (15)	185–200	555–600[c]
ASW (+Ca/−Mg)	440	–
PBS	0	–
ASW (−Ca/+Mg)	0	1178[c]
ASW (−Ca/−Mg)	0	–
PW	–	–
PW + Mg	–	1178[c]

[a] Literature values. Taken from Millero (2003).
[b] Literature values. Taken from Frink and Norvell (1984).
[c] Calculated.

2.2.2. Treatments with decreasing concentrations of Ca^{2+} salts

To further examine the underlying causes of false positives, calcium chloride was added to Milli-Q water to make solutions of different calcium levels. Five treatments were prepared, including: 400 parts per million (ppm), 200 ppm, 100 ppm, and 50 ppm $CaCl_2$. A blank (0 ppm) was also prepared. All treatments were sterile-filtered using Stericups® (Millipore Corp.), and aliquots (150 μl) of each were inoculated into MT2 MicroPlates. Plates were processed, read, and incubated as previously described in Section 2.1. Forty-eight replicates (wells) of each treatment were inoculated. Salinity and pH of treatments were measured as indicated in Section 2.1. Calcium concentrations for each treatment were measured (Red Sea Aquariums Inc.; Millipore Corp.) to ensure that they matched calculated values. Another plate containing 400-ppm and 200-ppm treatments only was prepared and read at time zero and 24 h. After the 24 h reading, all materials were removed from the wells and centrifuged for 2 min at an RCF of 1300 × *g* (Eppendorf). The supernatant was then re-inoculated into the original microplate and a new reading was taken.

2.3. Microplate data analysis

Absorbance data initially were analyzed using BioTek's Gen 5 software package. For statistical analysis, a general linear model (GLM) was used (Systat13). All data were tested for normality and homogeneity of variance prior to statistical analysis. Those that did not meet underlying assumptions were transformed (square or square root) to improve normality and homoscedasticity. Two-way, Repeated Measures Analysis of Variance (ANOVAR) was used to examine the effects of treatment and time on readings from EcoPlate control wells. Only days 1–5 of data were used because of missing values for some treatments. Due to the non-homoscedastic nature of the data, Greenhouse–Geiser and Huynh–Feldt epsilons were examined and in all cases adjusted *p*-values were used. A two-way ANOVAR was also used to examine the effects of treatment and time on readings from MT2 MicroPlates using all seven days of data. If there was a significant interaction effect, a one-way ANOVAR was run to evaluate the effects of time on absorbance readings for each treatment. Paired *t*-tests were used to analyze absorbance data of well fluids before and after centrifugation. To better demonstrate differences between treatments, readings from each well (EcoPlate control wells, MT2 MicroPlate wells) in each treatment were averaged across days one through five. Treatments were then compared using a one-way ANOVA followed by Tukey's Honestly-Significant Difference post-hoc test.

3. Results

In both the EcoPlate and MT2 Microplate experiments, treatments sorted into three groups (see below) and absorbance generally decreased after day four (Fig. 1). Results of the two-way ANOVARs revealed both treatment and time effects on absorbance for the EcoPlates ($p < 0.01$; Greenhouse–Giesser epsilon = 0.533, Huynh–Feldt epsilon = 0.795), but no interaction effects. Results for the MT2 MicroPlates also showed treatment and time effects, as well as a significant interaction effect ($p < 0.01$; Greenhouse–Giesser epsilon = 0.247, Huynh–Feldt epsilon = 0.256, 2-way ANOVAR). One-way ANOVAR results for MT2 MicroPlates showed a significant effect ($p < 0.05$) of time on absorbance in all treatments except PBS and ASW (+Ca/−Mg).

Averaging absorbance data for each well over time allowed for a more direct comparison among treatments. For the EcoPlate experiments, treatment means partitioned into three distinct, statistically different groups ($p < 0.05$, 1-way ANOVA; Fig. 2a). The first group, high absorbance, comprised treatments containing cation concentrations