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Practical applications of contaminant-free *Symbiodinium* cultures grown on solid media

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Abstract. Symbiotic dinoflagellates in the genus *Symbiodinium* are critical to the success of scleractinian reef corals in shallow tropical seas. These symbionts are commonly isolated from hosts and cultured separately in liquid media (f/2 or ASP8a), but initial isolations can be prone to abundant contaminants that can persist long-term in culture. To help remove these contaminants, we developed a solid growth substrate composed of 1% agar in f/2 medium, supplemented with a variety of antibiotics, to help isolate individual clones and establish new "axenic" cultures. We found that an antibiotic cocktail of kanamycin (50 μgmL^{-1}), ampicillin (100 μL^{-1}) and streptomycin (50 μgmL^{-1}) was the most effective at eliminating visual signs of contamination without apparent harm to a variety of *Symbiodinium* in culture. Photophysiological measurements of *Symbiodinium* grown on agar plates, taken using an Imaging Pulse Amplitude Modulated (I-PAM) fluorometer, were comparable with those grown in liquid f/2 medium, both with and without antibiotics. *Symbiodinium* cultures grown on solid substrates supplemented with antibiotics are useful for: (1) isolating individual cells or clones for subsequent applications; (2) establishing and maintaining "axenic" cultures, free of observable contaminants; and (3) directly comparing (on the same plates) the photophysiology of different cultures using an I-PAM fluorometer.

Key words: *Symbiodinium*, coral, physiology, culture, I-PAM fluorometry

Introduction

Scleractinian reef corals depend on symbiotic dinoflagellates in the genus *Symbiodinium* for their metabolic needs (Muscatine 1967). Currently, 8 clades (A-H) are recognized, each of which contains multiple sub-types (Coffroth and Santos 2005; Pochon et al. 2006). This genetic diversity could provide a mechanism for corals to adapt or acclimatize to environmental changes, provided that genetic diversity in the symbionts translates to different physiological capabilities for the coral host. Studies of symbiont photophysiology may therefore help provide insight as to whether symbiont diversity can help corals survive environmental change.

Cultures of *Symbiodinium* isolated from different invertebrate and protist hosts are usually established and maintained in liquid media (f/2 or ASP8a), but are often prone to abundant contaminants that can be difficult to remove. Antibiotics and repeated dilutions have been used to eliminate some contamination; however, this process is time consuming and inconsistent (Anderson and Kawachi 2005). In addition, isolating a single cell of interest is difficult using traditional methods, which involve using capillary action to isolate a cell in an ultrafine glass pipette (Guillard 2005). Agar blocks set onto liquid media have previously been used to select for single transgenic *Symbiodinium* cells, but this method required daily additions of fresh liquid media and antibiotics (ten Lohuis and Miller 1998).

Agar plates have been routinely used for culturing microalgae, but only rarely for dinoflagellates (Schoenberg and Trench 1980; Guillard 2005). Growing *Symbiodinium* spp. on agar plates resolves some of the issues mentioned above, and agar plates with host homogenate have been successfully used to isolate new types of *Symbiodinium* in clades C and D from the giant clam, *Tridacna crocea* (Isikura et al. 2004).

Here we show how growing *Symbiodinium* on a solid medium of 1% agar, supplemented with f/2 medium and a combination of antibiotics, helps isolate single cells, eliminate microbial contaminants, and facilitate photophysiological measurements using an Imaging Pulse Amplitude Modulated Fluorometer (I-PAM: Walz, Inc., Effeltrich, Germany).

The I-PAM is a convenient tool for measuring and visualizing photosynthetic parameters such as effective quantum yield (α), electron transport rate (ETR), and quantum yield of Photosystem II (as the ratio of variable fluorescence to maximum fluorescence, or F_v/F_m). Together, these parameters

have proven useful in assessing photosynthetic response to light limitation, the ability of symbionts to tolerate changes in light over a short period of time, and overall photosynthetic efficiency (Rolfe and Sholes 1995; Ralph and Gademann 2005).

Material and Methods

Maintenance of algal cultures

Algal cultures, supplied by S. Santos (Auburn University, USA), were maintained in liquid f/2 media or solid media (described below). Incubator settings were 25.5°C, 12h L/D cycle. Liquid media were changed monthly. Cultures were re-plated monthly, while still keeping the original plates.

Production of agar plates

A solid growth medium was made by autoclaving 1% bacto-agar (Difco) in 20 mL filtered seawater. Once the mixture had cooled to <55°C, 0.4 mL of 50X f/2 medium (Sigma) was added, and the mixture was then poured onto plates (100mm diameter) and allowed to cool. After 24h, 10-30 µL aliquots of 19 different *Symbiodinium* cultures were spread onto the plates (Table 2). All plates were sealed with parafilm.

Testing resistance to antibiotics

After one month growing on the solid medium, we tested the effectiveness of antibiotics in removing contaminants in 10 cultures with the highest levels of microbial contamination. With a bacterial loop, we removed approximately 10 µL of *Symbiodinium* from each plate (together with their associated contaminants), mixed this with 200uL filtered seawater, and spread this inoculate onto fresh plates to which we added a variety of antibiotics (Table 1). These plates were prepared as outlined above, with the antibiotics added with the f/2 medium as the agar cooled. We also inoculated control f/2 plates with no antibiotics. The relative growth of microbes and *Symbiodinium* was recorded after 3, 7 and 14 days, and periodically for 5 months thereafter.

Elimination of contaminants

Symbiodinium colonies growing on the antibiotic treatment showing the least contaminants (a cocktail of kanamycin, ampicillin and streptomycin, "KAS", see Table 1) after 2 weeks were picked with a bacterial loop and re-streaked onto fresh KAS-treated plates. After 1-3 weeks, if contamination persisted, single *Symbiodinium* cells were picked from plates, using a straight wire under a microscope, and then re-streaked onto new plates.

Photophysiological comparisons of symbionts

We compared the photophysiological parameters of *Symbiodinium* culture Zs (in clade A) grown in liquid f/2 (n=5) with the same culture grown on f/2 plates (n=2) and f/2 plates with KAS (n=5). Cultures were grown from contaminant-free clones picked from a KAS plate.

Table 1: Growth of *Symbiodinium* and bacterial contaminants after 3-14 days under different antibiotic applications: + medium growth, ++ high growth, – low growth, 0 no visible growth.

Growth of *Symbiodinium* and bacterial contaminants under different antibiotic

Antibiotic	Concentration (µg/mL)	Bacteria	*Symbiodinium*
Erythromycin	50	+	-
Amph B	300	+	+
	30	+	+
Chloramphenicol (C)	50	0	0
	25	0	0
	5	-	-
Doxycycline	50	+	-
Tetracycline	50	+	-
Streptomycin (S)	50	+	+
Ampicillin (A)	3,000	+	+
Pen Strep	300	+	+
	100	+	+
Kanamycin (K)	3000	+	+
	300	+	+
	30	+	+
G418	3,000	+	+
	1,000	+	+
	300	+	+
	100	+	+
K+A	50/100	-	+
K+A+S	50/100/50	-	++
K+A+S+C	50/100/50/5	-	-
None	0	+	+

I-PAM measurements were taken after 19 days. Cultures were dark adapted for 20 min before induction curves were measured, and then returned to the incubator for 20 min before rapid light curves were measured.

The maximum electron transport rate (ETR_{max}) and the effective quantum yield (α) were calculated by fitting rapid light curves to a double exponential decay curve in Sigma Plot (Ralph and Gademann 2005). Photosynthetic efficiency (F_v/F_m) was obtained from induction curves. For solid plates, we compared photosynthetic parameters extracted from the whole plate with those obtained from the average of 5 random points on the plate. For the liquid medium we measured parameters from the whole culture (Fig. 1). Data were compared using a one-way ANOVA followed by post-hoc Tukey-Kramer HSD tests if significant differences were found.

Results

Resistance to antibiotics

Most liquid stock cultures are prone to high levels of microbial contamination that cannot be controlled with a single antibiotic application. Cultures maintained on f/2 plates with KAS remained free of contaminants for at least 5 months, with no visual signs of bacterial growth. Agar plates with KAS show the highest relative growth for symbionts but the lowest bacterial growth (see Table 1). In addition, bacterial growth was easily reduced by physically removing symbionts from bacteria by picking clones and re- streaking. There was 100% mortality for both symbionts and bacteria on chloramphenicol plates after 3 days. Kanamycin and G418 had a high relative growth for at least one bacterial morphotype (based on colony shape and color), even at 3 $mgmL^{-1}$. Some cultures on KAS plates still showed some bacterial contaminants after 5 months; however, contamination was approximately an order of magnitude less on a per-cell basis than the same culture grown in control f/2 media (solid or liquid) with no antibiotics (see Table 1).

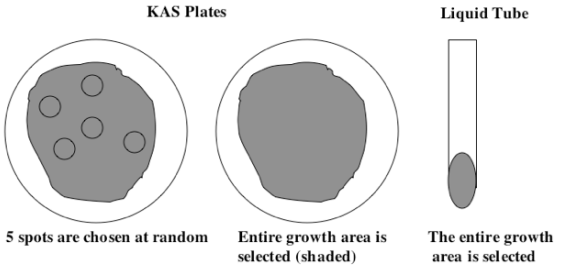

Figure 1: Two methods for IPAM measurements were used: selecting 5 spots at random of growth area, or selecting the entire growth area referred to as "whole plate." The entire liquid area (20 ml) was selected for liquid tube I-PAM measurements. Lids were removed from plates under the IPAM.

Many types of *Symbiodinium* can be grown on agar plates (Table 2), but in this study, members of clades A and C had the highest growth rates. Most of the 19 *Symbiodinium* types we tested on agar plates showed positive growth on this medium. Some were difficult to maintain long-term, particularly those in clade D. However, the majority of cultures were easily maintained on agar plates for >5 months and repeatedly used to create new stocks.

Photophysiological comparisons

The two sampling methods for taking I-PAM measurements on plates (5 random spots vs. whole plate), produced comparable mean values of ETR_{max} and α (P> 0.7292 and P> 0.9998, determined by a one- way ANOVA, respectively), both on f/2 only plates and f/2 + KAS plates. Further, following the ANOVA test, the F_v/F_m, values as shown by a post hoc test(Tukey-Kramer HSD test) displayed no significant difference within the same type of plates for either of the two sampling methods (Fig. 2). In contrast the post hoc test did show a significant difference between plates and liquid tubes for F_v/F_m Variability in all parameters was less using the "random spot" method, compared to "whole plate" method (error bars in Fig. 2). F_v/F_m values were significantly lower for solid media on plates,

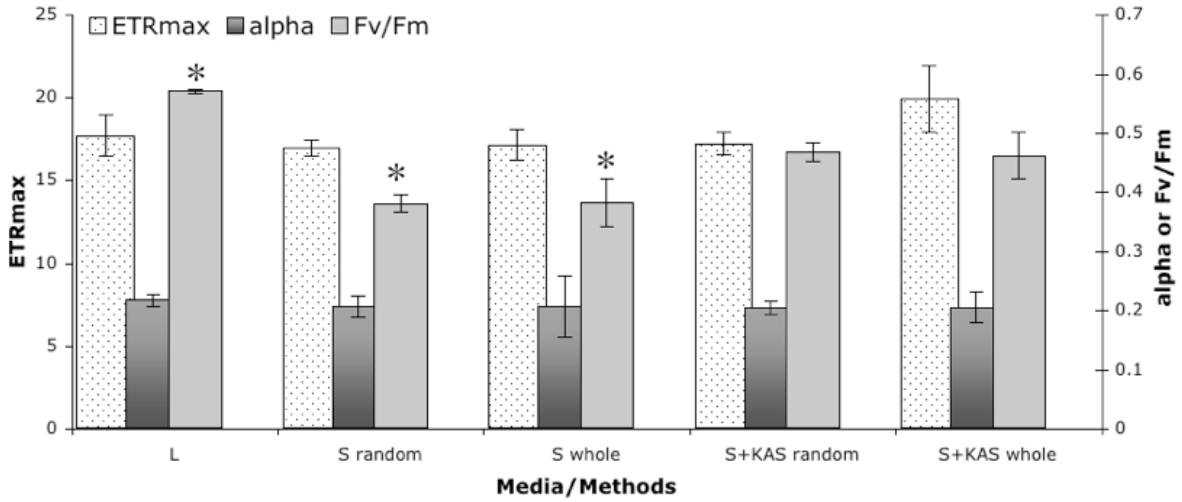

Figure 2: Comparison of photophysiological parameters (ETR_{max}, α and F_v/F_m) for *Symbiodinium* culture Zs (clade A) grown on three media types: L (liquid f/2), S (solid agar f/2) and S+KAS (solid agar f/2 supplemented with KAS) (whole plate and 5 spots). Error bars represent standard error of the appropriate mean value. A Tukey-Kramer HSD test revealed significant differences between treatments for parameters marked with an asterisk (*). Values not marked with an asterisk are not significantly different among methods and media.

compared to liquid media in tubes (P>0.0007; Fig. 2). All other comparisons showed no statistical differences from each other.

Discussion

General plate applications

Our results indicate that growing *Symbiodinium* on sold agar plates supplemented with f/2 and antibiotics is useful for the following applications: isolating single cells and clones, purifying cultures and reducing contamination, and undertaking comparative photophysiological studies. Current techniques to remove microbial contamination include repeated antibiotic doses applied on a weekly, or even daily, basis. This can be expensive and time-consuming. Using f/2 + KAS plates, the use of antibiotics is limited to 2-3 applications over a >5 month period. Transferring symbionts to new media is only necessary when plates begin to dry out (which typically occurs after a few months), and is mainly done as a precautionary step to avoid dessication and and self-shading of cultures. In addition, microbial contamination can be readily detected (by eye or microscope) and removed. In liquid media, contamination is much harder to detect, and can be done only by determining relative "cloudiness." Our cultures remained completely free of any visible contamination (via microscope) for >5 months on the same KAS plates, and remained sufficiently viable to establish new cultures at the end of this period. A potential pitfall of any plate-based method is local depletion of either antibiotics or nutrients over time, leading to mortality or decreased growth rates. More studies are needed to determine the long-term viability of *Symbiodinium* maintained on the same plate. Another potential problem is that sub-culturing from plate to plate may result in genetic drift. This might be reduced by mixing multiple clones picked from plates to establish new cultures.

Table 2: Types of *Symbiodinium* grown on agar plates, with their invertebrate host and geographic location. * denotes contaminate free cultures for over 5 months, + denotes slow growing, - denotes low amounts of contaminants.

Types of Symbiodinium cultures grown on agar plates

Culture ID/Clade	Invert Host	Geo Location	Status
FLAp1/A	*Aiptasia pallida*	Caribbean	*
T/A	*Tridacna gigas*	Indo-Pacific	-
719/A	*Pseudoplexaura porosa*	Caribbean	*
Zs/A	*Zoanthus sociatus*	Caribbean	*
Y109/A	*Unknown host*	W. Pacific	-
Mf 12.5f/B	*Montastraea faveolata*	Caribbean	-
Pd/B	*Pocillopora damicornis*	C. Pacific	-+
703/B	*Plexaura kuna*	Caribbean	-
PurPflex/B	*Plexaura flexuosa*	Caribbean	*
Mf 1.56/B	*Montastrea faveolata*	Caribbean	-
Mf 6.1T/C	*Montastraea faveolata*	Caribbean	-
Mv/C	*Montipora verrucosa*	C. Pacific	*
PtBr/C	*Briareum* sp.	Caribbean	*
Pa 45a/C	*Porites astreoides*	Caribbean	-
Mf10.8a/D	*Montastraea faveolata*	Caribbean	-
A008/D	*Acropora sp-4*	W. Pacific	-+
A013/D	*Porites annae*	W. Pacific	-+
Ap37/D	*Unknown anemone*	W. Pacific	-
CCMP421/E	*Free living*	W. Pacific	-

An additional potential application of agar plates and antibiotics is the selection of genetically transformed *Symbiodinium*. Chloramphenicol plates were very effective in killing *Symbiodinium* (and bacteria), and might therefore be used as means of selecting for successful transformants.

I-PAM specific plate applications

ETR_{max} and α were not significantly different for tubes or plates (with or without antibiotics), indicating that the plate method is equivalent to the traditional liquid tube method for rapid light curve measurements. We recommend that the "whole plate" method be used for large-scale experiments with >5 replicates, since the method is more rapid while still yielding results that are comparable to the "random spot" method. For studies with few replicates, the "random spot" method should be used to reduce error.

We found plates easier to manipulate than liquid media since there was no need to separate clumps and the entire plate could be visualized using the I-PAM. Additionally, individual clones grown on plates can be selected by the I-PAM as "Areas of Interest", allowing within-population comparisons to be made. In liquid media, where symbiont populations are mixed, these distinctions cannot be made.

Solid plates also allow clones with certain characteristics (such as high growth rate, varying F_v/F_m rates etc) to be selected from a population. Finally, different types of *Symbiodinium* can be compared side-by-side on the same plate (we have cultured up to 4 cultures in different quadrants of the same plate) for physiological comparisons among types. This helps reduce error within treatments.

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