Differential grazing of selected marine algivorous amoebae on microalgae and bacteria

Daniel G. Burton 1, 2, Charles O'Kelly 1

Blinks Research Fellowship 2012

1 Friday Harbor Laboratories, University of Washington, Friday Harbor, WA 98250
2 Department of Biology, Gallaudet University, Washington, DC 20002

Contact Information:
Daniel Burton
Gallaudet University
800 Florida Ave
Washington, DC 20002
Daniel.burton@gallaudet.edu

Abstract

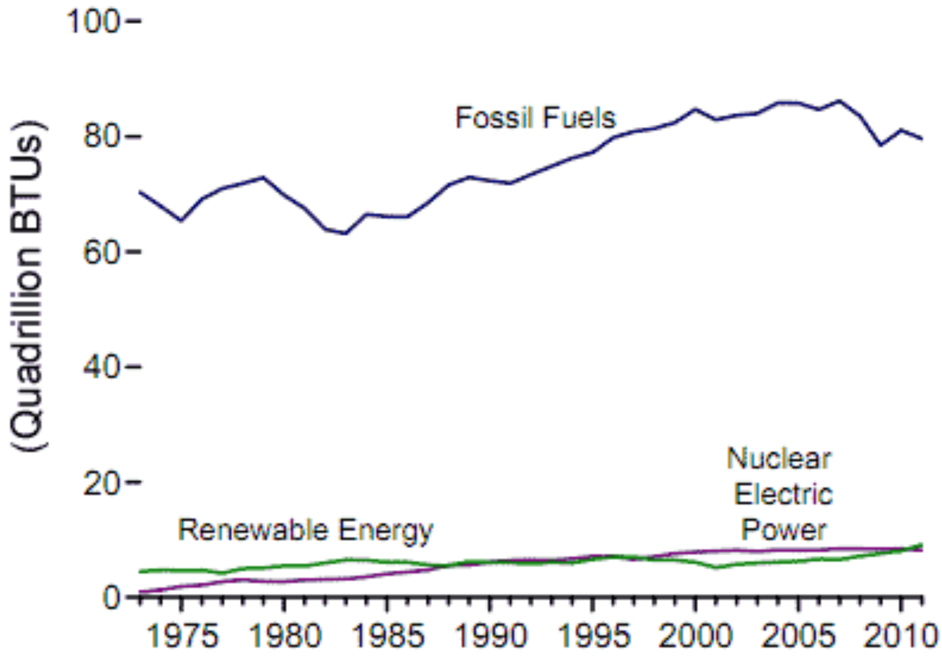
Production of biofuels derived from the mass of photosynthetic organisms is gaining attractiveness as climate change, resource shortage, and pollution events make it less sensible to continue current modes of fossil fuel consumption. The high oil-to-weight ratio of algae yields potential as a sustainable source of biodiesel, but current cultivation systems are plagued with contamination events leading to crop failure. Protozoic amoebae are the culprits of this invasion and while their feeding preferences or abilities are well known in regards to bacteria, with algae they are not. To begin implying an index of susceptibility between amoebic strains and marine algae, isolated *Neoparamoeba sp.*, *Paramoeba sp.*, and *Thecamoeba sp.* specimens from Hawai'i were given treatments of marine algae (*Chaetoceros sp., Pleurochrysis carterae, Nanochloropsis sp., Skeletonema marinoi, Isochrysis sp.*) in f/2- (silicon absent) media and algal density correlated to spectrophotometer absorbance values. Also, bacterial treatments were smeared in a uni-directional "raceway" on f/2- agar and the distance that amoebae from one end of the strip recorded as average migration rate per day. These methods proved successful in illustrating that the feeding abilities or preferences of marine amoebae vary greatly across algal and bacterial species. This valuable insight may inform managerial decisions in the event of contamination discovery and highlight directions for further research into the specific relationships between marine algae, bacteria, and amoebae that might either support or suppress the successful commercial cultivation of algae as a feedstock for biodiesel.

Introduction

Average global atmospheric CO_2 is at a record high of the last 650,000 years (Siegenthaler et al. 2005). Between the start of the industrial revolution in 1750 and present day of August 2012, this average has increased from 280 to 396 ppm. A major cause of this trend is the propagation of a carbon-intensive model by the world economy is overwhelming the limited ability of land and ocean CO_2 sinks to absorb emissions (Canadell, 2007). The inability to abandon this economic model in light of a finite resource and evidence connecting it to climate change stresses the need for sustainable alternatives.

An immediate option is in the form of biofuels derived from the mass of biological carbon fixation processes, i.e. living things (Demirbas 2009). Field et al. estimated in 1998 the annual CO_2 fixation by land plants as between $56 \text{ x } 10^9 \text{ tons/year}$. Much of this carbon is used in the synthesis of biomolecules such as carbohydrates and fatty acids, both of which have become the focus of biofuel research and generating popular products such as ethanol and biodiesel (Schubert, 2006). If grown in a sustainable way, atmospheric CO_2 contributed by biomass combustion has no impact on current CO_2 balance as photosynthetic fixation performed by the biomass cancels out the effect (Hall et al. 1991, Macedo 1992). However, as much that biofuels promise a renewable alternative to fossil fuel usage, commercial production in the U.S. is a long way from displacing current levels of consumption (Figure 1).

Figure 1: U.S. energy consumption by fuel type, 1975-2010 (source: EIA 2012)



Manufacturing efficiency, crop contamination, economic stimulus, and competition for natural resources are all aspects of biofuel production that affect commercial success. Corn is the dominant source of ethanol. 73% of the plant oil harvested in 2005 came from soybeans, oil palm, coconuts and rapeseed (Carlsson et al. 2007). The popularity of corn and soybean is due to the convenient accommodation of existing agricultural infrastructure already in place. However, in light of dwindling land and water resources, land-use priority is given to food crops, emphasizing the need to shift away from terrestrial plants for fuel production. Marine and freshwater algae support this trend, removing strain from land resources with the ability to inhabit a wide range of aquatic conditions. Combined with carbon-fixing metabolisms which exceed that of higher-trophic plants, many species of algae are attractive as a possible feedstock for biodiesel (Demirbas 2007, Carlsson et al., 2007). However, microalgal cultivation for the production of food additives, nutritional supplements, and cosmetics is also a growing industry (Spolaore 2006), and the financial gain between a nutritional crop and a biodiesel crop (\$1,000's ton^{-1} vs \$100's ton^{-1}, respectively) increases the demand for predictable results. Labor and harvesting costs (Christi 2008, Borowitzka 1997), insufficient of standardized data in literature dealing with species selection (Griffiths 2009), and lack of cohesion between laboratory results and large-scale culture caused by environmental variables such as evaporation, flooding, temperature and light fluctuations, contamination (Pulz 2001, Lee 2001), are all persistent challenges on the path towards successfully producing microalgae at the volume necessary to sustain a profitable venture (presently ca. 100 tonnes/hectare/year) (Day et al. 2012).

The scalability of open-pond production methods will likely be utilized, and one model has been proposed by Huntley and Redalje (2007). Uni-algal cultures are first reared in nutrient-replete conditions within a photo-bioreactor in order to promote rapid cell division, then transferred to nutrient-restricted open ponds in order to stress them into increased synthesis of cellular lipids. While nutrient limitations may discourage cellular division of the target organism as well as any potential competitor species, it does little to protect it from predatorial micro-organisms which instead graze on algae, such as amoebae.

In nature, amoeba and algae rarely interact due to their presence in a complex food web where there exist a multitude of higher and lower-trophic organisms such as flagellates, ciliates, and bacteria. It is only in an artificial system that amoebae are promoted to top predator, and their presence is pervasive. Design considerations of a commercial model are impacted by the availability of either freshwater or marine resources. While a freshwater system may be supplied with pre-treated municipal water, contamination by air transport remains a risk as freshwater amoebae revert to microbial cyst form when triggered by extreme environmental conditions, i.e. dessication periods. In a marine system, amoebae bypass UV radiation and filtration checkpoints of seawater treated en-route to ponds. Modern detection techniques are limited to concentrations of 10^3 cells/L, a number already dangerously close to the tipping point (Day et al. 2012). Being the elephant in the room of industrial drawing boards, the behavior of amoebae in dense microalgal culture is very little understood. Detailed models of amoebic grazing rates on various algae will be an indispensible tool in the anticipation of contamination events, which have been known to cause total crop failure in a matter of days.

In the study described below, we begin to sift the complexity of interactions between predator and prey by testing first the hypothesis that amoebae which prefer algae will eat any type of algae. Algivorous amoebae isolated from two field sites in Kailua-Kona Hawai'i are inoculated with both algal and bacterial prey. Rates of change in algal density due to predation are observed via spectrophotometry absorbance at 680 nm, the wavelength which corresponds most closely to chlorophyll. In the second stage, we determine whether amoebae with the ability to graze on bacteria will graze on any bacteria. A "raceway" experiment is designed using twenty species of bacteria (Table 2) smeared into a narrow strip on f/2- (silicon absent) agar. Collection of this data allowed us to evaluate whether amoebae that graze on bacteria will graze on only bacteria. Our amoebic strains exhibited highly diverse levels of heterotrophy, supporting the null hypotheses in each case. This data contributes to building a repository of information about the trophic preferences of amoebae on bacterial and algal species, providing the opportunity to direct future research towards the nature of specific interactions, possibly leading to the dependable cultivation of marine algae for a biodiesel product due to improved management decisions.

Materials & Methods

Isolation and Cultivation

Uni-algal cultures were sustained on f/2- (silicon-absent) media in a temperature-controlled environment of 23° under fluorescent illumination (46 µmol photons $\cdot m^{-2} \cdot s^{-1}$ irradiance, 12:12^{-h} light:dark photoperiod). Amoebae were cultivated in 60 x 15 mm petri dishes containing agar supplemented with 2216 nutrient and a narrow smear of heterotrophic bacteria, under identical conditions. Table 1 lists species of algae and amoebae used in this experiment.

Table 1. Algae and amoeba species tested

Prey Algae		Source
F641	Chaetoceros sp.	Ex. Strathmann
F644	Nannochloropsis	
F037	Pleurochrysis carterae	UW 15-6-21s
F164	Skeletonema marinol	UW-Friday Harbor Laboratory
F645	Isochrysis sp.	Tahiti
Amoebae		
03a	Neoparamoeba sp.	Kona Demonstration Facility, Cellana LLC, Kailua-Kona, HI, Nov.2009
R1	Neoparamoeba sp.	
KPF3	Neoparamoeba sp.	
08pp	Paramoeba sp.	Royal Kona Resort lagoon, Kailua-Kona, HI, Oct.2011
8t	Thecamoeba sp.	

Spectrophotometry

Algal concentration was initially estimated as the mean of three .01 µL subsamples screened according to the instructions of a Bright-Line hemacytometer and diluted with f/2- media in 5 mL screw-top test tubes to a density of $10^5 \text{cells mL}^{-1}$. Amoebae were transplanted to liquid culture by suspending and re-suspending 100 µL of sterile f/2- media on the surface of each respective petri dish. Each algae / amoebae combination was replicated three times, including non-amoeba controls, (total 64). Absorbance was measured at 680 nm using a UV/VIS spectrophotometer (Hach DR5000, serial 1307295). These initial cultures yielded negligible absorbance readings, positive readings were achieved by reducing sample volume to 3 mL with a ratio of 2:5 between algae stock/media (.864 mL to 2.14 mL, respectively). The media component of this final run was divided into: a) 1 mL from previous run and b) 1.14 mL of fresh media, in order to conveniently supply each culture with amoebae.

F645 was observed separately and subject to a slightly modified experimental design. Amoeba were isolated from agar stock and allowed to multiply in liquid f/2- media supplied with SS1 bacteria that had been washed of 2216 media and re-suspended in f/2- then added in high and low concentrations to the media component of algal cultures diluted to give an initial absorbance reading of ca. 0.1. Three replications for each amoeba/algae combination were started, and 100µL from one of these three replications was withdrawn and treated with a drop of iodine for cell enumeration via hemacytometer. Two control samples received the same treatment - one was left alone, another supplied aliquots for enumeration.

Bacterial Raceways

Bacteria specimens (Table 2) were deposited on f/2- agar in narrow “raceways” approx 30 mm in length and 15 mm apart. Amoebae were transplanted using a dental pick to remove a ca. 1 mm³ cube from its stock agar to one end of bacterial streaks. The distance that amoebae migrated from its starting points to the other end of the raceways was recorded every 24 hours for 4 days.

Table 2. Bacterial and amoebic species tested

Bacteria		Amoeba	
1	PBR 2E	1	03a (Neoparamoeba sp.)
2	PBR 2D	2	R1 (Neoparamoeba sp.)
3	PBR 2BW	3	KpF3 (Neoparamoeba sp.)
4	Ty A no 1	4	8t (Thecamoeba sp.)
5	Ty B no 2		
6	Ty D no 11		
7	Ty G no 16		
8	Pond 4D i		
9	Pond 4D g		
10	Pond 4D h		
11	Pond 4D e		
12	Ty L no 24		
13	SS1		
14	Red P		
15	Bact x6H6		
16	Ty M no 26		
17	PBR A		
18	Ty O no 29		
19	Bact ex A		
20	Bact x6H10		

DNA extraction, direct PCR amplification, and sequencing

Genomic DNA was extracted from 20 bacterial samples using the Quiagen DNEasy Microbe Mini-kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions. 1 µL reaction volume was used for direct PCR of small subunit (SSU) rRNA genes using primers (Table 3) 16S63F and 16S1387R

(Marchesi et al. 1998). Electrophoresis was performed on Bio-Rad Mini Sub-Cell GT (Bio-Rad Laboratories, Hercules, CA, USA) with 1.5% agarose/TBE gel.

Table 3: SSU rRNA primer sequences

16S63F	5' CAG GCC TAA CAC ATG CAA GTC 3'
16S1387R	5' GGG CGG WGT GTA CAA GGC 3'

Data Analysis

Spectrophotometry

Absorbance data from the most dependable sample (steepest negative slope) per combination of algae/amoebae were used to graph data in a scatter plot (Figures 2-6) and the slopes recorded to indicate predation rate.

Bacterial raceways

Bacterivoric preference was illustrated by bar-graphing the mean distance that amoebae travelled per-day (mm).

Results

Spectrophotometry

Figure 2: Spectrophotometry absorbance of strain F644 at 680 nm

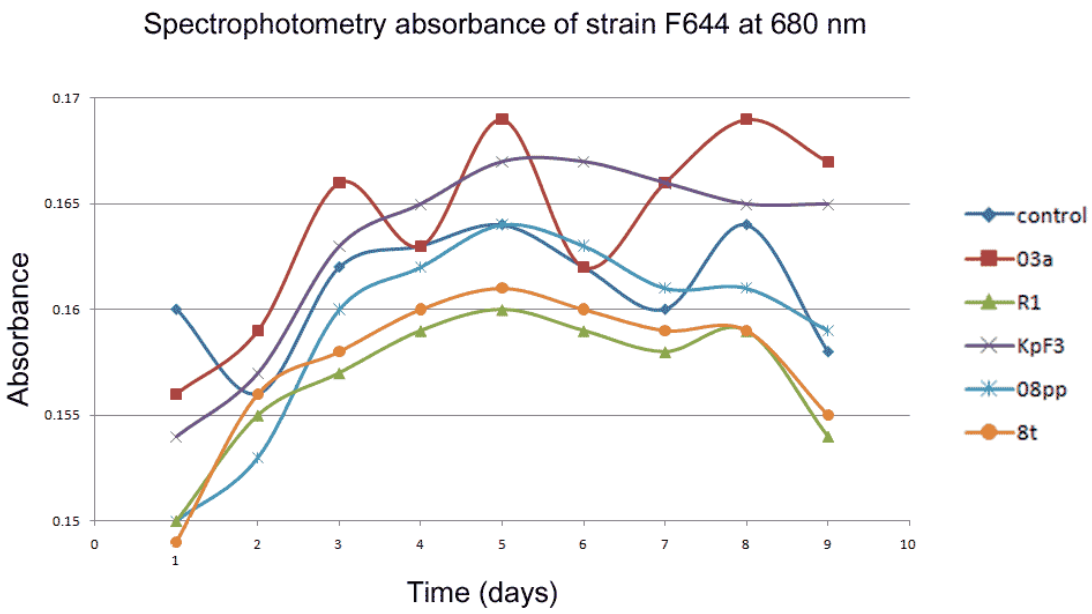


Figure 3. Spectrophotometry absorbance of strain F641 at 680 nm

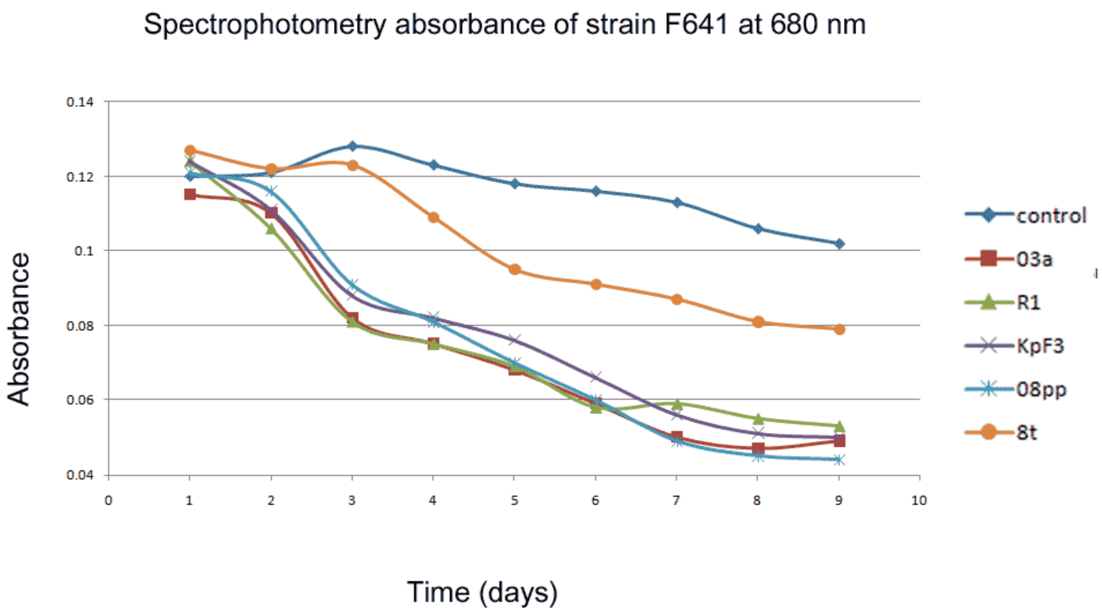


Figure 4. Spectrophotometry absorbance of strain F162 at 680 nm

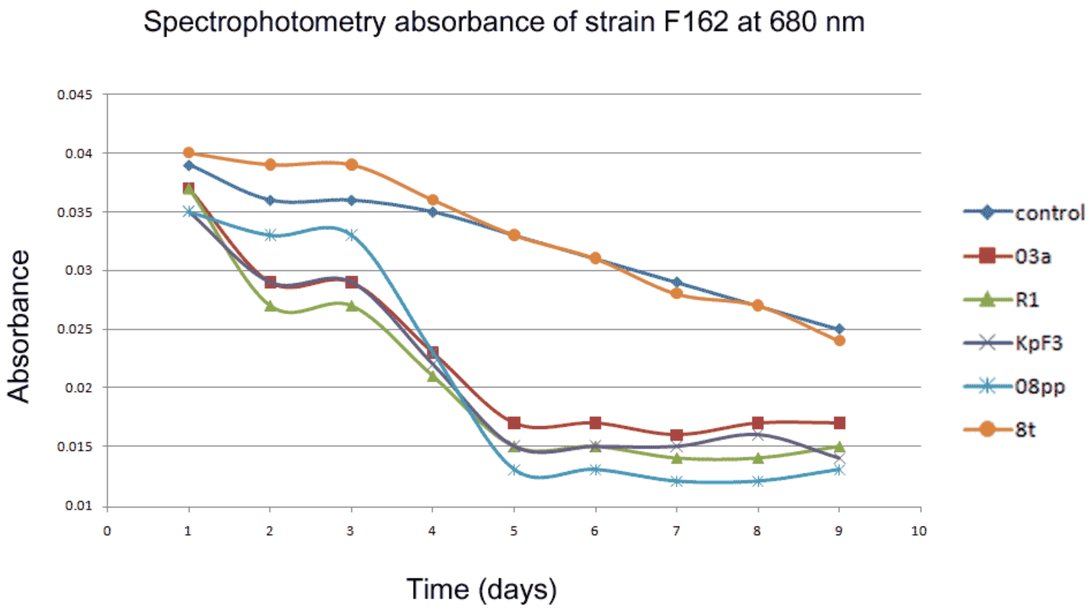


Figure 5. Spectrophotometry absorbance of strain F036 at 680 nm

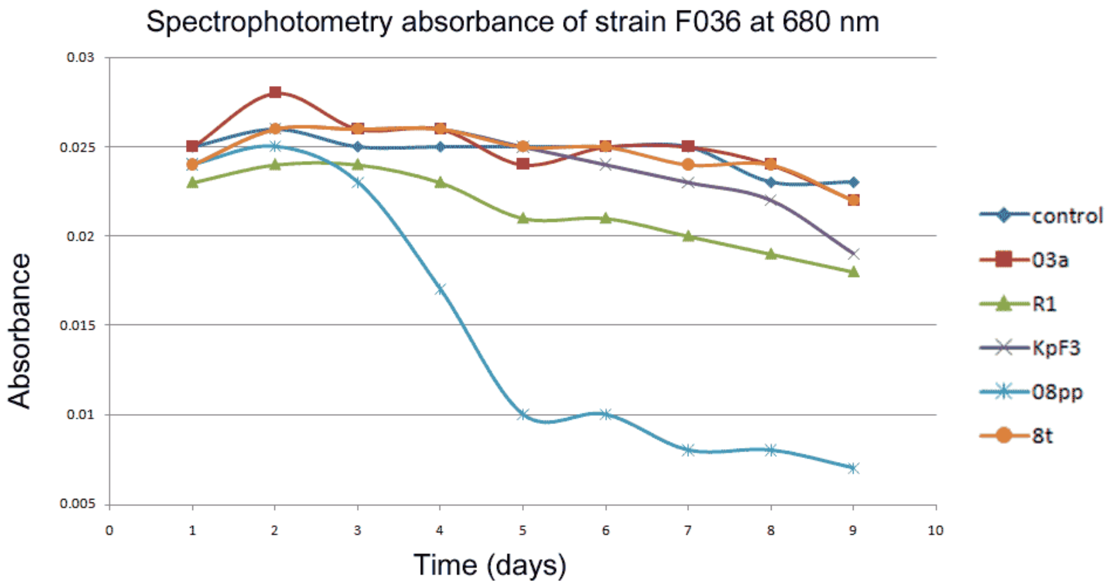


Figure 6. Spectrophotometry absorbance of strain F645 at 680 nm

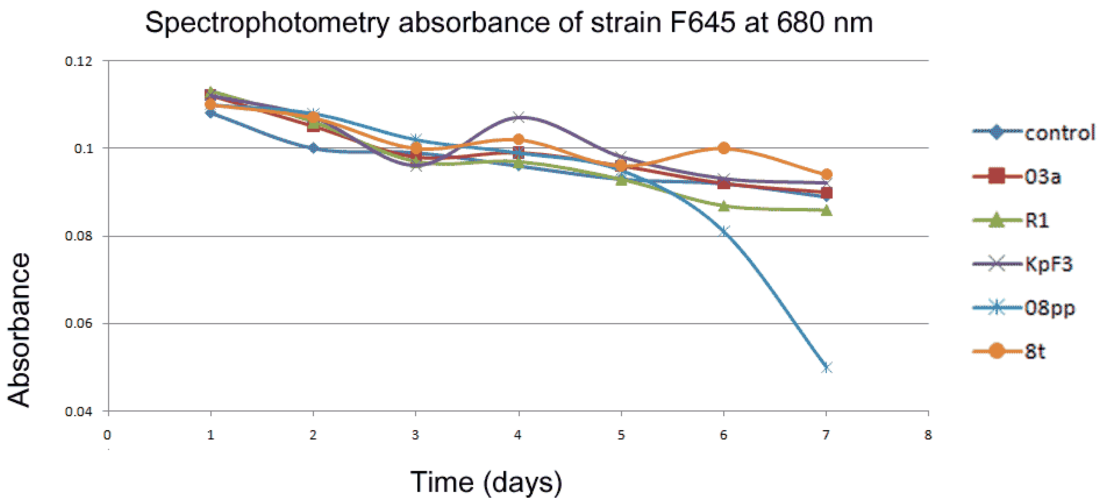


Table 3. Linear slopes of experimental cultures vs. controls

	F641	F644	F162	F036	F645
Control	-0.002	0	-0.001	0	-0.002
03a	-0.035	0	-0.012	0	-0.002
R1	-0.043	0	-0.012	0	-0.002
Kpf3	-0.03	0	-0.014	0	-0.002
08pp	-0.035	0	-0.02	-0.013	-0.008
8t	-0.019	0	0	0	-0.002

DNA extraction, direct PCR amplification, and sequencing

DNA extraction was successful, although PCR products yielded no results after electrophoreisis.

Bacterial raceways

Figure 7. Average 24-hour migration rate of 03a, R1, Kpf3 (mm)

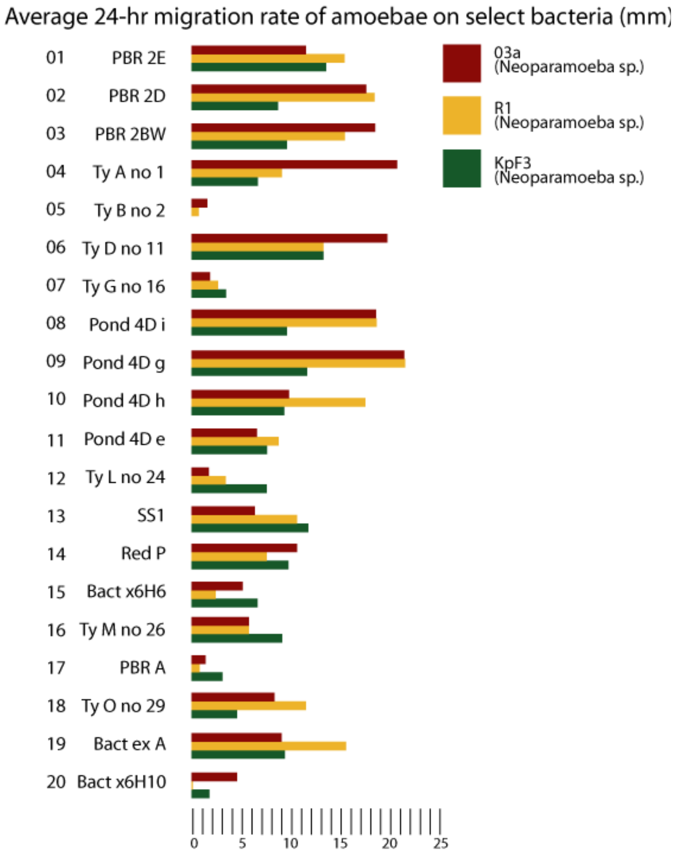
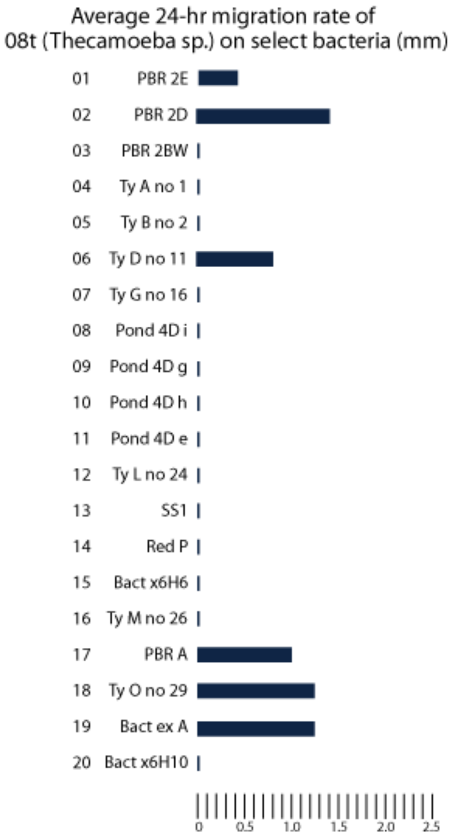


Figure 8. Average 24-hour migration rate of 08t (mm)



Discussion

Results show varying degrees of heterotrophy between both marine amoebae/algae and marine amoebae/bacteria, and there seem to be some specialized abilities of both algae and bacteria to resist grazing. Predation on algae strain F644 (*Nannochloropsis*) was negligible throughout amoebic specimens, while amoebic strain 08pp exhibited an enhanced ability to graze on strains F036 (*Pleurochrysis carterae)* and F645 (*Isochrysis sp.)* (-0.013 and -0.006, respectively, Table 3). The contamination threshold of 08pp with strain F645 appears delayed in comparison to F036, indicating that the increased locomotion of F645 in culture may contribute to grazing resistance. The steepest slopes of the experimental data points (Table 3) may be of mathematical interest, potentially used to calculate a susceptibility index in the future. Using absorbance in lieu of population could be interpreted as a function of time with the equation:

$$\frac{dA}{dt} = -(m + g(A)z)A$$

Where *m* represents mortality, *A* is absorbance, *g* is grazing rate, and *z* is the number of consumers (amoebae). An estimation of the product was acquired using experimental data in a second equation (below). The slope of natural log-transformed control data can be applied to *m* and added to the product of below equation to retrieve *g(A)z*, represented as the rate of grazing in relation to absorbance (*A*) and the number of consumers (*z*) and expressed as a more accurate susceptibility index (SI).

$$-m - g(A)z = \frac{\ln\,(At2 - At1)}{t_2 - t_1}$$

Bacterial specimens TyBno2 and TyGno16 were generally resistant to grazing by all amoebae specimens (Figures 7, 8), and 08t exhibited partial preference for strainsÉ. Without genetic data, there is little information to direct inquiries towards the physiological characteristics that may influence amoebic grazing rates on the bacteria. Future research may benefit from standardizing algal density across specimens before being subject to spectrophotometric analysis.

Acknowledgments

This research was supported by:

- The National Science Foundation (144 PE44)
- The University of Washington
- Cellana, LLC of Kailua-Kona, HI
- Royal Kona Resort of Kailua-Kona, HI

References

1. Borowitzka MA. Microalgae for aquaculture: opportunities and constraints. Journal of Applied Phycology. 1997. 9: 393-401.
2. Canadell JG, LeQuere C, Raupach MR, Field CB, Buitenhuis ET, Ciais P, Conway TJ, Gillett NP, Houghton RA, Marland G. 2007. Contributions to the accelerating atmospheric CO2 growth from economic activity, carbon intensity, and efficiency of natural sinks. PNAS. 104, 47: 18866-18870.
3. Carlsson AS, Beilenvan JB, Moller R, Clayton D. 2007. In: Bowles D. (Ed.), Micro- and Macro-Algae: Utility for Industrial Applications. EPOBIO Report CPL Press.
4. Chisti Y. Biodiesel from microalgae beats bioethanol. Biotechnology trends. 2008. 26: 126-131.
5. Costa JAV, Morias MG. 2011. The role of biochemical engineering in the production of biofuels from microalgae. Bioresource Technology. 102: 2-9.
6. Demirbas A. Importance of biodiesel as transportation fuel. Energy Policy. 2007. 35: 4661-4670.
7. Griffiths M, Harrison S. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. Journal of Applied Phycology. 2009. 21: 493-507.
8. Hall DO, Mynick HE, Williams RH. Cooling the greenhouse with bioenergy. Nature. 1991. 353:11.
9. Huntley, ME, Redalje DJ. CO2 Mitigation and Renewable Oil from Photosynthetic Microbes: A New Appraisal. Mitigation and Adpotion Strategies for Global Change. 2007. 12: 273-608.
10. Lee YK. Microalgal mass culture systems and methods: their limitations and potential. Journal of Applied Phycology. 2001. 13: 307-315.
11. Macedo IDC. Energy from biomass and wastes. Biomass Bioenergy. 1992. 3: 77-80.
12. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Applied and Environmental Microbiology 64: 795-799 (corrigendum p. 2333).

13. Pulz O. Photobioreactors: production systems for phototrophic microorganisms. Valuable products from biotechnology of microalgae. Applied Microbiology and Biotechnology. 2001. 57: 287-293.
14. Schubert C. Can Biofuels Finally Take Center Stage? Nature Biotechnology. 2006. 24, 4: 777-784.
15. Siegenthaler U, Stocker TF, Monnin E, Luthi D, Schwander J, Stauffer B, Raynaud D, Barnola J-M, Fische H, Masson-Delmotte V, et al. 2005. Sable Carbon Cycle–Climate Relationship During the Late PleistoceneScience. Science. 310:1313–1317.
16. Spolaore P, Joannis-Cassan C, Duran E, Isambert A. 2006. Commercial applications of microalgae. Journal of Bioscience and Bioengineering. 101, 2: 87-89.