

**SINGLE CELL ACTIVITY OF AEROBIC ANOXYGENIC PHOTOTROPHIC
BACTERIA IN THE DELAWARE ESTUARY**

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Marine Studies

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BACTERIA IN THE DELAWARE ESTUARY**

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ABSTRACT

Aerobic anoxygenic phototrophic (AAP) bacteria are abundant in estuaries, coastal regions and in the open ocean, comprising 1-30% of total prokaryotes. To understand their distribution and potential contribution to the carbon cycle, single cell activity of the microbial community was determined by ^3H -leucine incorporation detected using microautoradiography combined with additional steps to identify AAP bacterial cells before and after microautoradiography. The approach was used on transects through the Delaware estuary in August and November 2011. The percent of active AAP bacteria was up to two fold higher than the percentage of active bacteria for the rest of the bacterial community in the estuary. Likewise, the silver grain area associated with active AAP bacteria was larger than that for the rest of the community, indicating higher rates of leucine consumption by AAP bacteria. The difference between the activity of AAP bacteria and other bacteria was greatest in high salinity waters and was lowest in freshwater. The incorporation of ^3H -leucine by AAP bacteria did not vary with light under ambient light conditions as well as in incubations with constant light and darkness. Our results suggest that AAP bacteria are more active than other bacteria in many parts of the estuary, just not in all locations.

Chapter 1

INTRODUCTION

Aerobic anoxygenic phototrophic (AAP) bacteria contain bacteriochlorophyll a that is used to capture energy from sunlight (Yurkov and Beatty 1998). AAP bacteria are abundant in all of the world's oceans ranging from the poles to the equatorial tropics in the open oceans (Beja 2002, Cottrell 2006, Salka et al. 2008, Sieracki et al 2006, Yutin 2005, Kolber 2000 and 2001, Koh 2011). In addition to being abundant in the open oceans, AAP bacteria are abundant in estuaries (Schwalbach and Fuhrman 2005, Cottrell et al. 2008, Waidner and Kirchman 2008, Zhang and Jiao 2007, Lami et al. 2007). The abundance of AAP bacteria averages 5-10% of the prokaryotic community (Yutin 2008).

Data about growth rates may help us understand the abundance and potential contribution of AAP bacteria to biogeochemical processes. A few studies have found that AAP bacteria have a higher growth rate than other bacteria. Koblizek et al. (2007) used the turnover rate of bacteriochlorophyll to calculate the growth rates of AAP bacteria in the eastern Atlantic Ocean. They determined that AAP bacteria have a growth rate as high as 1.08 day^{-1} while the rest of the prokaryotic community has a growth rate around 0.1 day^{-1} . This technique assumes that bacteriochlorophyll is not synthesized in light but

recent evidence suggests there might be low levels of synthesis in light (Spring et al. 2009). This would suggest that calculations based on the assumption that the bacteriochlorophyll was only synthesized in the light are not completely accurate.

A common approach to examine the activity of specific bacterial groups is fluorescent in-situ hybridization (FISH) in conjunction with microautoradiography (Brock 1967, Lee et al. 1999). This approach provides information on the activity of the bacteria targeted by FISH probes based on consumption of radiolabeled compounds such as ^3H leucine. However, this approach does not work for AAP bacteria because they are too diverse to be detected by a single FISH probe for rRNA (Yutin et al. 2007). Instead, AAP bacteria are identified by the infrared autofluorescence of bacteriochlorophyll (Schwalbach and Fuhrman 2005, Cottrell et al. 2006). However, this autofluorescence cannot be used in conjuncture with microautoradiography because bacteriochlorophyll does not survive the microautoradiography assay, preventing identification of AAP bacteria after microautoradiography. We needed to develop a way to identify AAP bacteria before and after microautoradiography.

In this study we developed a method that combines IR epifluorescence and microautoradiography and used it to examine the growth-related activity of AAP bacteria in the Delaware Bay. AAP bacterial abundance usually ranges from 5-15% of the total prokaryotic community in the Delaware estuary which

is higher than the average for the open ocean (Cottrell et al. 2006). AAP bacteria have been observed to be associated with particles in the turbid portions of the Delaware estuary and similar waters (Lamy et al. 2011, Waidner and Kirchman 2007). The composition of the AAP bacterial community varies in the estuary (Waidner and Kirchman 2008). We examined the seasonality of AAP growth activity and the effects of light and other environmental conditions on single-cell growth. We hypothesized that AAP bacteria would be more active than the rest of the bacterial community. We found that AAP bacteria are more active but the difference in activity compared to the rest of the bacterial community varied in the estuary.

Chapter 2

METHODS

Sample Collection

Water samples were collected aboard the RV Hugh R Sharp during two cruises in August and November 2011 (Appendix Table 1). Additional samples were collected during monthly sampling trips in Delaware coastal waters (Appendix Table 1). Samples were processed on board or immediately upon returning to the laboratory. Environmental parameters such as light attenuation and salinity were measured and collected for a variety of other measurements. Samples were collected for chlorophyll a by filtering 100 mL seawater through 25mm GF/F filters. 150mL of seawater was filtered through ashed 47mm GF/F filters and frozen for analysis with a SEAL autoanalyzer for nitrate, ammonium, phosphate and silicate. Total bacterial production was determined from the incorporation of ^3H -leucine (Kirchman 2001).

Samples for AAP bacteria-microautoradiography (AAP-MAR) were incubated with ^3H leucine at a final concentration of 20 nmol/L in polycarbonate bottles. The assays used [4,5- ^3H] leucine with a specific activity of between 50-60 Ci/mol obtained from Amersham. Incubations lasted one hour and were conducted under ambient light conditions. These incubations were ended with the addition of paraformaldehyde (PFA) at a final concentration of 2%. An

hour after the addition of PFA, samples were filtered through black polycarbonate filters (25 mm with a pore size of 0.2 μm) and frozen at -80°C .

Effect of light on AAP bacterial activity

Two types of experiments were conducted to examine the effect of light on AAP and total bacterial activity. Samples were collected 4 and 9 hours after dusk and then the activity of AAP bacteria and the total bacterial community was assayed in dark incubations. The second experiment was conducted in 10 L carboys. Whole water was collected in the 10 L carboys before dawn in bottles that were blacked out with heavy-duty foil and in other bottles with no covering which were exposed to light. After 6 hours in the light or the dark, 5 mL of sample water from each treatment was incubated with ^3H leucine for an hour at a final concentration of 20 nmol/L and ended with 2% PFA. The samples were frozen at -80°C .

Enumeration of AAP bacteria and microautoradiography

A section of the filter was stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes and then mounted on a microscope slide. The sample was analyzed with a microscope with an automatic stage capable of moving in the x-y plane with a precision of 0.2 μm . Before the analysis began the coordinates of the apex and one corner of the filter were recorded (for details see Appendix). These points were used as fixed points for the relocation of the fields of view. Four images of each field of view were taken using the excitation/emission parameters detailed in Cottrell et al. (2006) for DAPI,

bacteriochlorophyll a (Bchl a), chlorophyll a (Chl a), and phycoerythrin.

MicrobeCounter, an image analysis software, identified the cells by edge detection, and AAP bacteria were identified as cells that were positive for the DAPI stain and Bchl a fluorescence and negative for Chl a and phycoerythrin.

After the AAP bacterial count the filter pieces were removed from the microscope slide, dipped in 100% acetone to remove the oil, and dried (Appendix). Microautoradiography was performed using Kodak NTB emulsion, developer and fixer following published protocols (Cottrell and Kirchman 2003). Exposure in the emulsion lasted 24 hours. The coordinates of the apex and corner of filter section were recorded before the reanalysis. The coordinates of the previously analyzed fields of view were calculated using the location of the field in relation to two anchored coordinates. This calculation is possible because the angle formed by the lines connecting the anchor points and the apex is the same for the field of view determined before and after microautoradiography. The stage was moved to the new calculated coordinates to view the first field analyzed for AAP bacteria. Once the field was manually focused on the DAPI cells, images of DAPI fluorescence and silver grains (bright field) were acquired. The silver grain image was acquired using transmitted light. The stage was then moved to the next field using calculated coordinates and continues until all of the fields have been examined. After all fields of view have been analyzed ImagePro was used to align DAPI images from before and after the microautoradiography assay.

The user determined whether the images were properly aligned based on the number of cells shared between the two images. If the images matched, then the two DAPI images were merged to create a composite image that accounts for all cells. The MicrobeCounter program counts all cells and cells with silver grains, and calculates cell size, cell volume, and the area of the silver grains.

Chapter 3

RESULTS

Total bacterial abundance was highest near the mouth of the bay, averaging 3.35×10^6 cells mL^{-1} in August (Table 1). Bacterial abundance decreased up the estuary to 1.87×10^6 cells mL^{-1} farthest from the mouth of the bay. During November the bacterial abundance did not vary substantially, remaining around 2.15×10^6 cells mL^{-1} in the entire estuary. Salinity and the light attenuation coefficients increased down the estuary in both months (Table 1). Chlorophyll a concentrations varied slightly across the estuary, being highest up the river, reaching $17 \mu\text{g/L}$ in August. Nutrients also varied throughout the estuary with nitrate ranging from $132 \mu\text{M}$ in the river to about $2 \mu\text{M}$ near the mouth of the bay during both months (Table 1). Phosphate concentrations were slightly lower in the mouth of the bay during both months, averaging 0.8 to $1.9 \mu\text{M}$ in the entire estuary.

The abundance of AAP bacteria varied with location in the estuary and between the two months. Abundance of AAP bacteria was highest in the waters farther from the mouth of the estuary, reaching 12% in August and 16% in November (Figure 1). AAP bacterial abundance was lowest at the mouth of the estuary, as low as 1.2% in August and 2.3% in November. AAP bacterial abundance was slightly higher in November than in August (Figure 1). There

was a significant negative correlation between the relative abundance of AAP bacteria and salinity ($r = -0.69$; $p < 0.0001$; $n = 26$) and between AAP bacterial abundance and light attenuation ($r = 0.46$; $p = 0.030$; $n = 21$).

Bacterial production of the entire community was higher in August than November (Figure 2). In August, leucine incorporation varied throughout the estuary and was higher farthest from the mouth of the bay, reaching $518 \text{ pmol Leu L}^{-1} \text{ h}^{-1}$. Production decreased with distance down the bay to a low of $86 \text{ pmol Leu L}^{-1} \text{ h}^{-1}$. Leucine incorporation was lower in November than in August, and it was highest farthest from the mouth of the estuary, reaching $460 \text{ pmol Leu L}^{-1} \text{ h}^{-1}$.

The percent of bacteria incorporating leucine varied throughout the estuary. The percent of all bacteria that incorporated leucine was 15-25% in August and 5-17% in November (Figure 3A). AAP bacteria were more active than the rest of the community near the mouth of the estuary (Figure 3B). In August the AAP bacteria were up to 13% more active than the rest of the community near the mouth of the bay. In November, the AAP bacteria were only 7% more active than the rest of the community near the mouth of the bay. Around 60 km from the mouth of the bay, however, there was little difference between the activity of the total community and the AAP bacteria; other bacteria were even more active than AAP bacteria in some locations. In August, other bacteria were up to 11% more active than the AAP bacteria around 100 km from the mouth of the bay in waters with a salinity of 3. In

freshwaters, there was little difference between the percent activity of AAP bacteria and the total community (1.5% and 0.5%).

The area of silver grains created during microautoradiography is a quantitative indication of leucine incorporated (Cottrell and Kirchman 2003, Sintes and Herndl 2006). The average silver grain size varied in August from 0.15 to 0.78 μm^2 for the total community (Figure 4A). In November the average silver grain area was smaller, ranging from 0.2 to 0.55 μm^2 . Overall, AAP bacteria had larger silver grains than the average bacterium near the mouth of the estuary (Figure 4B), although farther up the estuary AAP bacteria did not always have larger silver grains. Near the mouth of the bay the silver grain area associated with the AAP bacteria were up to 0.8 μm^2 larger than those with the rest of the bacteria, in August. In November, the difference in silver grain size was smaller (only 0.2 μm^2) than in August.

The silver grains associated with AAP bacteria increased down the estuary (Figure 5). Silver grains associated with AAP bacteria were larger closest to the mouth of the bay than in the estuary. The silver grain area decreased up the river and was smallest in low salinity waters.

The percent of AAP bacterial cells that were active had a significant negative correlation with light attenuation, nitrate and silicate (Table 3). The area of silver grains associated with AAP bacteria was negatively correlated with the distance from the mouth of the bay, nitrate and phosphate. The silver grain area associated with AAP bacteria was also positively correlated with

salinity. The difference in percent active cells between AAP bacteria and the rest of the prokaryotic community was negatively correlated with distance to the mouth of the bay and light attenuation. The difference in silver grain area associated with AAP bacteria and the rest of the prokaryotic community was negatively correlated with Chl a and nitrate.

Experiments were conducted to determine the effect of light on the activity of AAP bacteria (Table 2). The time of day when the samples were collected, day or night, had no effect on the activity of AAP bacteria. Two experiments were conducted at different locations beginning before sunrise and lasting 6 hours. The AAP bacteria were almost twice as active as the total bacteria in both experiments. However, there was no significant difference in the activity of AAP bacteria in the light versus dark incubations.

Multiple samples were collected at the mouth of the bay (FB station) over time, allowing us to examine bacterial activity from October to December. The percent active cells was highest in October (30%) and lowest in December (6%) (Figure 6). In August and October, the percent of active AAP bacteria was similar to the rest of the bacterial population. However, the percent of active AAP bacteria was 4% higher than the rest of the bacteria in December. In August silver grain area was larger associated with AAP bacteria than for the rest of the community. The silver grain area was not larger for AAP bacteria, however, in waters outside the estuary from November to December 2011.

Table 1. Biogeochemical parameters of the Delaware estuary in August and November 2011. The bay was split into three sections: mouth of the bay=0-40km from the mouth of the bay, middle bay=40-80 km from the mouth of the bay, up river= 80-120km from mouth of bay. For the August cruise samples n=10 for mouth of bay, n=6 for middle, n=7 for up river and for the November cruise n=11 for mouth of bay, n=7 for middle and n=7 for the river. Atten= attenuation coefficient and Temp= temperature.

	August						November					
	Mouth Bay		Middle		River		Mouth Bay		Middle		River	
	<u>Mean</u>	<u>SD</u>	<u>Mean</u>	<u>SD</u>	<u>Mean</u>	<u>SD</u>	<u>Mean</u>	<u>SD</u>	<u>Mean</u>	<u>SD</u>	<u>Mean</u>	<u>SD</u>
Total bacteria (10 ⁶ cells mL ⁻¹)	3.35	0.6	2.78	0.6	1.87	0.9	2.13	0.8	2.13	1.0	2.19	0.7
Salinity	28.3	2.5	17.0	2.9	3.6	4.0	26.4	4.7	12.4	3.9	3.1	2.5
Temp (°C)	23.8	2.4	27.2	0.4	28.1	0.2	13.3	0.4	12.3	1.0	11.8	1.0
Light Atten (m ⁻¹)	-0.8	0.2	-1.25	0.04	-2.7	1.4	-0.69	0.1	-1.6	0.6	-2.9	1.6
Secchi (m)	2.1	1.9	1.0	0.1	0.6	0.4	2.1	0.4	0.9	0.3	0.7	0.5
Chl a (µg/L)	4.0	2.1	4.6	0.9	8.9	5.1	4.8	1.2	3.1	1.0	8.5	3.1
Nitrate (µM)	34.0	73.4	63.3	14.3	92.7	31.6	17.4	12.4	55.0	13.8	99.7	16.7
Ammonium (µM)	5.0	2.3	4.1	2.2	2.4	3.2	4.2	1.4	3.5	0.7	7.0	8.9
Phosphate (µM)	0.9	0.6	1.5	0.5	1.9	0.2	0.8	0.3	1.5	0.1	1.4	0.1
Silicate (µM)	33.3	41.1	48.6	40.8	14.8	7.1	21.1	13.0	35.4	12.2	51.5	51.5

Table 2. Activity of AAP bacteria and the total community in light and dark experiments. Seawater was collected before sunrise and incubated for 6 hours under light or dark conditions. The leucine incorporation of cells was determined for the initial time point and after exposure to 6 hours of light or complete darkness. Experiment #1 was conducted on water from 63.5 km from the mouth of the bay and 14.4 salinity water on August 9, 2012. Experiment #2 was conducted on water from 10 km from the bay with 31.2 salinity water on August 8, 2012.

		% Active Cells				Silver Grain Area (μm^2)	
		<u>Total Bact</u>	<u>SD</u>	<u>AAP Bact</u>	<u>SD</u>	<u>Total Bact</u>	<u>AAP Bact</u>
Exp 1	Initial	12.4	5.5	22.0	16.5	0.64	1.12
	Light 6 h	8.6	3.5	15.7	10.3	0.47	0.70
	Dark 6 h	20.2	11.9	30.8	16	0.77	1.45
Exp 2	Initial	21.5	15.5	44.8	20.9	0.54	1.03
	Light 6 h	24.0	6.6	50.8	24.5	0.98	1.70
	Dark 6 h	22.8	15.0	51.0	26.3	0.83	1.60

Table 3. Correlation between aspects of AAP bacterial activity and environmental parameters. Pearson correlation coefficients were calculated for percent active AAP bacteria, the area of silver grains associated with AAP bacteria, the difference in percent active cells and the difference in silver grain areas (SGA). *p<0.05; **p<0.01.

	<u>% Active AAP Bacteria</u>	<u>AAP Bacterial SGA</u>	<u>Difference in % Active (AAP-DAPI)</u>	<u>Difference in SGA (AAP-DAPI)</u>
Distance to Mouth	-0.389	-0.500*	-0.466*	-0.170
Salinity	0.378	0.444*	0.233	-0.049
Temperature	0.176	0.021	-0.202	-0.047
Bacterial Production	0.191	0.061	-0.330	-0.052
Secchi Depth	0.098	0.355	0.281	0.274
Light Attenuation	-0.642*	-0.510	-0.636**	-0.460
Chlorophyll	-0.274	-0.449	-0.494	-0.624*
Nitrate	-0.704**	-0.713**	-0.463	-0.660*
Ammonium	-0.221	-0.248	0.191	-0.179
Phosphate	-0.467	-0.583*	-0.463	-0.514
Silicate	-0.616*	-0.271	-0.060	-0.196

Figure 1. Abundance of AAP bacteria across the Delaware estuary. The solid line is the regression line for the August data and the dashed line the November data.

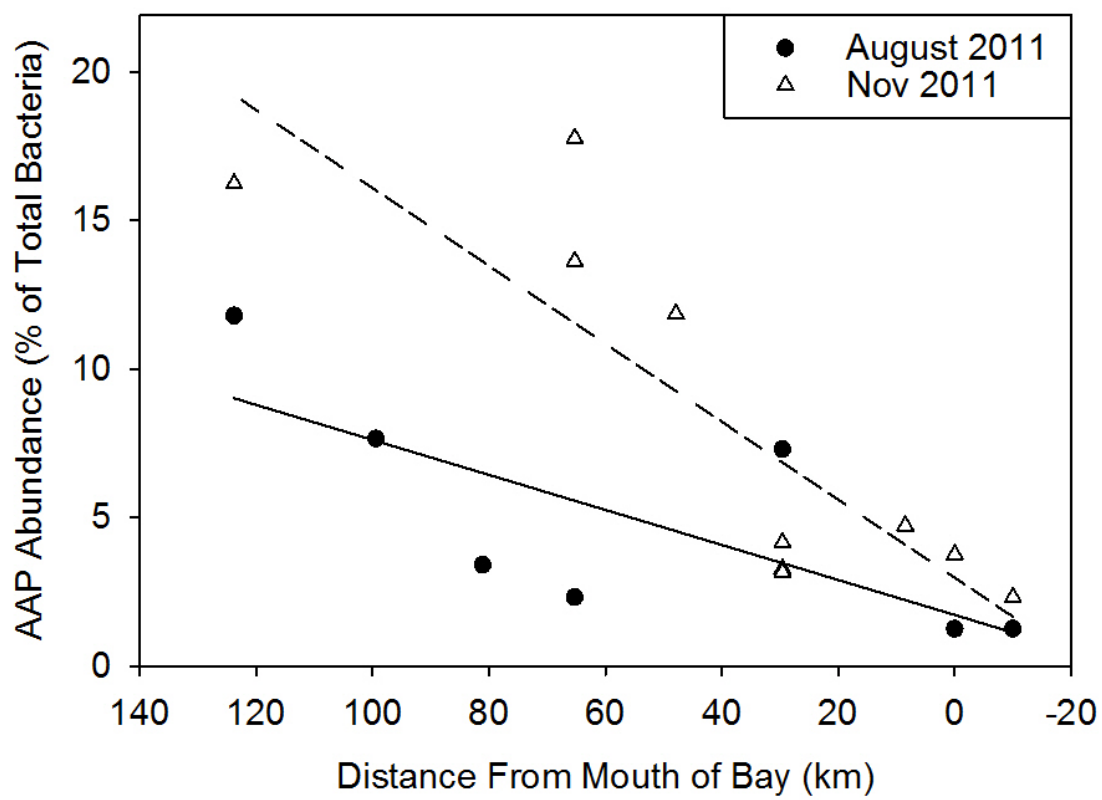


Figure 2. Leucine incorporation in the Delaware Estuary. The solid line is the regression line for the August cruise and the dashed line is the November cruise

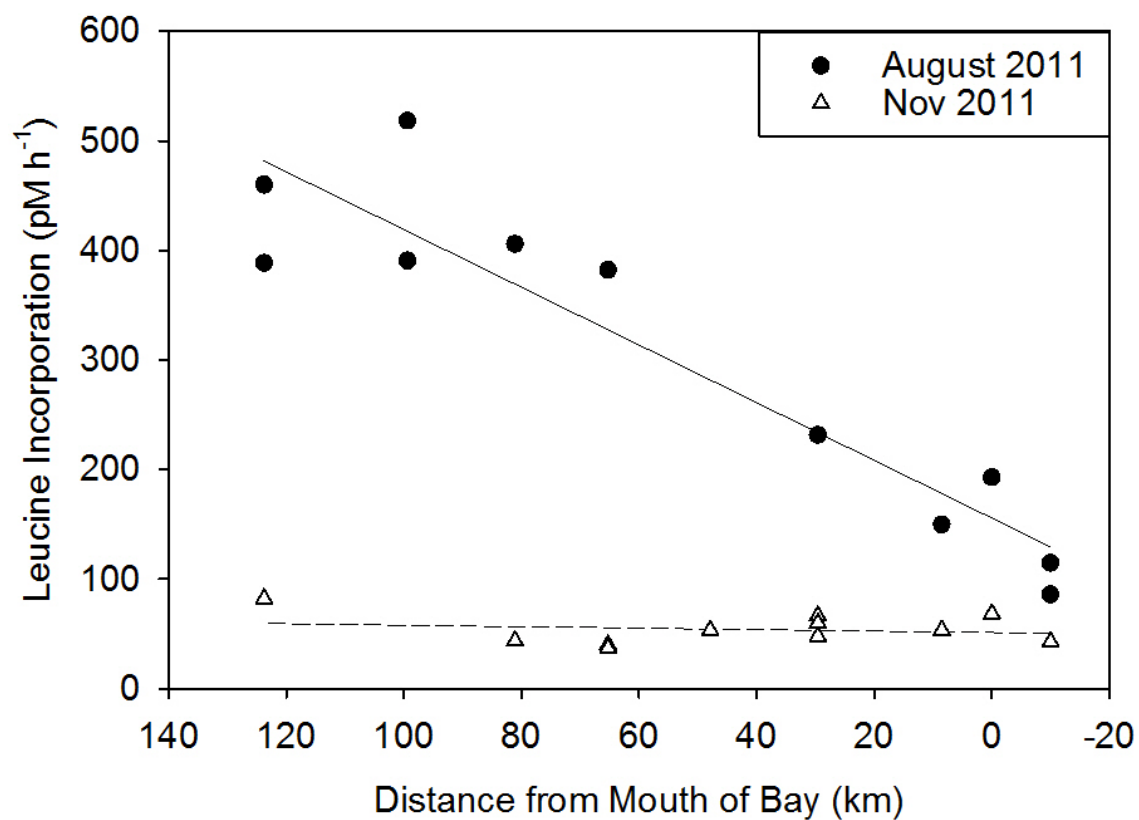


Figure 3. Percent activity of bacteria in the Delaware Estuary assayed by leucine incorporation. (A) The percent active cells in the total bacterial community. (B) The difference in percent activity between AAP bacteria and the total population. The difference was percent of active AAP bacteria minus percent of total active bacteria.

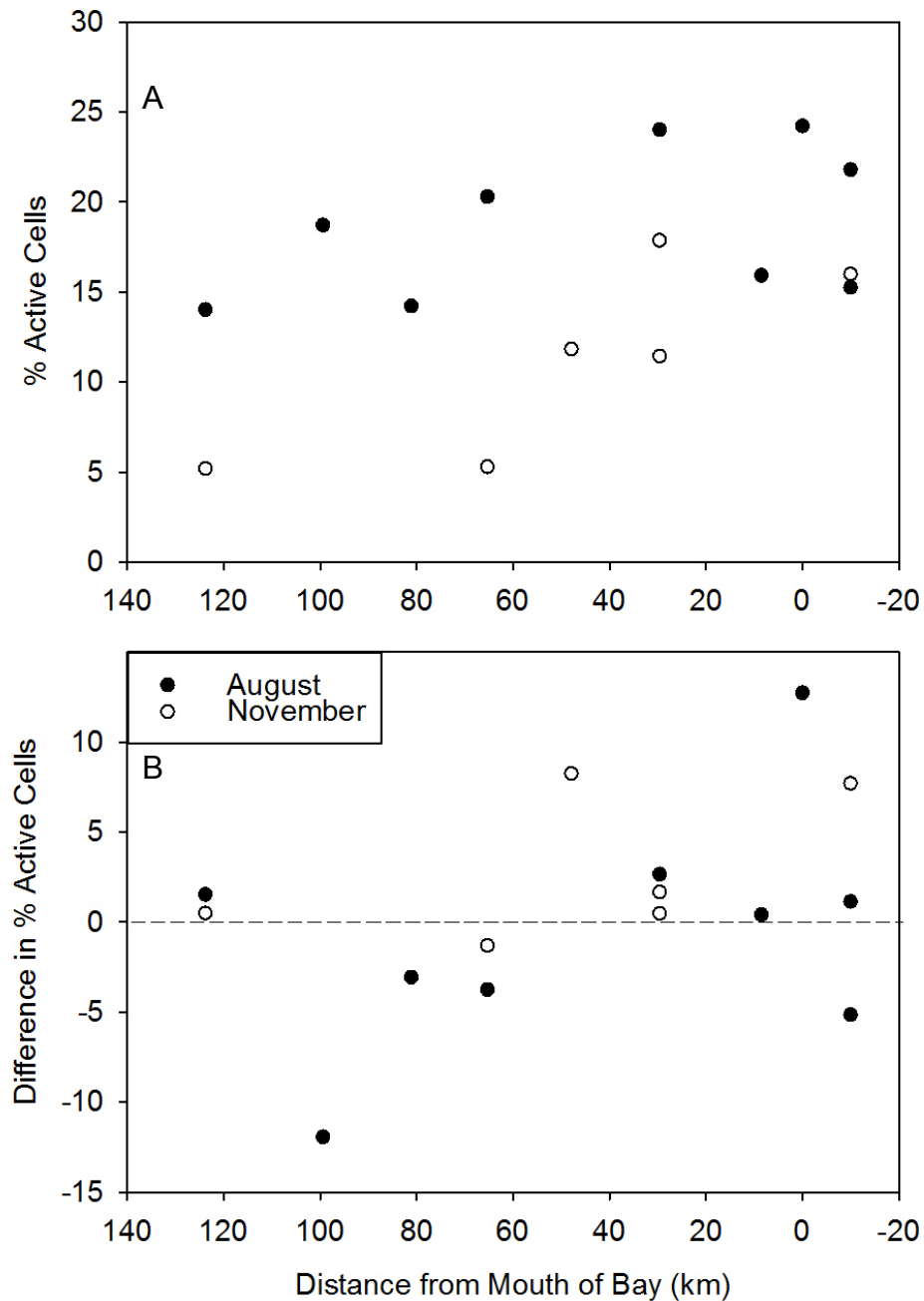


Figure 4. Area of silver grains associated with leucine-active bacteria in the Delaware Estuary. (A) The average silver grain area for the entire bacterial population. (B) The difference in silver grain areas associated with AAP bacteria and the rest of the population. The average silver grain area associated with the total bacterial community was subtracted from the AAP bacteria at a sample site.

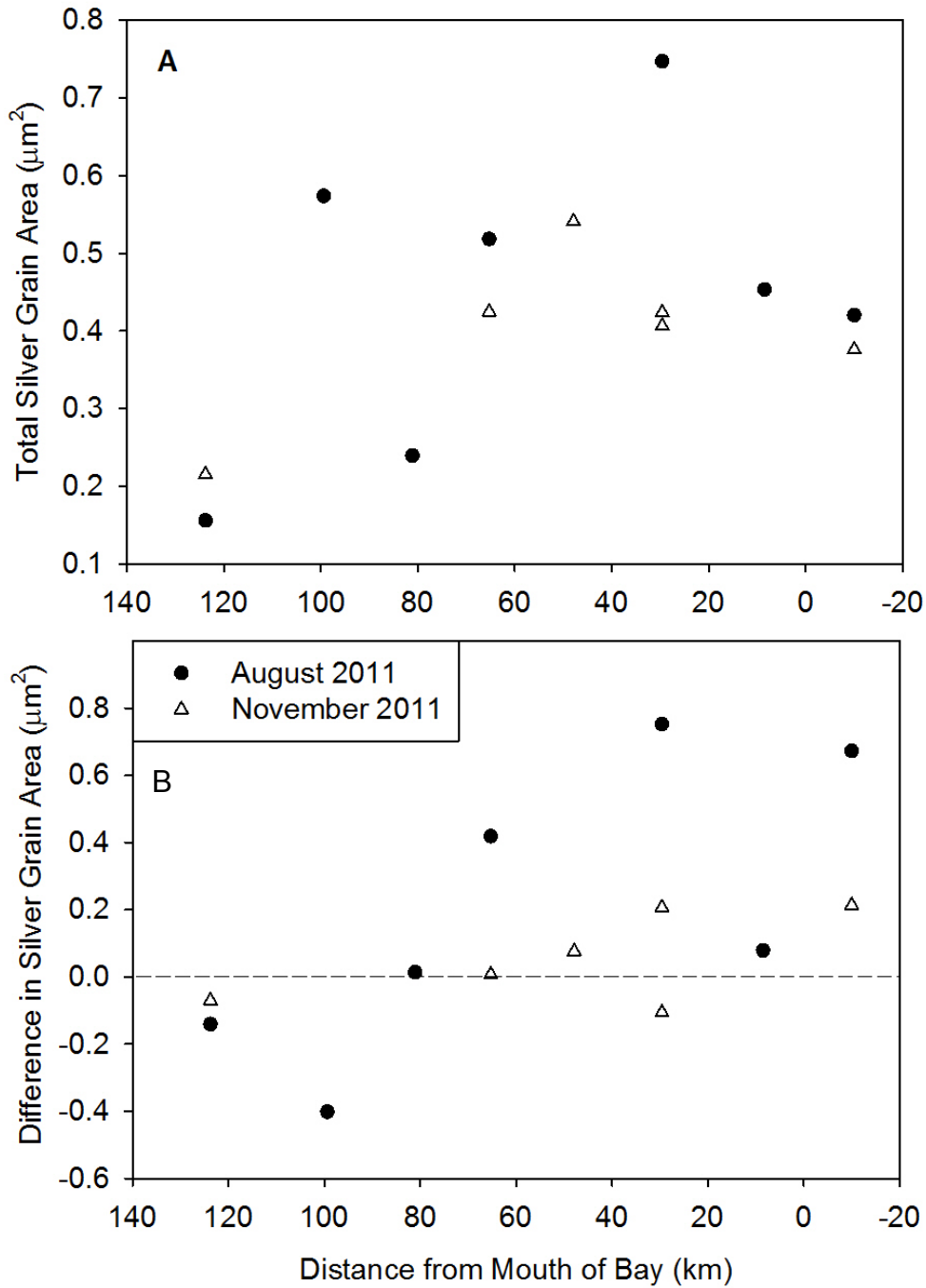


Figure 5. Silver grain area associated with AAP bacteria active for leucine incorporation in the Delaware estuary. The line was calculated from a regression analysis.

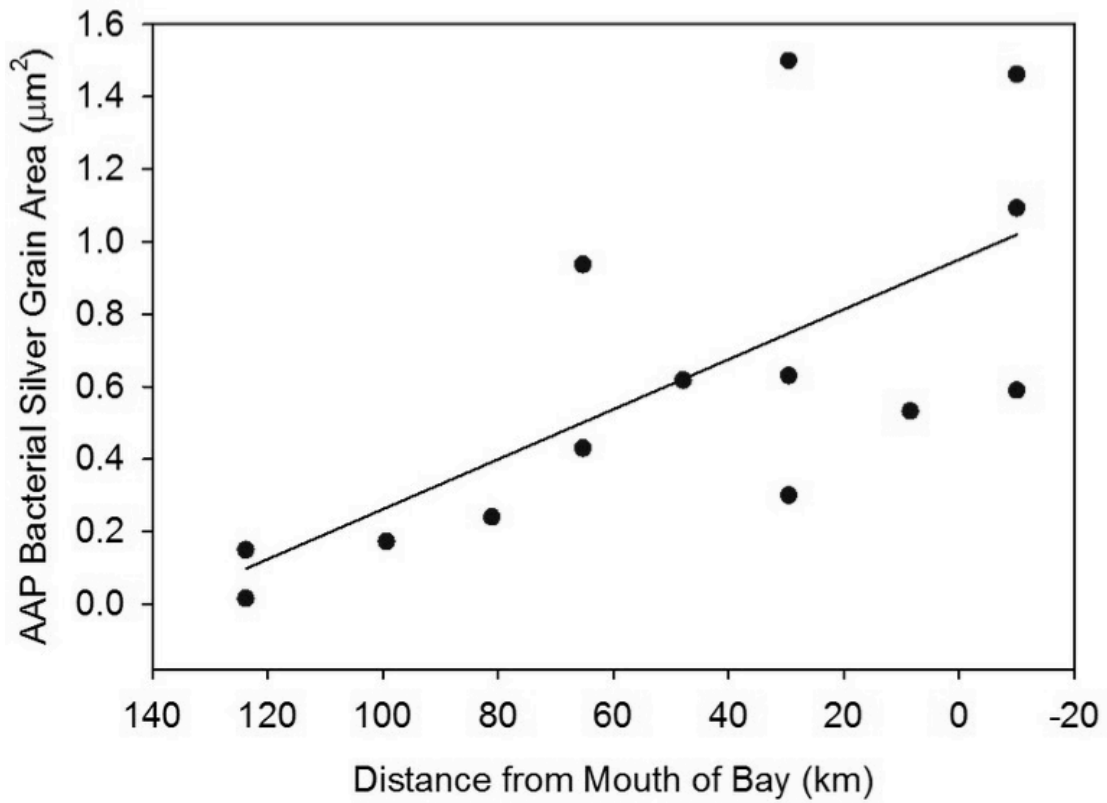
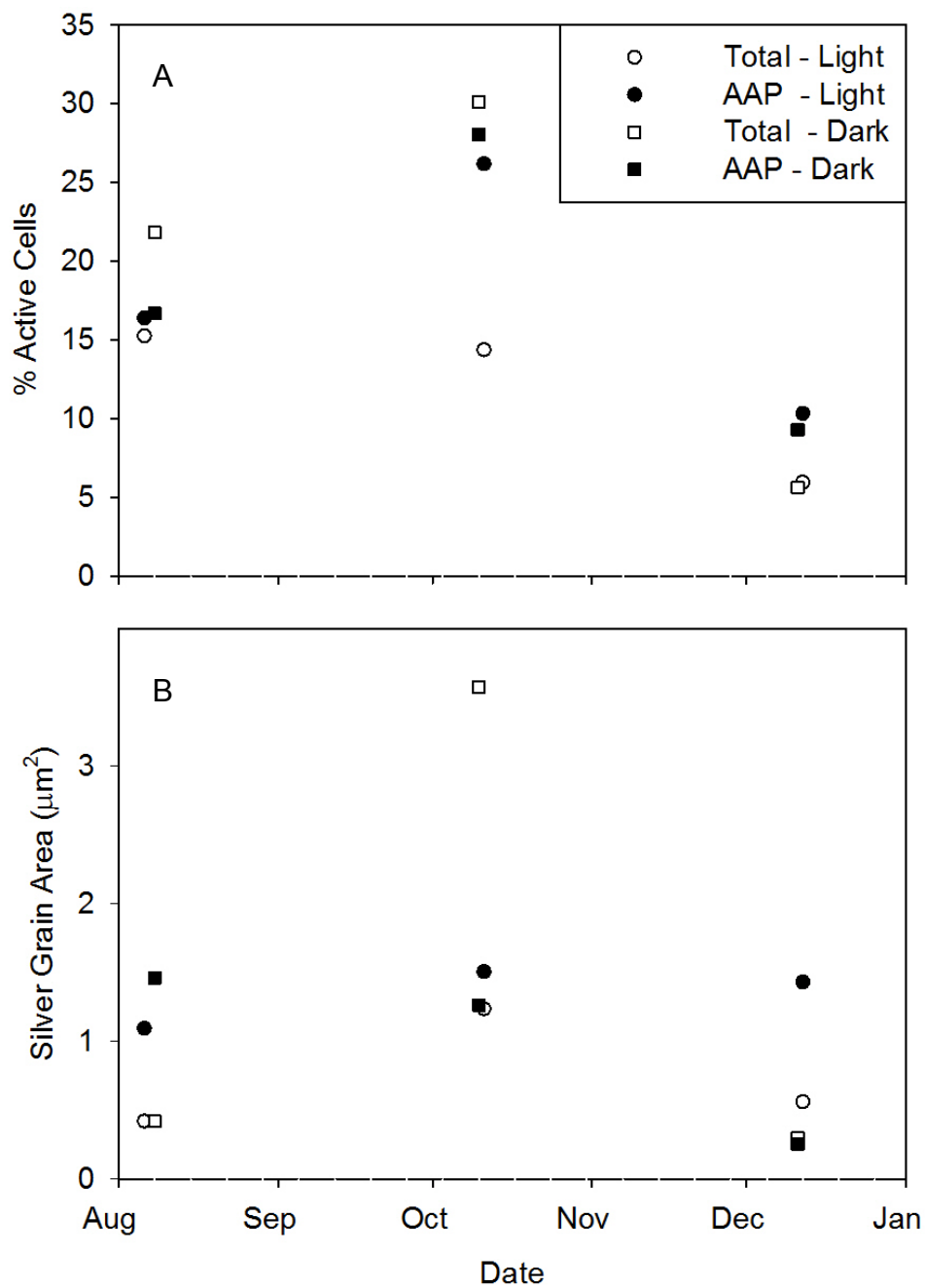


Figure 6. Activity of AAP bacteria and total bacterial community over time at mouth of bay (FB station). (A) The percent of cells active in the total bacterial community are open symbols and the AAP bacteria are black symbols. The circles represent samples that were incubated under ambient light conditions and the squares were incubated in the dark. (B) The average silver grain areas associated with AAP and total bacterial communities.



Chapter 4

DISCUSSION

We investigated the activity of AAP bacteria, based on the incorporation of leucine, during transects of the Delaware Bay in 2011. A new variation of microautoradiography was developed to identify the activity of individual AAP bacterial cells. Our samples were incubated under ambient light conditions instead of traditional dark conditions to determine the activity of AAP bacteria. We hypothesized that AAP bacteria would be more active than other bacteria throughout the Delaware estuary. We found that AAP bacteria are more active than other bacteria but the difference varied in the estuary.

On average AAP bacteria incorporated more leucine than other bacteria, consistent with our hypothesis that phototrophy gives AAP bacteria a metabolic advantage over heterotrophic bacteria. However, AAP bacteria were not always more active than other bacteria in all locations and times in the Delaware Bay. Our results are different from those found by Koblizek et al. (2007), which determined that AAP bacteria are always more active than the rest of the community. Koblizek et al. (2007) demonstrated that AAP bacteria were more than five-fold more active than other bacteria in open ocean waters. We did not find a difference near that anywhere in the Delaware estuary. At most AAP bacteria incorporated leucine about three-fold faster than other bacteria.

There are several possible explanations for the difference between the Koblizek et al. study and our work. We used different techniques to determine the activity of AAP bacteria. Our experiments have a much shorter time scale. Incubations in our study only required one hour as opposed to 18-72 hours needed to detect the activity of AAP bacteria through the change in bacteriochlorophyll a concentration (Koblizek et al. 2005, 2007). Also, the Koblizek et al. approach is based on the assumption that bacteriochlorophyll cannot be synthesized in light. However, some synthesis in the light has been discovered to occur at low levels (Spring et al. 2009). Leucine used in our assays may not track all active bacteria (del Giorgio and Gasol 2008). In addition, we examined an estuary with high nutrients that changed greatly, whereas Koblizek et al. examined ocean waters where nutrients are usually low.

Several hypotheses may explain the observation that the activity of AAP bacteria varied throughout the estuary, with the lowest activity in the river and the highest in high salinity waters. This pattern is the opposite from that predicted by the hypothesis that AAP bacteria favor particles (Lamy et al. 2011, Waidner et al. 2007). If particles provided an advantage, then AAP bacterial activity would be higher in the river because of the large particle load but our results do not support this hypothesis. Another hypothesis is that AAP bacterial activity varies because of grazing. AAP bacteria are potentially an easy target for grazers (Fenchel et al. 1986) because they are larger than

other bacteria (Sieracki et al. 2006). However, it is unclear if this top down control varies systematically in the estuary (Jurgens and Matz 2002). The concentration of dissolved organic carbon (DOC) is another parameter that changes constantly throughout the estuary system, which may affect AAP bacterial activity. However, DOC concentrations in the Delaware (Sharp and Pennock 1994) vary the opposite of AAP bacterial activity, suggesting that DOC does not control the activity of AAP bacteria.

The relative percent of active of AAP bacteria did have a negative correlation with light attenuation; activity and light are both low in low salinity waters and high in high salinity waters. This potential factor controlling activity of AAP bacteria is supported by the observation of light effects in pure cultures (Cooney et al. 2006, Fuchs et al. 2007, Spring et al. 2009, Holert et al. 2001, Tomasch et al. 2011). However, we did not see a significant effect of light on AAP bacterial activity in short term incubations. These experiments included testing the effect of light on bacterial communities collected before dawn after being in natural darkness for 9 hours. The activity of AAP bacteria was also not affected by light when communities were collected during the day and incubated with or without light. It may be hard to determine an effect of light on bacteria in a natural setting in a short incubation because of the low energy apparently gained by AAP bacteria via phototrophy (Kirchman and Hanson 2013). The lack of a light effect in our experiments is consistent with previous

work in Delaware and elsewhere in which light effects were also hard to demonstrate (Straza and Kirchman 2011, Alonso-Saez et al. 2006).

We demonstrated that AAP bacteria incorporate more leucine than other bacteria, although the difference varied in the estuary. This suggests that AAP bacteria have a slight metabolic advantage. Because AAP bacteria grow faster and are bigger than other bacterial cells, they may consume more DOM. They may also use different types of DOM. More work is needed to determine the connection between growth and DOM uptake by AAP bacteria.

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Appendix A
SUPPLEMENTAL DATA

Table 4.

<u>Date</u>	<u>Station</u>	<u>Latitude</u> (°N)	<u>Longitude</u> (°W)	<u>Estuary</u> <u>Section</u>
8/5/11	12	39 51.4	75 13.91	River
8/5/11	16	39 37.32	75 34.95	River
8/5/11	18	39 22.58	75 28.09	River
8/5/11	20	39 21.83	75 26.96	Middle
8/6/11	FB	38 46.85	74 55.17	Mouth
8/6/11	Bay	38 50.86	75 06.52	Mouth
8/6/11	26	38 55.77	75 04.77	Mouth
8/6/11	24	39 04.82	75 10.56	Middle
8/7/11	14	39 48.01	75 24.99	River
8/7/11	S5	39 34.47	75 33.15	Middle
8/8/11	FB	38 47.19	74 55.54	Mouth
10/10/11	FB	38 47.19	74 55.54	Mouth
10/10/11	Bay	38 50.86	75 06.52	Mouth
10/11/11	FB	38 47.19	74 55.54	Mouth
10/11/11	Bay	38 50.86	75 06.52	Mouth
11/7/11	26	38 54.98	75 06.16	Mouth
11/7/11	24	39 05.28	75 10.85	Mouth
11/7/11	20	39 22.03	75 26.9	Middle
11/8/11	14	39 47.97	75 24.93	River
11/8/11	S6	39 23.83	75 29.72	River
11/8/11	S16	38 50.98	75 06.48	Middle
11/8/11	Bay	38 50.86	75 06.52	Mouth
11/9/11	FB	38 46.75	74 54.84	Mouth
11/9/11	S24	39 00.79	75 08.75	Middle
11/9/11	S15	39 14.04	75 18.32	Middle
11/9/11	S4	39 24.16	75 35.03	River
12/12/11	FB	38 47.19	74 55.54	Mouth
12/12/11	Bay	38 50.86	75 06.52	Mouth

Detailed Protocol for AAP-Microautoradiography

A. Incubation with Radiolabeled Compound

B. Filtration

C. AAP Bacterial Enumeration

D. Autoradiography

E. Development

F. Microautoradiography Analysis

A. Incubation with Radiolabeled Compound

1. Fill three clear Exetainer vials with 5 ml seawater (this will yield three replicate filters) and a killed control of the same volume of water that is killed with 2% PFA (final concentration). (This volume of seawater is for the Delaware estuary.) Wait 5 minutes after poisoning the killed control to proceed to ensure that all cells in the sample are dead and fixed.
2. Add radiolabeled compound to all vials.
3. Incubate with ambient light and in situ temperature. (Ideally the incubation is done in a flow through incubation table) Samples were incubated for 1 hour in estuary waters with ^3H leucine a low molecular weight compound. Incubations with other ^3H compounds can last up to 12 hours and ^{14}C compounds over night.
4. At the end of the incubation transfer the sample into a 20 mL plastic scintillation vial for fixation. Add PFA to a 2% final concentration and fix for 1 hour at 4 °C. Never use the Exetainer vials for fixation so that they can be reused for live samples. Any vials exposed to PFA should not be used for live incubations.

B. Filtration

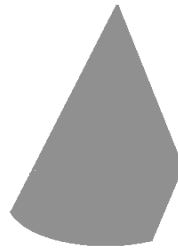
1. Set up a 25mm filter manifold with a 0.45 μm nitrocellulose filter as a backing filter to ensure even filter coverage and aid in decreasing cell clumping. Place a drop of deionized water on the backing filter and then place a 0.22 μm black polycarbonate filter on top of the water. Add suction to the tower to flatten the clack filter.
2. Place the sample water to be filtered (a volume that should be determined by direct counts – cells should be spread out enough to avoid false positive cells)
3. Filter the sample onto the filter. DO NOT RINSE WITH DEIONIZED WATER.

4. Remove the filter from the manifold while the pump is still running to ensure the filter is dry and to minimize the filter folding onto itself.
5. Place filter in a 1.5 mL CryoVial, so that the filter is hugging the inside of the vial, with cell side in (the non-cell side touching the vial) without wrinkles.
6. Store at -80 °C.

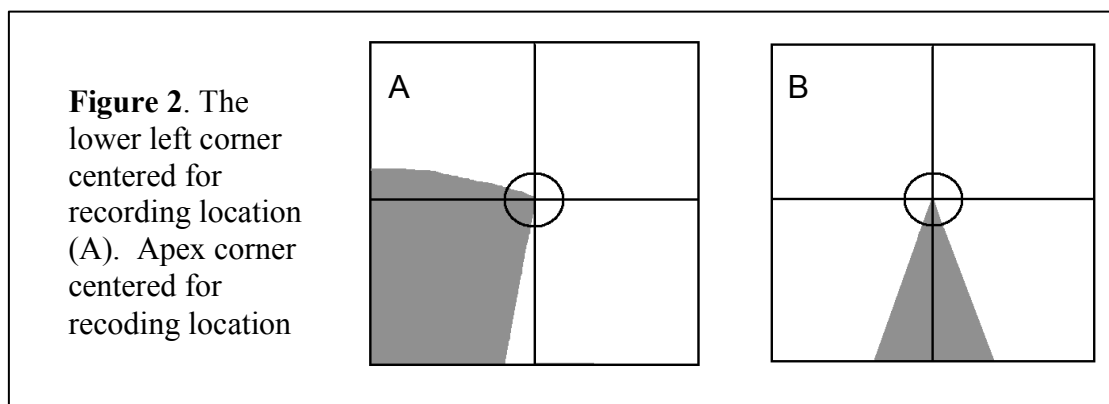
C. AAP Bacterial Enumeration

1. Cut $\sim 1/10^{\text{th}}$ of a piece of filter and snip the bottom right hand corner of the filter to indicate which side of the filter is cell side up. (Figure 1.)

Figure 1.
Diagram of filter
wedge with right
corner cut to
indicate cell side
up



2. Stain with DAPI (1 $\mu\text{g/mL}$) in 1X PBS for 5 minutes.
3. Remove the filter from the stain and place on a piece of Whatman paper to remove excess liquid but do not allow filter to dry out.
4. Mount filter on a slide with oil, making sure to keep the cell side of the filter oriented facing up.
5. Focus of the cells manually before proceeding to the cell counting software.
6. Select the AAP-MAR (AAP) program and enter all pertinent data for the samples.
7. Turn on transmitted light and switch objective to 10x. While in 10x locate the lower left corner of the filter (LLC), center it in your field of view and return to 100x objective. Turn on the crosshairs and center the corner of the filter and record the location (Figure 2A).
8. Repeat this step for the apex point of the filter (Figure 2B).
9. Move objective away from corner, towards the center of the filter, before beginning to ensure that the cells are not cover in the final analysis.



10. Set the exposure times for the sample and proceed to image capture phase.
11. Capture 20 fields of view of images. The coordinates are recorded in .txt file by the microscope program. Slides can be kept at -20 °C until next step.

D. Autoradiography

****All work with photographic emulsion is done in a dark room with a red safe light.****

1. Dilute Kodak NTB emulsion from stock vial 2:1 water to emulsion in a black film canister at least 24 hours before first use to reduce chance of bubbles. On day of use set emulsion canister in a 43 °C water bath and let heat for at least 30 minutes.
2. Dismantle the slides and remove the oil from the filter pieces using 100% EtOH. Dip each filter piece individually in EtOH and dry it using Whatman paper. Make sure that all of the oil and EtOH are off of the filter piece.
3. Place the dry filter pieces each in a well of a 12-well culture plate and take to dark room.
4. Set an aluminum block on ice.
5. Place samples near safe light on lab bench.
6. Dip a pre-labeled glass slide in emulsion, wipe back of the slide off on a paper towel.
7. Work quickly under the side light, place a filter piece cell side down on the emission. It is important to keep the filter from wrinkling when it is placed in the emulsion. Keep the filters pointing up
8. Place to slide on the aluminum block.
9. Leave the slide on the block for 15 min to allow the emulsion to gel.
10. Transfer slide to dark boxes

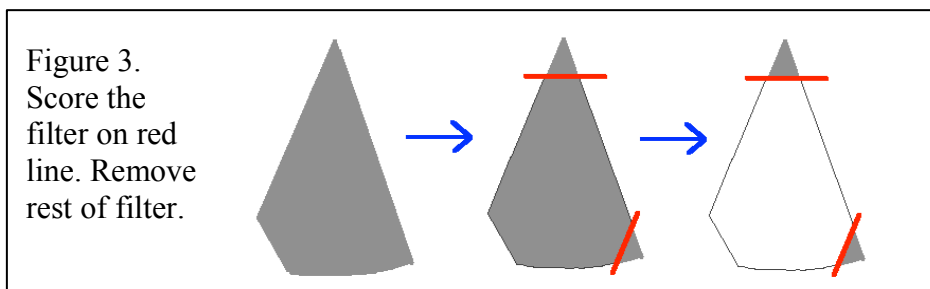
11. Place dark boxes in refrigerator for exposure time (24 hours for leucine).

E. Development

1. Prepare Kodak developer and fixer.
2. Place ~500 mL of developer, water (stop), fixer and more water in 4 containers in a row.
3. Develop slide for 2 min, stop for 30 sec in water, fix for 6 min and wash for 6 min in water.
4. Dry overnight in a dark vacuum desiccator for no more than 24 hours.

F. Microautoradiography Analysis

1. Remove slide from vacuum desiccator and trace the outline of the filter piece on the back of the slide.
2. Using an Xacto knife, score the filter as shown in Figure 3 (red lines).



3. Peel the large section of the filter off of the slide, leaving the corners in the emulsion.
4. Mount a cover slip using an antifade mount containing 0.5 ng/ μ L DAPI
5. Focus the microscope on the DAPI stained cells.
6. Select the AAPMAR (MAR) program and enter all pertinent data for the samples. Make sure that you set to program to play a .txt file and select the file made during the AAP enumeration.
7. Switch the objective to 10x and save the locations of the corner and the apex same as in AAP enumeration.
8. Set exposure times for DAPI image and silver grains.
9. After exposure times are set the program will announce that the stage is about to move to the first field. Click ok and proceed to image acquisition.
10. Focus on the cells and click acquire. The program will move the stage to the next field of view after it has completed image capture.
11. After all of the images have been taken for the 20 fields of view the program will analyze the photos. The program flips and rotates the

DAPI images from the AAP enumeration to align them with the DAPI image taken during the silver grain acquisition.

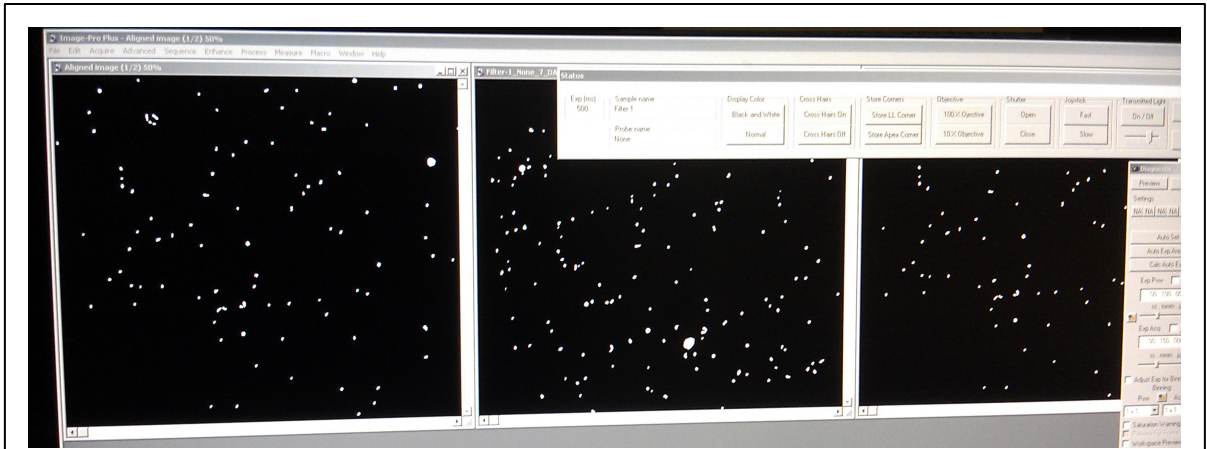


Figure 4. DAPI image alignments. Left image is composite image. DAPI images from 2 analyses are to the right.

12. The user is presented with three images. The left image is a composite of the two DAPI images, which are presented next to it. The composite image is rapidly flashing between the two DAPI images. Cells that are solid are present in both DAPI images and ones that are flashing are only present in one. Select YES for the alignment if the majority of the cells are solid, indicating a good alignment and NO if the alignment was unsuccessful.
13. The program will analyze the images that were successfully aligned.
14. Set the program to run Volumes overnight. This will calculate the size of the silver grains detected.