

**Chemotaxis Assays for Marine and Freshwater Amoeba**

**Jessica Rivera Pérez**  
**Department of Biology**  
**University of Puerto Rico Rio Piedras Campus**  
**San Juan, Puerto Rico**

Marine Biological Laboratory  
Microbial Diversity Summer Course  
2015

## **Abstract**

Chemotaxis, i.e. the ability to detect and respond to gradients in a heterogeneous environment, is a powerful fitness advantage for microbes. Although this process has been extensively studied in model bacteria such as *Escherichia coli*, and pathogenic amoeba such as *Entamoeba histolytica*, it has only been assessed to a much lesser extent in a handful of environmental amoeba, including *Dictyostelium discoideum* and *Acanthamoeba*. In fact, little is known about this process in environmental eukaryotes. Recent studies have alluded to the capabilities of amoeba tracking their microbial prey, however the precise mechanisms governing this process remain unknown. This study attempts to evaluate chemotaxis capabilities in several marine and freshwater free-living amoebas.

## **Introduction**

Extensive studies on pathogenic amoebas such as *Entamoeba histolytica* suggest these protozoa may be capable of chemotactic behavior towards their target cells<sup>1</sup>. Ironically however, this process has barely been studied in free-living environmental amoeba, which would undoubtedly also benefit from this fitness advantage while living in such heterogeneous environments. One study on the soil-amoeba *Dictyostelium discoideum* suggested a strong chemotactic response towards folate, however the exact mechanisms by which this occurs remains unknown<sup>2</sup>. In fact, many questions remain in regards to this interaction. For example, can amoeba “sense” gradients alluding to the position of their prey in the environment? Are these gradients mainly made up of abiotic factors that also attract bacteria and other microbes, like for example high nutrient concentrations? Do specific compounds secreted by their prey act as chemo-attractants? Is there specificity or preferences between amoebas or are their compounds that act universally as chemoattractants for these microbes? Do amoeba respond to prey motility? In order to begin answering these questions in environmental amoeba, I will expose individual amoeba cultures to pre-determined concentrations of folic acid, amino acids and other compounds to see if their feeding behavior varies in response to chemoattractant gradients. The hypothesis of this study states those environmental free-living amoebas are chemotactic towards specific compounds presumably associated to their prey.

## **Methods**

**Amoebae.** The following amoeba strains were used in this study: *Corallomyxa sp.*,  $\Delta$ TR2, *Neovahlkampfia damariscottae*, unidentified marine amoeba (lovingly nicknamed Sloth), and an unidentified freshwater amoeba (namely, FLA).

**Culture media.** Two modifications of Sea Water Complete media were used to limit nutrient concentrations in cultures. The first, named MSCW1, contained 1000mL Seawater base 1X, 0.5g Bacto Peptone (BD Biosciences), 0.2g Yeast Extract (BD Biosciences), 5ml MOPS (5mM) and 3ml glycerol. The second modified media, MSCW2, lacked Bacto-Peptone and Yeast Extract. Both media were sterilized by autoclaving at 121°C for 20 min and stored at room temperature. Solid media was prepared daily by adding agarose (Difco) to reduce the presence of salts and metals coming from the agaropeptin present in agar (e.g. sulfate and pyruvate groups).

**Preparation of compounds.** Chemoattractant stock solutions were initially prepared using autoclaved Milli-Q water, filter sterilized (with the exception of folic acid) and stored at 4°C in 15ml falcon tubes until use. Stock solutions were diluted to experimental concentrations using SWCM2 prior to use (Table 1). Experimental concentrations were determined based on previous chemotaxis studies<sup>2</sup>. Compounds used were: Casamino acid, Bacto Peptone, Yeast Extract, Sucrose/Saccharose (Sigma-Aldrich), Folic acid (Sigma-Aldrich), D-Biotin (Sigma-Aldrich), Riboflavin (Sigma-Aldrich),  $\beta$ -NADH (Sigma-Aldrich), Spent culture, Glycine, Tryptophan (Sigma-Aldrich), Proline (Sigma-Aldrich) and Wolfe's vitamin mix. Wolfe's vitamin mix was prepared as according to manufacturer's instructions (ATCC).

**Table 1.** List of chemoattractants evaluated in this study.

<b>Compound</b>	<b>Stock Concentration</b>	<b>Experimental Concentration</b>
Casamino acid	10%	>1%
Bacto Peptone	10%	>1%
Yeast Extract	10%	>1%
Sucrose/Saccharose	10mM	0.1-1mM

Folic acid <sup>1</sup>	5mM	0.1mM
D-Biotin	10mM	0.4mM
Riboflavin	10mM	5uM
β-NADH	10mM	0.1-1mM
Spent culture <sup>2</sup>	100%	>1%
Glycine	100mM	20mM
Tryptophan	5mM	0.01mM
Proline	10mM	0.1mM
Wolfe's vitamin mix	1000x	1x

<sup>1</sup>Folic acid was sterilized by autoclaving and stored in the dark at 4°C.

<sup>2</sup>Indicates the supernatant of *Maribacter spp* cultures grown for >24h then centrifuged at 20000 rpm for 1min.

**Under-agarose well assay.** A 3-D printer was used to create customized molds for the well assay. Molds created 3 equally spaced wells, each 2mm wide, 1.2mm deep and 12mm of length. Solid SWCM1 and SWCM2 media (0.5% agar) was poured to petri dishes containing the molds (100mm x 15mm) and left in a sterile biosafety cabinet until solidified. Molds were then carefully extracted from the media, leaving wells as intact as possible (see Fig1A). Two hundred microliters of fresh free-living amoeba culture were placed in the middle well and allowed to grow overnight. Chemoattractants (same volume) were placed in the right exterior well. SWCM2 was used as a control and was placed in the left well. Assays were sealed with parafilm to prevent dehydration and incubated at room temperature. Amoebae were observed under the microscope after 1h, 4h, 12h and 24h for cell migration.

**Agarose block assay.** Agarose was added to SWCM2 (final concentrations 0.5% and 0.75%) and supplemented with chemoattractants for this assay (see Table 1 for final concentrations). Blocks were aseptically cut using razors sterilized with 70% ethanol (block dimensions: 8mm X 5mm). Amoeba cultures were grown overnight in petri dishes with SWCM1 and posteriorly checked for cysts before use. A chemoattractant-infused agar block was then placed on one side of the petri dish and left incubating at room temperature (see Fig1B). Assay was checked under the microscope after 1h, 2h, and 24h of incubation.

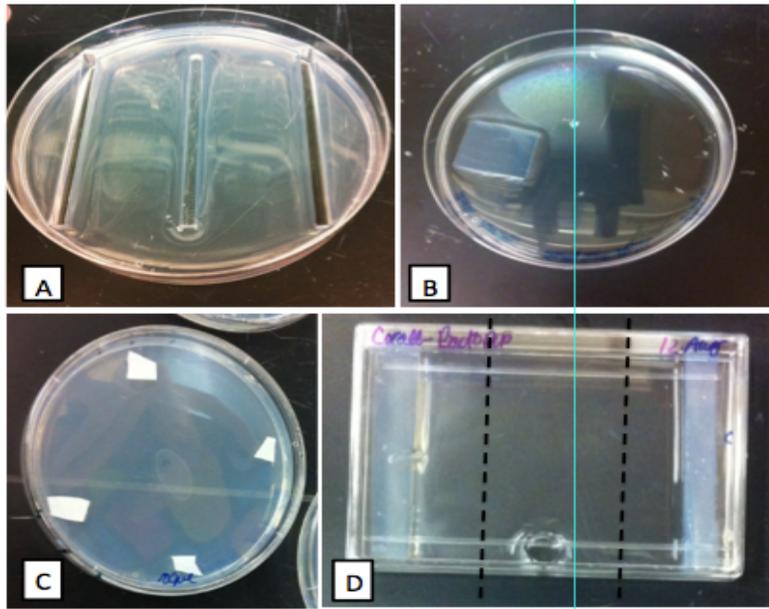
**Chemoattractant-infused filter assay.** Polycarbonate membrane filters (0.2 $\mu$ m porosity) were cut into 4 pieces using a sterilized razor and infused with a chemoattractant for 3h in a 2ml Eppendorf tube. Three filters with chemoattractants and one control (with SWCM2) were then placed onto solidified SWCM1 media (0.75% and 1% final concentration of agarose). Filters were left for 30 minutes at room temperature to allow for a gradient to occur (see Fig1C). Afterwards, 50 $\mu$ l, 100 $\mu$ l or 200 $\mu$ l of fresh amoeba culture were inoculated at the center of the petri dish and left overnight for cell migration.

**Agarose plug assay.** Rectangular polystyrene petri dishes (50mm X 75mm) were used for this assay. Specifically, chemoattractant-infused SWCM2 was solidified using agarose (final concentration 0.5%) and cut into 12mm wide strips using sterile razors. Solidified SWCM2 was also used as a control. Eight milliliters of SWCM1 and 1ml of non-cyst amoeba cultures were inoculated into the media and left overnight for growth at room temperature. Cultures were checked under the microscope for cysts before conducting the experiment. Chemoattractant-infused and control agarose strips were placed at opposite ends of the petri dish and left overnight for cell migration (see Fig1D).

**Capillary Assays.** Glass capillary tubes were used to assess amoeba chemotaxis in liquid media. Glass bottles and 1L plastic cell culture flasks with 10 equidistant holes were inoculated with non-cyst amoeba cultures in 10ml of SWCM1 and left overnight for growth and bacteria depletion. Capillary tubes were filled with chemoattractant or control media by capillary action and sealed with plasticine (see Fig2A and 2B). Capillary tubes were held in place using plasticine molded around the tube and also sealing the holes in the flask.

**Agar pad slide assay.** A microscope slide was inoculated with 10 $\mu$ l of molten agar (final concentration 2%) infused with chemoattractant. A coverslip was placed on top of the agar until it solidified (see Fig3), then 10 $\mu$ l of fresh amoeba culture was placed beside the agar pad and sealed with a coverslip and V-LAP. Slide was monitored by microscopy after 1h and 24h of exposure.

## Results and Discussion



**Figure 1. Experimental setups for chemotaxis assays using agarose.** (A) Under-agarose well assay, (B) Agarose block assay, (C) Chemoattractant-infused filter assay and (D) Agarose plug assay.

**Agarose-based chemotaxis.** An under-agarose well assay was used to force amoeba to crawl under the solid media until they reached the chemoattractant (Fig1A). Although this assay has been previously used to assess chemotaxis in *Dictyostelium discoideum*<sup>3</sup>, it did not work with the strains evaluated in this study because they were not visible when observed under the microscope. The reasons for this remain unknown, however I suspected it could have something to do with the density of the media and used different concentrations of agarose (0.5%, 0.75%, 1%, 1.5%) as well as different volumes of media in the petri dishes to no avail. One possible explanation could be that the amoeba were crawling up the walls of the wells and therefore could not be observed.

Afterwards, I conducted an agarose block assay (Fig1B) to test for chemotaxis in these microbes. The first round of this experiment suggested that Sloth amoebas in particular were chemotactic in response to Casamino acids and Bacto Peptone, because they were all congregated alongside the agar block despite having *Maribacter sp* present in the inoculum. However, subsequent repetitions of this experiment were not able to observe the same tendencies again. One possible

reason for the lack of reproducibility was the movement of the petri dish, and thus the chemoattractant gradient, while transporting and observing these assays under the microscope. This experiment was posteriorly repeated with an agarose base, but failed miserably. Then I evaluated the chemotactic capabilities of these amoebas via a filter assay on solid SWCM1 (Fig1C). Although no conclusive results were obtained, it seemed the *Maribacter spp.* present in the inoculum were chemotaxing towards the Casamino acid solution. Finally, an agar-plug assay was conducted as shown in Fig1D. Results for the first run of this experiment are shown in Table 2. This assay suggested the amoeba could respond to different chemoattractants. For example, Sloth seemed to chemotax to Bacto Peptone and Folic acid, whereas *Corallomyxa sp.* appeared responsive towards Casamino acids. However, repetitions of the experiment resulted inconclusive, as these results were never reproduced effectively. Upon closer look at the amoeba cultures I noticed that these had a large concentration of *Maribacter sp.* This could have affected the experiment negatively as the amoebas were probably receiving multiple chemotactic signals at once. I tried to wash off the *Maribacter sp.* in subsequent experimental repetitions but was not successful.

**Table 2. Observed results in agar plug assays.**

Amoeba	Compound	A Count <sup>1</sup>	C Count <sup>2</sup>
Sloth	Bacto Peptone	160	63
	Casamino acid	48	13
	Suc/Sacch	40	13
	Folic acid	122	3
<i>Corallomyxa sp.</i>	Bacto Peptone	43	60
	Casamino acid	106	44
	Suc/Sacch	37	72
	Folic acid	109	136
FLA	Bacto Peptone	200	124
	Casamino acid	209	371
	Suc/Sacch	182	130
	Folic Acid	109	136
<i>N. damariscottae</i>	BactoPep	32	30
	Casamino	76	82
	Suc/Sacch	0	58
	Folic Acid	156	165

List of cell counts surrounding the agarose plug (up to 12mm radius from plug) after 24h of incubation. <sup>1</sup>Indicates area surrounding chemoattractant. <sup>2</sup>Indicates area surrounding control.

**Capillary assays.** Glass capillary tubes were used to assess amoeba chemotaxis in liquid media. However, after initial incubation in glass bottles (Fig2A) the cultures had all gone into cyst form, including strains that had previously not been observed in cysts, such as Sloth (Fig3). Thinking that the reason for this was the coating of the glass bottles, the experiment was repeated using cell culture flasks, which contain a special coating for eukaryote cells (normally used for immune cells) (Fig2B). Despite this however, the cultures went into cyst form again. The reasons for this remain unknown, but microscope observations of the culture indicated that the amoeba did not lack bacteria as food source. One possibility is that small quantities of the plasticine covering the holes seeped into the culture media and triggered amoeba cysting, however this was not verified in depth.

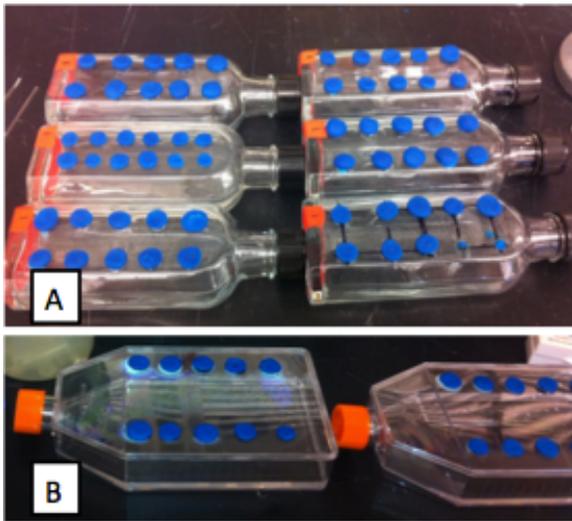


Figure 2. Capillary tube assays used with glass bottles (A) and plastic cell culture flasks (B).

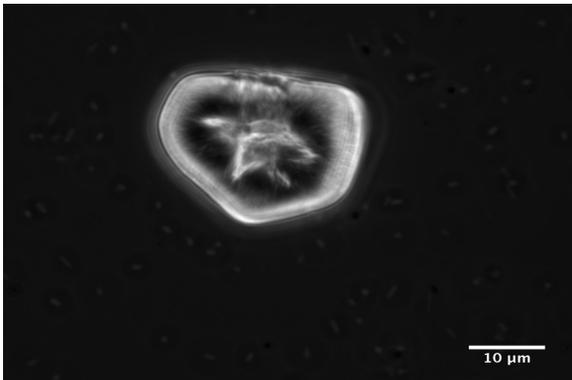


Figure 3. Cyst form of Sloth amoeba culture.

Finally, agar pads infused with chemoattractants were placed onto microscope slides to assess migration (similar to Fig4). Unfortunately, absolutely no cell migration was observed during this assay. It is possible that the amoeba were inactivated by the V-LAP used to seal the coverslips or the lack of gas exchange the seal eventually might have caused.

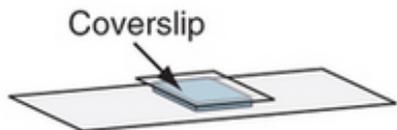


Figure 4. Agar pad microscope slide. (Taken from Skinner et al, 2013, Nature Protocols)<sup>4</sup>

## **Conclusions**

Although in some of the assays conducted in this study the Sloth amoebas seemed to be chemotactic towards amino acids and Bacto Peptone, results were not reproducible and were therefore unsatisfying. In conclusion, these assays are useful for testing chemotaxis in amoeba but require much more troubleshooting than what can be done effectively in two weeks time.

## **Acknowledgments**

Thank you to all the TA's, Jared and Dianne for making this wonderful experience possible. My gratitude especially goes to Scott, Teamoeba, James and Emily for their help and extraordinary attitude towards the long hours in the lab and Swope food. Also, many thanks to the William Townsend Porter Scholarship and the National Science Foundation for funding my participation in the Microbial Diversity course.

## **References**

1. Blazquez, S. *et al.* Chemotaxis of *Entamoeba histolytica* towards the pro-inflammatory cytokine TNF is based on PI3K signalling, cytoskeleton reorganization and the Galactose/N-acetylgalactosamine lectin activity. *Cell. Microbiol.* **10**, 1676–1686 (2008).
2. Maeda, Y., Mayanagi, T. & Amagai, A. Folic acid is a potent chemoattractant of free-living amoebae in a new and amazing species of protist, *Vahlkampfia* sp. *Zoolog. Sci.* **26**, 179–186 (2009).
3. Laevsky, G. & Knecht, D. a. Under-agarose folate chemotaxis of *Dictyostelium discoideum* amoebae in permissive and mechanically inhibited conditions. *Biotechniques* **31**, 1140–1149 (2001).
4. Skinner, S. O., Sepúlveda, L. A., Xu, H. & Golding, I. Measuring mRNA copy number in individual *Escherichia coli* cells using single-molecule fluorescent in situ hybridization. *Nat. Protoc.* **8**, 1100–1113 (2013).