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Filtration-induced release of dissolved free amino acids: application to cultures of marine protozoa

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ABSTRACT: We examined effects of filtration on measuring dissolved free amino acids (DFAA) in cultures of bacterivorous marine flagellates *Paraphysomonas imperforata* and ciliates *Uronema* sp. Based on comparisons of several filtration techniques, we found that syringe filtration using Acrodiscs (Gelman Sciences) did not cause an artificial increase of DFAA for cultures of both species. Filtration caused release of DFAA from *Uronema* sp. when cells collected on the filter surface of the Acrodiscs were exposed to air. Filtration with GF/F glass fiber filters (Whatman) also caused release of DFAA from *Uronema* sp., leading to overestimation of DFAA concentrations by a factor of 1.7. These results indicate that Acrodiscs with syringe filtration can be used to collect DFAA samples from cultures of protozoa and probably of other microorganisms and from natural aquatic environments.

INTRODUCTION

Filtration is a common technique in chemical measurements of dissolved organic matter (DOM) in seawater. Shear force and hypotonic osmotic shock (Goldman & Dennett 1985) associated with filtration rupture fragile cells, leading to an artificial increase in DOM concentrations. Fuhrman & Bell (1985) observed an increase of dissolved free amino acids (DFAA) by filtration with some types of filters, indicating possible breakage of planktonic organisms during filtration. Previous studies have also indicated that filtration causes artificial release of dissolved organic carbon from fragile phytoplankton (e.g. Goldman & Dennett 1985).

Recent research has suggested that heterotrophic protozoa release DOM (Taylor & Lean 1981, Taylor et al. 1985), including dissolved free amino acids (DFAA) (Andersson et al. 1985, Flynn & Fielder 1989, Nagata & Kirchman unpubl.). This DOM is probably a significant bacterial substrate in marine systems (Andersson et al. 1985, Taylor et al. 1985, Hagström et al. 1988, Nagata & Kirchman unpubl.). The possibility that filtration causes release of DOM from protozoa has not yet been examined critically. Because protozoa are fragile (Taylor & Lean 1981), filtration might seriously affect measurements of DOM release.

The objective of this study was to assess the effects of filtration on the measurement of DFAA in marine protozoan cultures. We examined syringe filtration by using Acrodiscs (Gelman Sciences). We can process samples quickly with less risk of contamination by the use of prepacked filters (Flynn 1988). Fuhrman & Bell (1985) suspected that syringe filtration could generate a large pressure differential and easily damage plankton, but did not test this directly. Our results suggest that Acrodisc-filtration does not cause artificial increase of DFAA in protozoan cultures.

MATERIALS AND METHODS

Cultures. We used a heterotrophic flagellate, *Paraphysomonas imperforata*, and a ciliate, *Uronema* sp. Both strains were originally isolated from the Delaware Bay, USA, and identified by David J. Patterson, University of Bristol, UK. Experimental batches were prepared by adding a stock culture of protozoa to a bacterial suspension. The bacterial suspension was a mixed assemblage of bacteria from the Delaware Bay. The bacteria were grown in a peptone-yeast extract medium, and harvested and washed by centrifugation. Batches were incubated at room temperature (23 °C) in the dark. Bacteria were enumerated by the acridine orange direct count method (Hobbie et al. 1977). Ciliates and flagellates were counted using a hemacytometer. Protozoan cultures in the logarithmic, transition or stationary phase were used for experiments (Table 1).

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Table 1. *Uronema* sp. and *Paraphysomonas imperforata*. Abundances of protozoa and bacteria in each experiment

Expt	Species	Incubation period (h)	Protozoan growth phase[a]	Protozoan abundance (cells ml^{-1})	Bacterial abundance[b] (cells ml^{-1})
1	*Uronema* sp.	50	T	2.8×10^4	1.2×10^7
2	*Uronema* sp.	52	T	2.9×10^4	1.2×10^7
3	*Uronema* sp.	93	LS	5.2×10^3	5.3×10^7
4	*P. imperforata*	53	Log	3.2×10^5	6.5×10^7
5	*P. imperforata*	67	ES	5.0×10^5	5.7×10^6
6	*P. imperforata*	98	LS	4.8×10^5	9.5×10^6

[a] T: transition; LS: late stationary; ES: early stationary
[b] Initial bacterial abundance was 1×10^8 cells ml^{-1}

Filtration. We used several filtration techniques for preparing samples for measuring DFAA (see below). Each experiment was completed within 15 min. The volume of sample water filtered for each filtration was 1.5 to 4 ml. All the experiments included Acrodisc filtration (diameter 25 mm; pore size 0.2 µm, product # 4192; Gelman Sciences). We used plastic syringes with rubber plungers (3 or 5 ml) for the filtration. Syringes were rinsed with methanol, followed by thorough rinsing with HPLC grade water (Fisher Scientific). Filtration speed was about 0.05 ml s^{-1} unless described otherwise. All filtrate was collected in precombusted glass vials. To check contamination during filtration, we filtered 3 to 4 ml of HPLC grade water through an Acrodisc, and measured DFAA in the filtrate. We found that DFAA contamination was less than 2 nM. We also checked adsorption of DFAA to Acrodiscs. We filtered a standard solution of DFAA (Pierce, diluted to 20 nM with HPLC grade water) through an Acrodisc, and measured DFAA in the filtrate. We did not find significant decrease of DFAA concentration after filtration. In Expts 1, 3 and 5, samples were filtered through glass fiber filters (GF/F, diam. 25 mm, Whatman) using a glass filtration manifold and a vacuum pump. Cells were exposed to air by GF/F filtration. The GF/F filtrate was filtered through an Acrodisc in order to eliminate bacteria before DFAA analysis.

In Expts 1, 3, 4, and 6, protozoan cells were eliminated by reverse-flow filtration (Dodson & Thomas 1964). A filtration apparatus was made from a plastic scintillation vial (7 ml). A Nuclepore filter (pore size of 0.8 µm for flagellate, 3 µm for ciliate) was fixed to the vial using a screw cap with a hole. The bottom of the vial was cut out to make an opening for sampling. We dipped the filtration apparatus into a protozoan culture in a beaker, keeping the opening above the water surface. The filter surface inside the vial was moistened with 200 µl of seawater filtered through Acrodiscs to help water to start passing through the Nuclepore filter. Bacteria but not protozoa were found in the filtrate. To eliminate the bacteria, the filtrate was further filtered through an Acrodisc.

DFAA analysis. DFAA concentration was measured with an HPLC using the precolumn derivatization method with *o*-phtaldialdehyde (Mopper & Lindroth 1982) after the modification of Fuhrman & Bell (1985). Alpha amino butyric acid was the internal standard. Alanine and tyrosine, and glycine and threonine coeluted, and we could not determine leucine, isoleucine, ornithine and lysine concentrations because a large peak of ammonium masked these peaks. Care was taken to avoid DFAA contamination during preparation of samples. We combusted all glassware and glass fiber filters (500 °C, > 1 h) before use. Plastic ware was rinsed with HPLC-grade methanol, followed by thorough rinsing with HPLC-grade water.

RESULTS AND DISCUSSION

Reverse-flow filtration

Although investigators have considered filtration-induced release of DOM by planktonic organisms (Fuhrman & Bell 1985, Goldman & Dennett 1985), lack of a proper control has hampered critical assessment of filtration effects. The investigator needs to demonstrate the lack of cell breakage in the control. We examined here the reverse-flow filtration technique (Dodson & Thomas 1964) to measure DFAA. We checked the breakage of protozoan cells during this filtration by comparing the protozoan abundance in the beaker (10 ml) after the filtration to that in the control beaker without filtration. If no breakage occurs, cell abundance in the beaker after the filtration is expected to be concentrated by the factor of C/(C−F), where C is the

total volume of sample water in the beaker and F is the volume of filtrate. Based on microscopic examinations, protozoan cells attach to the filter surface, leading to the underestimation of protozoan abundance after filtration (data not shown). We solved this problem by taking out the filtration apparatus after fixing the sample with glutaraldehyde. This fixation detached protozoa from the filter. We found that the filtration caused an increase of protozoan abundance as expected from the concentration factors (Table 2), indicating that this filtration technique was gentle enough to avoid rupturing protozoa. It is reasonable to use this filtration procedure as a control for evaluating other filtration techniques. This technique may also be applicable to evaluating the effect of filtration on measuring DOM release by fragile phytoplankton and estimating DFAA concentrations in natural waters.

Filtration effects on DFAA measurement

The DFAA concentrations measured after various types of filtration are summarized in Table 3. DFAA concentrations in the reverse-flow filtered samples from *Uronema* sp. cultures did not differ significantly from that in the Acrodisc-filtered samples (Expts 1 & 3). DFAA concentrations did not differ significantly between the samples filtered with Acrodiscs at different speeds (0.05 ml s^{-1} versus 1 to 2 ml s^{-1}) (Expt 2). We also examined the effect of exposing cells to air, because cells collected on filters are exposed to air in common filtration techniques using filtration manifolds. DFAA concentrations in samples with air exposure (247 ± 61 nM) were significantly higher than that of reverse-flow filtered samples (136 ± 15 nM) (Expt 1). We also examined filtration with GF/F glass fiber filters. In Expt 1, DFAA concentrations in samples after GF/F-filtration were higher than that of reverse-flow filtered samples by a factor of 1.4. Similar results were observed in Expt 3: DFAA concentrations in GF/F filtered samples (> 10 cm Hg) were higher than that in the control by a factor of 1.7. We found that GF/F-filtration at a pressure differential of 5 cm Hg did not increase DFAA concentrations over the control (Expt 3).

DFAA concentrations in the reverse-flow filtered samples from *Paraphysomonas imperforata* cultures did not differ significantly from that in Acrodisc-filtered samples (Table 3; Expts 4 & 6). In contrast to *Uronema* sp., we detected no increase of DFAA concentrations after Acrodisc-filtration with exposure of cells to air (Table 3; Expt 4). In Expt 5, DFAA concentrations in the GF/F filtrate did not differ significantly from that in the Acrodisc-filtered sample (Table 3).

The above results suggest that Acrodisc-filtration does not cause an artificial increase of DFAA in protozoan cultures, if cells are not exposed to air. We recognize that the sensitivity in detecting filtration effects by our assay depends on protozoan abundance and DFAA concentrations in the culture. However, we used relatively dense protozoan cultures (10^4 cells ml^{-1} for ciliate; 10^5 cells ml^{-1} for flagellate; Table 1) with relatively low DFAA concentrations (10 to 700 nM; Table 3), which maximizes the sensitivity of our tests.

The use of prepacked syringe-type filters for DFAA analysis of seawater samples has been increasing (Flynn 1988), mainly because it is relatively easy to avoid contamination with these filters. We know from our own experience that we can process many samples more quickly with less risk of contamination by using Acrodiscs than by using a filtration manifold with a vacuum pump. However, the possibility of breaking fragile cells by this filtration technique has not been examined until this study. Although Fuhrman & Bell (1985) suspected that syringe filtration could generate a large pressure differential and easily damage planktonic organisms, our results indicate that this is not the case. We suggest that filtration with Acrodiscs is appropriate for estimating DFAA in samples containing fragile microorganisms.

Table 2. *Uronema* sp. and *Paraphysomonas imperforata*. Changes in abundance of protozoan cells after concentrating the culture by reverse-flow filtration

Species	Concentration factor[a]	Protozoan abundance[b] ($\times 10^4$ cells ml^{-1})		
		Control	Concentrated	Expected[c]
Uronema sp.	2.0	2.7 ± 0.9	4.8 ± 1.0	5.4
P. imperforata	1.7	5.1 ± 1.0	8.0 ± 1.3	8.7

[a] Concentration factor = C/(C−F), where C is the total volume of culture in the beaker and F is the volume of filtrate
[b] Protozoan cells were counted using a hemacytometer. Errors are standard deviations (n = 12 for *Uronema* sp.; n = 8 for *P. imperforata*: n represents number of slide per filtration)
[c] (Expected abundance) = (abundance of cells in the culture) × (concentration factor). Observed abundance in the concentrated sample was not significantly different from the expected abundance for both species ($p > 0.05$, Student's t-test)

Table 3. Total DFAA concentrations in protozoan cultures measured by using different filtration techniques

Expt	Filter	Treatment[a]	DFAA[b] (nM)	n[c]	p[d]
1	Nuclepore	Reverse-flow	136 ± 15	3	
	Acrodisc		119 ± 23	3	ns
	Acrodisc	Air-exposure	247 ± 61	4	<0.05
	GF/F	10 cm Hg	190 ± 55	3	<0.05
2	Acrodisc		247 ± 22	3	
	Acrodisc	1–2 ml s^{-1}	220 ± 40	4	ns
3	Nuclepore	Reverse-flow	10 ± 2	3*	
	Acrodisc		12 ± 1	3*	ns
	GF/F	5 cm Hg	10 (9, 10)	2*	
	GF/F	10 cm Hg	17 (16, 17)	2*	
	GF/F	20 cm Hg	17 (17, 16)	2*	
	GF/F	60 cm Hg	17 (15, 18)	2*	
	GF/F	10–60 cm Hg	17 ± 0	3	<0.001
4	Nuclepore	Reverse-flow	696 ± 67	4	
	Acrodisc		659 ± 76	4	ns
	Acrodisc	Air-exposure	712 ± 18	4	ns
5	Acrodisc		90 ± 16	3*	
	GF/F	5 cm Hg	95 (95, 96)	2*	
	GF/F	10 cm Hg	90 ± 2	3*	
	GF/F	20 cm Hg	120 ± 15	3*	
	GF/F	55 cm Hg	89 ± 10	3*	
	GF/F	10–55 cm Hg	100 ± 18	3	ns
6	Nuclepore	Reverse-flow	23 ± 3	3	
	Acrodisc		22 ± 1	3	ns

[a] In the Acrodisc-filtration without description of treatment, care was taken to avoid air-exposure of cells collected on filter surfaces, and filtration speed was about 0.05 ml s^{-1}. In Expts 1 & 4, cells collected on the filter surface of Acrodiscs were exposed to air by a syringe (air-exposure). In Expt 2, samples were filtered at a speed of 1 to 2 ml s^{-1}. Vacuum pressure differentials (cm Hg) applied to the filtration are shown for GF/F filtration. See text for explanations

[b] DFAA concentration ± standard deviation; numbers in parentheses are duplicate values

[c] Number of replicates for filtration. Numbers with asterisk indicate replicate measurements for a single filtration of each treatment

[d] DFAA concentration for each treatment was compared to that of reverse-flow filtered sample (Expts 1, 3, 4 & 6) or Acrodisc-filtered sample (Expts 2 & 5). For Expts 3 & 5, the average DFAA concentration in the GF/F filtrate with pressure differentials greater than 10 cm Hg was compared to the concentration in the reverse-flow filtered or Acrodisc-filtered sample, because replicate filtration for each treatment was not carried out for these experiments. Significance of the difference was tested by Student's t-test. ns: not significant

The internal soluble pool of *Uronema* sp. was apparently released when cells were exposed to air (Table 3). The release may be attributable to cell breakage caused by hypotonic osmotic shock of cell surfaces (Goldman & Dennett 1985). We did not detect release of DFAA from *Paraphysomonas imperforata*. The difference between *Uronema* sp. and *P. imperforata* cannot be explained simply from a difference in biomass; total biovolume of the *P. imperforata* culture (6×10^6 to 1×10^7 μm^3 ml^{-1}) was similar to that of *Uronema* sp. (2×10^6 to 2×10^7 μm^3 ml^{-1}) in our experiments. *P. imperforata* are probably structurally more rigid and less susceptible to breakage during filtration than *Uronema* sp.

The composition of DFAA in the filtrate of protozoan cultures differed depending on the species and growth stages. Generally, molar percentages of glycine/threonine, alanine/tyrosine and glutamate were high in log, transition and early stationary phases, and glutamate was most abundant in late stationary phase. In Expt 1, the DFAA composition in reverse-filtered samples was similar to that of artificially increased DFAA through the filtration with air-exposure (Fig. 1). The same trend was observed in Expt 3; glutamate was most abundant for both reverse-flow filtered samples and GF/F-filtered samples (pressure differentials 10 to 60 cm Hg). The similarity may indicate that release of DFAA by *Uronema* sp. is not selective for some specific

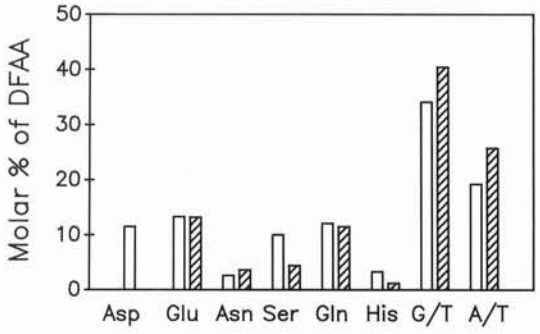

Fig. 1. *Uronema* sp. Molar % of DFAA in reverse-filtered sample of culture (open bars) and that which was artificially increased through exposure of cells to air (air-exposed sample – reverse-flow filtered sample) (hatched bars). Asp: aspartate; Glu: glutamate; Asn: asparagine; Ser: serine; Gln: glutamine; His: histidine; G/T: glycine/threonine; A/T: alanine/tyrosine. See text for explanations

amino acids in the internal pool, but rather it is a nonspecific process, probably related to egestion (Andersson et al. 1985, Nagata & Kirchman unpubl.).

In conclusion, we recommend the use of prepacked filters such as Acrodiscs for the analysis of DFAA in protozoan cultures, because it is easy to avoid exposure of cells to air. Moreover, we can process samples quickly and reduce the risk of contamination by using Acrodiscs. Acrodiscs are probably useful for preparing DFAA samples from natural waters and cultures of fragile phytoplankton.

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