DETERMINATION AND SIGNIFICANCE OF SUSPENDED PROTEIN IN WASTEWATER

EMILE VAN HANDEL[1,2,3]

ABSTRACT. A simple method is presented to measure the nutrient load of wastewater. Suspended particles, including microorganisms, are collected on a membrane filter by vacuum filtration, followed by colorimetric protein assay. Soluble proteins can be determined in the filtrate by precipitation. In domestic sewage and agricultural dairy wastewater, the largest portion of protein was retained on the membrane. Young larvae of *Culex nigripalpus*, *Cx. quinquefasciatus* and *Aedes aegypti* pupated when maintained in wastewater or its sediment but not when maintained in a membrane filtrate. If mosquito larvae feed primarily on suspended particles, the assay can be used to identify major feeding areas and may result in more economic application of microbial larvicides that act as food poisons.

INTRODUCTION

Protein is the most important nutrient promoting animal growth. Mosquito larvae depend on sources of protein available in the habitat. These proteins are locked in the cells of other aquatic organisms such as bacteria, algae, protozoa and small metazoa. If the larvae were dependent on dissolved proteins for their energetic and developmental requirements, each larva would have to isolate all the proteins of several hundred ml of habitat water at the prevailing concentrations of a few mg per liter. Raising adult mosquitoes under aseptic conditions in the laboratory on dissolved high quality proteins or their constituent amino acids has succeeded only rarely (Akov 1962, Dadd 1978, Friend and Dadd 1982), and the concentrations needed were several hundred times those found in the dirtiest wastewater. It is unlikely that a similar high concentration of dissolved protein, in the absence of cellular material, would ever occur in a natural breeding area.

In order to identify potential mosquito breeding sites, I have developed a rapid and sensitive method to determine cellular protein dispersed in the habitat. It depends on filtering wastewater through a membrane, dissolving the collected proteins in alkali followed by colorimetric assay. The soluble proteins in the filtrate can be precipitated and assayed separately.

MATERIALS

Microfiltration assembly (Spectrum Medical Industries Inc., Los Angeles, CA 90054 or Fisher catalog). Vacuum pump. Membrane filters (47 mm diam. 0.45 or 0.20 μm pore size). Culture tubes 16 × 100 mm; mark at 5 ml level. Centrifuge with swinging bucket rotor.

Protein standard (1 mg/ml). Dissolve 100 mg bovine serum albumen and 200 mg sodium azide in 100 ml water.

Reagent A. Dissolve 4 g sodium hydroxide and 20 g anhydrous or 54 g crystalline sodium carbonate in a liter of water.

Reagent B. Dissolve 0.5 g copper sulfate and 1.5 g sodium potassium tartrate in 100 ml water. Filter occasionally.

Reagent C. Folin-Ciocalteau reagent.

Standard and reagents are stable at room temperature.

EXPERIMENTAL

SUSPENDED PROTEIN. Strain wastewater through an 80 or 100 mesh sieve to remove larvae and large particles. Place membrane on top of the filter holder and screw on funnel (follow manufacturers' instructions). Connect filter assembly with vacuum pump. Filter an amount of sample that contains between 0.1 and 1 mg protein. This may require between 1 and 1000 ml, depending on the nutrient load. As the load on the membrane increases, the filtration rate decreases. When filtration becomes inconveniently slow, one can stop the filtration, then pour off and subtract the unfiltered portion from the sample volume. With a glass rod, push an unused membrane (blank) and the sample membrane to the bottom of a culture tube. When all samples have been filtered, add reagent A to the 5 ml mark. Let tubes stand for 10 to 15 min with occasional vigorous mixing to dissolve the proteins. Discard the membranes and centrifuge to remove material not dissolved in the alkaline solution. Transfer 1 ml of the supernatants of blank and samples to a clean culture tube, fill to the mark with reagent A, add 0.2 ml reagent B, mix, add 0.2 ml reagent C and mix immediately. A blue color develops that reaches a maximum in 10–20 min and is

---

[1] Florida Medical Entomology Laboratory, Institute of Food and Agricultural Sciences, University of Florida, 200 9th Street S.E., Vero Beach, FL 32962.

[2] The assistance of Mrs. Hilda Lynn is acknowledged.

[3] University of Florida, Institute of Food and Agricultural Sciences, Experiment Station Journal Series No. 6135.

stable for 1 to 2 hr. Read optical density (OD) at 650 nm against the membrane blank. Repeat the color development, substituting the 1 ml for a more appropriate aliquot so that the reading lies between 0.3 and 0.5 OD.

Standard curve. Use 0, 50, 100 and 200 μg (=μl) of the standard solution, add reagent A to the mark, then 0.2 ml reagent B and 0.2 ml reagent C and proceed as above. This is a modification of the well known protein assay of Lowry et al. (1951). The OD lies between 0.32 and 0.36 for 100 μg protein.

Soluble and total protein. For the assay of soluble proteins (the membrane filtrate) and total proteins (the original sample, passed through an 80 or 100 mesh sieve) the method of Bensadoun and Weinstein (1976) is recommended. Twenty-five ml of water (reagent blank) or of sample solution are mixed with 1 ml sodium desoxycholate (5g/liter) in 50 ml (25 × 115 mm) glass tubes, followed by 2 ml 100% trichloroacetic acid solution. Thorough mixing, preferably by inversion, is essential. The tubes are centrifuged for about 30 min, the supernatants carefully poured off and the precipitates dissolved in 5 ml reagent A by vigorous mixing. Undissolved material should be removed by centrifugation. Appropriate aliquots should be taken for color development as described above. For membrane filtrates it is usually necessary to react the entire 5 ml solution (25 ml of the original filtrate), but it is not necessary to centrifuge.

RESULTS

CHEMICAL ASSAYS. A variety of wastewater samples and infusions prepared in the laboratory were analyzed for suspended and soluble protein. To test completeness of recovery, total protein was also determined. The membrane always produces some blue color. Washing with water or reagent A did not remove this interference. It amounts to 0.20 - 0.25 OD for 47 mm and about 0.10 OD for 25 mm membranes.

Raw sewage entering the municipal wastewater treatment plant (influent) and sewage leaving the plant after treatment by an activated sludge process (effluent) was periodically analyzed in 1985 (Table 1). Total protein of influent fluctuated between 20 and 35 mg/liter and of effluent between 1.5 and 3.5 mg/liter. Between 70 and 95% of the influent consisted of suspended proteins, and about 90% of suspended proteins were removed by the treatment. Treatment was more efficient for suspended than for dissolved proteins (Table 1).

Table 2 lists the dispersed and soluble proteins of two dairy lagoons in Okeechobee County, Florida. The settling lagoon (60 × 100 m) receives wastewater directly from a barn where 480 to 600 cows are milked daily. At irregular intervals, some of the wastewater is pumped into a nearby holding lagoon. The settling lagoon has an abundant seasonal *Culex* population (O'Meara and Evans 1983). Wastewater ponds from a variety of citrus processing plants varied between 10 and 100 mg per liter total protein, and in each case, dispersed protein exceeded the soluble protein several fold. These ponds often breed *Culex* mosquitoes. On the other hand, oak leaves and hay incubated in the laboratory were much richer in soluble proteins (Table 3).

BIOASSAYS. As an example of the potential value of the proposed method to field application, newly hatched mosquitoes (10/250 ml) were maintained in wastewater from the dairy lagoon (80 mg protein per liter), in its ultrafiltrate (5 mg protein per liter), or in a sediment. This sediment was obtained by centrifugation of 250 ml of untreated wastewater portions at 12,000 rpm, and dispersing it in 250 ml tap water. The results show that pupation takes place in the intact waste and in the sediment, but not in the filtrate (Table 4). Apparently the filtrate is not much more nutritious to the larvae than tap water. Even when this wastewater was filtered through 5 μm instead of 0.2 μm membranes, none of these three mosquitoes pupated, although protein increased from 5 to 8 mg per liter.

Table 1. Protein content (mg/liter) of sewage.

	Influent			Effluent		
Date (1985)	Total	Filtrate	Membrane	Total	Filtrate	Membrane
Jan 14	30	4.0	24.0	3.5	1.3	2.0
Feb 7	20	6.0	13.0	3.0	1.0	2.5
July 23	20	2.5	20.0			
Aug 30	24	5.0	16.0	1.5	1.2	0.4
Sept 4	35	5.0	26.0			
Oct 22	20	5.5	13.5	2.5	1.5	0.8
Oct 24	25	6.0	15.0	3.0	1.7	0.7
Nov 28	30	1.5	26.0	3.5	0.5	2.0

Table 2. Protein content (mg/liter) of dairy lagoons.

Date	Settling lagoon			Holding lagoon		
	Total	Filtrate	Membrane	Total	Filtrate	Membrane
Sept 25, 1985	58	6.5	47	18.0	6.0	14
Oct 15, 1985	52	3.5	47	15.0	2.0	10
Feb 10, 1986	120	4.0	110	12.5	1.5	11
March 5, 1986	48	3.5	40	18.0	3.0	15
March 24, 1986	120	3.5	105	26.0	1.5	20

DISCUSSION

Instead of a 47 mm, a 25 mm filtration assembly may be used. In this case the amount of collected cellular protein is smaller and not always sufficient to replicate the color development. Effectively purified wastewater may be so low in protein that neither the Kjeldahl method (1883) nor precipitation from large volumes with trichloroacetic acid yields reliable results. It can be rapidly filtrated through a membrane in large quantities determining levels of 0.1 mg (approximately equivalent to 0.02 mg organic nitrogen) per liter and lower. Instead of 0.2, 0.45 μm membranes may be used with little difference in results or filtration rate.

Protein of untreated wastewater of agricultural and domestic origin is largely in cellular or particulate form and only a few mg per liter passes through a 0.2 μm membrane filter. Secondary treatment largely reduces cellular proteins. The proposed assay is much easier to carry out, and more sensitive and specific than the time honored Kjeldahl method for total nitrogen that includes ammonia and possibly other components without nutritional value for mosquitoes.

The same settling lagoon (Table 2) has been analyzed with the Kjeldahl method both for total nitrogen and ammonia by O'Meara and Evans (1983). The difference represents organic nitrogen. Their values measured in 1979 and 1980, varied between 150 and 300 mg protein (calculated from 25–50 mg organic nitrogen) per liter, compared to 50–120 mg protein per liter determined with the present method (Table 2). Carlson (1982) sampled an evaporation-percolation pond of Clemans School in Indian River County, Florida. This pond receives secondarily treated effluent from activated sludge wastewater treatment. In 9 trials (1980–81) he lists organic (Kjeldahl) nitrogen as 0.0 – 0.0. This testifies to the insensitivity of the method for organic nitrogen that was obtained by subtracting ammonia nitrogen (0 – 27 mg) from total nitrogen (0 – 27 mg). With the present method, I determined in the same school pond (3 trials) 1.5 – 3 mg for soluble and 0.3 – 1.5 mg per liter for protein retained on the membrane. These low values seem unfavorable for mosquito production. Indeed, at the time of sampling there was no larval activity and addition of *Culex quinquefasciatus* Say and *Cx. nigripalpus* Theobald in the laboratory failed to yield pupae. However, during 1980–81, there was abundant breeding of *Culex quinquefasciatus* and *Cx. nigripalpus* (Carlson 1982). Both O'Meara and Evans (1983) and Carlson (1982) reported similar numbers of immature mosquitoes (up to several hundred per dip) in spite of very different values for organic nitrogen. Secondarily treated water seems too low in nutrients for the production of adult mosquitoes. I have not been able to raise *Culex* larvae to pupation in the effluent from the sewage treatment plant where the total protein content does not exceed 4 mg per liter (Table 1). If heavy breeding occurs in habitats of seemingly low protein content, it must be assumed that the larvae derived nutri-

Table 3. Protein content (mg/liter) of infusions.

Infusion	Total	Filtrate	Membrane
Hay infusion (1 month old)	48	34	11
Oak leaf infusion (1 month old)	70	64	10
Oak leaf infusion (5 months old)	120	120	1

Table 4. Pupae produced in wastewater fractions.*

Species	Total	Filtrate	Sediment
Culex nigripalpus	9,9	0,0	9,9
Culex quinquefasciatus	10,9	0,0	7,8
Aedes aegypti	9,9	0,0	10,9

* Dairy (settling) lagoon wastewater. Each jar contained 250 ml of wastewater, untreated, filtered through a 0.2 um membrane, or the sediment from 250 ml, dispersed in the same volume of tap water. Ten recently hatched larvae were added per jar and pupation recorded over a 14 day period. Duplicate experiments.

ents from sessile sources that had escaped the sampling of surface water.

Wastewater collected in the field is always low in dissolved protein. Oak leaf infusions (used as oviposition attractant for *Culex* mosquitoes) prepared in the laboratory had a much higher content of dissolved protein (Table 3). Larvae of the three species maintained in an ultrafiltrate of oak leaf infusion (120 mg protein per liter) failed to pupate. This strengthens the hypothesis that cellular protein is needed for complete development. However, it is also possible that the eluted oak leaf proteins are nutritionally inferior to those from human or animal waste.

The present method makes it convenient to establish major concentrations of nutrients in habitats where the distribution is not homogeneous. This could lead to more selective and therefore more economic application of microbial larvicides (such as *Bacillus thuringiensis* var. *israelensis* and *B. sphaericus*) that function after ingestion rather than on contact. Untreated wastewater is not necessarily stable and may slowly deposit dispersed nutrients, thereby diminishing the ratio between dispersed and soluble protein. It should be passed through a 100 mesh sieve and be analyzed as soon as possible. Since sources of wastewater vary greatly, no general procedure will be recommended for prolonged storage.

References Cited

Akov, S. 1962. A qualitative and quantitative study of the nutritional requirements of *Aedes aegypti* L. larvae. J. Ins. Physiol. 8:319–335.

Bensadoun, A. and D. Weinstein. 1976. Assay of proteins in the presence of interfering materials. Analyt. Biochem. 70:241–250.

Carlson, D. B. 1982. Mosquitoes associated with evaporation-percolation ponds in Indian River County, Florida. Mosq. News 42:244–250.

Dadd, R. H. 1978. Amino acid requirements of the mosquito *Culex pipiens:* asparagine essential. J. Insect Physiol. 24:25–30.

Friend, W. G. and R. H. Dadd. 1982. Insect nutrition —a comparative perspective. Advances in Nutritional Research, vol. 4 (H.H. Draper, editor). Plenum Publishing Corp., p. 205–247.

Kjeldahl, J. 1883. Neue methode zur bestimmung des stickstoffs in organischen korpern. Z. Anal. Chem. 22:366–382.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.

O'Meara, G. F. and F. D. S. Evans. 1983. Seasonal patterns of abundance among three species of *Culex* mosquitoes in a South Florida wastewater lagoon. Ann. Entomol. Soc. Am. 76:130–133.