AD-A146 100 NEW AGAR TECHNIQUE COMPARED WITH SAND FLOTATION FOR OBTAINING SALT MARSH.. (U) SCIENCE AND EDUCATION ADMINISTRATION GAINESVILLE FL INSECTS A.. 1/1

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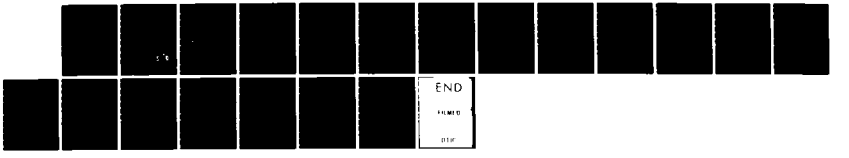

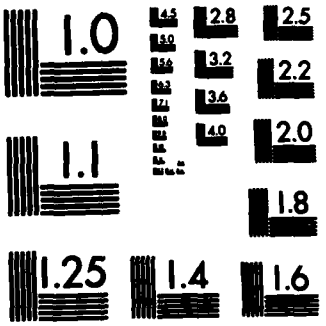

MICROCOPY RESOLUTION TEST CHART

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New Agar Technique Compared with Sand Flotation for Obtaining Salt Marsh *Culicoides mississippiensis* Hoffman (Diptera: Ceratopogonidae) Larvae[1]

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ABSTRACT

Sand flotation was compared with 1% and 2% (wt/v) agar formulations for extracting *Culicoides mississippiensis* Hoffman larvae from marsh soil samples. The 1% agar formulation yielded significantly less ($P<0.001$) larvae than the other 2 methods. The 2% agar method produced a greater number and healthier larvae, required less time, was cleaner, and provided a quantitatively equivalent and less variable estimate of the total larval population size than sand flotation.

Several methods have been used to recover larvae of biting midges (*Culicoides* spp.) from salt marsh substrate (soil) samples. They are: (1) sieve-flotation (Kettle and Lawson, 1952; Wirth, 1952; Kettle et al., 1956; Jamnback, 1965); (2) sand flotation (Bidlingmayer, 1957; Williams, 1960); (3) direct flotation (Linley and Kettle, 1964; Linley and Adams, 1972); and (4) Berlese funnels (Jamnback and Wirth, 1963; Jamnback, 1965). Kline et al., (1975) compared these methods and found that all were tedious, time-consuming, and had various other undesired qualities.

Sand flotation is the most commonly used method but it is messy and the extracted larvae are often damaged, making them unfit for use in rearing, insecticide, and pathogen studies. The need for a cleaner method of extracting healthy larvae in less time prompted us to search for an alternate procedure. Based on the investigations of Roberts (1966) and Kettle et al., (1975) in which tabanid and culicoid larvae were successfully reared in agar media, we decided that one possibility was to replace the sand with a layer of non-nutrient agar. We felt that since the agar media allowed the *Culicoides* larvae freedom of movement, perhaps they could be induced to move from the soil samples up into the agar layer. If so, then the agar layer could be removed to an examining pan and broken apart in water, thereby freeing the larvae for easy removal with a pipette.

To test this possibility we collected several soil samples from known *Culicoides* breeding habitats at Yankeetown, Florida. A 0.8% (wt/v) agar solution was prepared in an autoclave for 15 minutes at 15 psi. The agar was allowed to cool to ca. 47°C before being poured onto soil samples contained in quart size plastic pans. Three hundred ml of the solution was used to form a layer ca. 1 cm thick on each sample. After the agar formed a gel, the samples were covered and allowed to stand for 24 hrs. When the container lids were removed, several larvae were observed crawling on the surface even though they are photonegative. These trapped larvae were easily removed by rinsing the agar surface with a small amount of filtered estuarine water, decanting the water into a pan, and removing the larvae with a pipette. Most of the larvae remained in the agar layer and were removed when this layer was broken apart as planned. Unfortunately, this latter technique required considerably more time than the sand flotation method. However, the fact that some larvae were trapped on the surface of the 0.8% agar suggested the possibility that, if the right consistency (>0.8%) of agar were used, perhaps all the larvae in the substrate could be induced to crawl to the surface, trapped and recovered, and provide us with an acceptable alternative to sand flotation. Therefore, a comparison of sand flotation with 2 agar concentrations

(1 and 2%) was made to determine the efficacy of the technique, the number and condition of extracted larvae, and an estimate of larval population densities.

MATERIALS AND METHODS

Field-collected samples.--To determine the feasibility of using the agar techniques for routine sampling, marsh soil samples were collected from Yankeetown, Florida, during the winter and spring of 1978. Samples were taken with post hole diggers yielding ca. 0.8 liter soil cores (ca. 10 cm diam. X ca. 8 cm deep). Three adjacent samples were taken from areas which consistently produced large numbers of *Culicoides mississippiensis* Hoffman larvae. Each sample was transported to the laboratory in a closed plastic container (16 cm diam. X 11 cm deep). A replicate, chosen randomly, was used for each of the following treatments:

1. Sand flotation method.--Samples were retained in the field collection containers, covered with ca. 5 cm of prewashed sand, saturated with filtered estuary water, and allowed to stand at room temperature (ca. 21°C) for 24 hrs with the lid replaced. After 24 hrs the sand layer was removed and placed in a liter container. Saturated magnesium sulfate solution (500 ml) was added and the mixture thoroughly agitated. After agitation the sand was allowed to settle out and the liquid was decanted into a black-painted porcelain enameled pan for examination. The white larvae,

easily seen against the black background, were removed with a pipette. After removal of all visible larvae, the solution was returned to the container of sand and the mixture re-agitated. This process was repeated until 3 consecutive negative collections were made. A stopwatch was used to record the actual processing time, i.e., from removal of sand from the container until the last larvae was recovered. Each sample was then covered with a fresh layer of sand, saturated with filtered estuarine water and the extraction process repeated. This procedure was repeated daily until no larvae were recovered for 2 consecutive days, or for a maximum of 9 days.

2. Agar method.--Two agar concentrations (1 and 2%) were compared to determine if concentration has any significant effect on numbers of larvae moving up into the agar layer. For these tests the soil samples were retained in their field containers. The agar was prepared and allowed to cool to about 47°C, and then 300 ml of either a 1% (wt/v) or 2% agar solution was poured onto the samples. After the agar gelled the container lids were replaced. Twenty-four hrs later 200 ml of filtered estuarine water was poured onto the surfaces, swirled back and forth several times, and then decanted into a black-painted porcelain enameled pan. Larvae were removed with a pipette. After removal of all visible larvae, the 200 ml of estuarine water was again poured into

the container and the process repeated until 3 consecutive negative trials were obtained. The actual processing time, i.e., from the moment water was first poured onto the agar until removal of the last larva, was recorded. This procedure was repeated daily until no larvae were recovered for 2 consecutive days or for a maximum of 9 days.

Laboratory samples.--The field-collected samples were unsuitable to determine the relative efficiencies of each method since they contained unknown numbers of larvae. Therefore, the 2 methods were compared by setting up laboratory containers with known numbers of larvae. The samples contained 25 (5 replicates) or 50 (5 replicates) 3rd or 4th stage larvae which were added to specially prepared media in the plastic containers. The media for each container consisted of 254 grams of field-collected marsh soil that was first air-dried and then treated at 50°C for 24 hrs to assure that no viable *Culicoides* eggs or larvae remained. The soil was then saturated with filtered estuarine water and allowed to stand for 24 hrs, after which the *Culicoides* larvae were added, and *Panagrellus* sp. nematodes were liberally provided as food. Three different treatments (sand flotation, 1% and 2% agar) were set up and processed exactly as the field-collected samples.

RESULTS AND DISCUSSION

Larvae recovery.--Table 1 data show that the 2% agar formulation recovered a greater number or percentage of

larvae than the 1% agar concentration or the sand flotation method. Analysis of variance (ANOVA) of these data by sample source was not significant for the field-collected material but was for the laboratory samples ($P \leq 0.001$). Possibly the ANOVA of this data was not significant because the means contained variability due to the heterogeneity of soil and numbers of larvae. The 2 techniques which recovered the highest numbers of larvae, sand flotation and 2% agar, were not significantly different ($P = 0.05$, Duncan's multiple range test) for field or laboratory samples.

A possible explanation for the significant difference in number of larvae recovered from the laboratory samples between 1% agar and the other techniques lies in our hypothesis of how the agar works. We believe that agar extraction works because the agar layer interferes with oxygen exchange between the soil and air. As the agar concentration is increased, the air exchange becomes more difficult. Decreased oxygen or even anaerobic conditions in the soil forces the larvae to move upward and penetrate the agar layer, eventually burrowing through the surface. Larval penetration of the soil-agar interface is probably achieved by the larvae using the dense substrate to push against. The photonegative larvae become trapped on the agar surface even when the container lid is removed, allowing light to

strike the surface, because the larvae are unable to re-enter the agar layer due to the excessive surface tension and because the air-agar interface does not provide them with a dense surface to push against. Only those larvae close to a burrow are able to escape; the others are easily rinsed off the surface.

We believe that this hypothesis is supported by the data. Daily recovery rates (Table 2) show that larval recovery is greater for 2% than 1% agar, and that larvae are recovered for a longer duration (9 vs. 5 days). This may possibly be explained by a reduced interference with oxygen exchange with 1% agar, thereby reducing the need for the larvae to leave the soil, and also by a more rapid deterioration of the 1% agar gel. While both agar gel concentrations progressively deteriorate, it requires only 3-5 days for the 1% agar to break down (i.e., become mushy), while it takes 10-14 days for the 2% agar gel to noticeably lose its form. We feel that the softer 1% agar also allowed easier access to the photonegative larvae, especially with time, than the 2% agar. In order to make this determination several 1% and 2% agar layers from the field-collected samples were removed and examined after their allotted sampling time had elapsed. Many larvae were consistently recovered from the 1% agar layers, but only an occasional larva from the 2% agar layers.

Time requirements.--There are 4 basic steps required to obtain biting midge larvae: (1) collection of soil samples; (2) preparation of samples for larval extraction; (3) actual processing; and (4) clean-up. The time required for collection of the samples is the same no matter which method is used, but varies with method chosen for the other 3 steps.

Preparation of the sample requires that the soil be compacted within the container for removal of air pockets, and then the extraction layer is added (sand or agar). The actual preparation time required per sample is ca. 1-2 minutes for sand flotation and ca. 30-45 secs. for the agar methods. An advantage of the agar method is that compaction of the soil samples can be done while the agar is cooking and cooling, but with the sand flotation method compaction and addition of sand cannot be done simultaneously. This time differential becomes more significant as the number of samples collected for processing is increased.

Actual processing of the samples can be sub-divided into 3 steps: (1) removal of larvae from container; (2) picking larvae from the enameled pan; and (3) resetting those samples which are to be continued in the test. The time required per larva for removal from the enameled pan (step 2) is the same no matter which method is used. Thus, any time differential between methods is caused by steps 1 and 3.

As outlined in detail in the methods section, the sand flotation method requires that the sand layer be removed and transferred to another container where a flotation liquid is added. Then the sand and liquid are shaken for at least a minute before the liquid is decanted into the enameled pan for removal of the larvae. This procedure requires ca. 2-3 minutes per sample, whereas only ca. 30 secs. are required by the agar method to rinse the agar surface. Each sample may require this process to be repeated 6 times before the necessary 3 consecutive negative collections are made. This could result in a difference of up to 12 mins. per sample. Routinely, in our ecological study of larval habitats, we process 50 samples per wk. The other difference in time between methods is the resetting of samples. Each day a soil sample using sand flotation was used in this comparative study, it had to be covered again with a new layer of sand, and filtered estuarine water was added to saturate the sand. This step required 2-3 mins. per sample. In contrast, once the agar layer was established, it lasted for the duration of the study.

Cleanup requires at least twice as long for sand flotation because no matter how careful one is, the salt solution and sand end up all over the work area, on the processor's clothes, and on the floor. With the agar method, some water is occasionally spilled on the work area

or floor, but is easily sponged up.

Analysis of variance of actual processing time (excluding re-setting time) for the laboratory studies showed that there was a highly significant difference ($P \leq 0.001$) between the mean time required to process a sample by the sand flotation and agar techniques. Duncan's multiple range test ($p = 0.05$) showed that mean time required was significantly different for all 3 treatments (13.3, 18.1, and 23.7 mins., respectively, for 1% agar, 2% agar, and sand flotation).

Quality of larvae.--No actual quantitative measurement was made, but it was observed that larvae recovered by the agar methods appeared to be healthier and had less mortality than those extracted by sand flotation. Consequently, the larvae recovered by agar were more desirable for use in our rearing, insecticide, and pathogen studies.

Estimation of natural populations. If one knows that a particular technique recovers a certain proportion of the total larvae within a sample, then the inverse of that proportion times the number recovered provides an estimate of the total number within the sample. Table 3 presents the mean percentage recovered after 1 day extraction by sand, 1 and 2 days extraction by agar, the standard error of the mean ($S\bar{x}$), the inverse of the percentage recovered (multiplier) and the 95% confidence interval about the multiplier.

For example, multiplying the number of larvae actually recovered using the sand flotation method for 1 day by 1.39 provides an estimate of the total number of larvae in the sample. The sand method provides less variable estimates than 2% agar if both methods are used for 1 day only. However, a less variable estimate is provided by using the 2% agar technique for 2 days. Additionally, our data (Table 3) show that a 2 day extraction with 2% agar recovers the same percentage of larvae (72%) as sand flotation does in 1 day, but only requires ca. 62% of the time. Thus, the 2% agar method outperformed the 1% agar and sand flotation methods of extraction for *Culicoides*. It recovered a greater number of larvae, required less time, produced healthier larvae, was cleaner, and provided a more reliable estimate of larval population densities.

REFERENCES CITED

Bidlingmayer, W. L. 1957. Studies on *Culicoides furens* at Vero Beach. Mosq. News 17: 292-294.

Jamnback, H. A. 1965. The *Culicoides* of New York State (Diptera:Ceratopogonidae). N.Y. Stage Mus. Sci. Serv. Bull. 399, 154 p.

Jamnback, H. A., and W. W. Wirth. 1963. The species of *Culicoides* related to *obsoletus* in eastern North America (Diptera:Ceratopogonidae). Ann. Entomol. Soc. Amer. 56: 185-198.

Kettle, D. S., and J. W. H. Lawson. 1952. The early stages of British biting midges *Culicoides latreille* (Diptera: Ceratopogonidae) and allied genera. Bull. Entomol. Res. 43: 421-467.

Kettle, D. S., R. H. Nash, and B. A. Hopkins. 1956. Field tests with larvicides against *Culicoides impunctatus* Goetgh. in Scotland. Bull. Entomol. Res. 47: 553-573.

Kettle, D. S., C. H. Wild, and M. M. Elson. 1975. A new technique for rearing individual *Culicoides* larvae (Diptera:Ceratopogonidae). J. Med. Ent. 12: 263-264.

Kline, D. L., J. C. Dukes, and R. C. Axtell. 1975. Salt marsh *Culicoides* (Diptera:Ceratopogonidae): Comparison of larval sampling methods. Mosq. News 35: 147-150.

Linley, J. R., and D. S. Kettle. 1964. A description of the larvae and pupae of *Culicoides furens* (Poey), and *Culicoides hoffmani* Fox (Diptera:Ceratopogonidae). Ann. and Mag. Natur. Hist. Ser. 13: 129-149.

Linley, J. R., and G. H. Adams. 1972. Ecology and behavior of immature *Culicoides melleus* (Coq.) (Diptera: Ceratopogonidae). Bull. Entomol. Res. 62: 113-128.

Roberts, R. H. 1966. A technique for rearing the immature stages of Tabanidae (Diptera). Entomol. News 77: 79-82.

Williams, R. W. 1960. A new and simple method for the isolation of fresh-water invertebrates from soil samples. Ecology 41: 573-574.

Wirth, W. W. 1952. The immature stages of two species of Florida salt marsh sandflies (Diptera:Heleidae). Fla. Entomol. 35: 91-100.

FOOTNOTES

[1]The research reported in this manuscript was conducted in part with contract funds transferred from the Office of Naval Research, Department of the Navy.

Table 1.--Recovery of *Culicoides mississippiensis* Hoffman larvae from field-collected marsh soil samples and laboratory samples.[a]

Method	Mean recovery of larvae from Field samples[b] (No. collected)	Laboratory samples[c] (% recovered)
1% Agar	52.4a	43.4b
2% Agar	73.3a	88.1a
Sand flotation	67.0a	79.3a

[a]Means in the same column followed by different letters are different at the 0.05 level (Ducan's multiple range test).

[b]Based on 40 samples.

[c]Based on 5 samples with 50 larvae and 5 samples with 25 larvae.

Table 2.--Recovery of *Culicoides mississippiensis* Hoffman larvae by sand and agar methods (cumulative % recovery in parentheses) from laboratory samples.

Sampling day	Percent recovery by indicated method					
	Sand flotation		1% Agar		2% Agar	
1	72.1	(72.1)	27.4	(27.4)	42.2	(42.2)
2	6.8	(78.9)	10.4	(37.8)	29.8	(72.0)
3	0.4	(79.3)	2.6	(40.4)	7.4	(79.4)
4	0.0	(79.3)	1.4	(41.8)	3.4	(82.8)
5	0.0	(79.3)	1.6	(43.4)	2.6	(85.4)
6			0.0	(43.4)	1.4	(86.8)
7			0.0	(43.4)	0.6	(87.4)
8					0.3	(87.7)
9					0.4	(88.1)

Table 3.--Variability of population estimates based on sand flotation or 2% agar techniques.

Technique	Mean recovery rate (%)	$S\bar{x}$	Multiplier	Multiplier fiducial limits (95%)[a]
Sand flotation (1 day)	72.1	6.6	1.39	1.15-1.75
2% Agar (1 day)	42.2	4.5	2.37	1.91-3.13
2% Agar (2 days)	72.0	2.2	1.39	1.30-1.47

[a]Based on t_9, 05 = 2.262 and laboratory samples.

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