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Estimation of Lipid Reserves in Unstained Living and Dead Nematodes by Image Analysis

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Abstract: During storage, non-feeding stages of entomopathogenic nematodes become visibly more transparent due to depletion of energy reserves. Optical density per unit area (OD per area) of infective juveniles of *Steinernema carpocapsae* (All) and two *Heterorhabditis* isolates (UK211 and HF85) was measured with an image analysis system and compared with neutral lipid levels obtained by Oil Red O staining. Optical density (OD) measurements were compared with triglyceride levels of UK211 and HF85. Good correlations between OD per area and neutral lipids (0.90) and between OD and triglycerides (0.87) were found. Thus, OD reflects lipid levels and can be used as an indicator of lipid reserves in these nematodes. Heat-killing of nematodes had no significant effect on OD measurements, but length increased significantly. Storage in a triethanolamine in formaldehyde solution decreased the OD and OD/area by about 5% to 8%. An additional advantage of the image analysis method described is that repeated measurements can be performed on live nematodes.

Key words: entomopathogenic nematode, *Heterorhabditis*, image analysis, neutral lipid, Oil Red O, optical density, *Steinernema*, triglyceride.

Stored energy reserves, neutral lipids in particular, in the free-living stages of various plant- and animal-parasitic nematodes and entomopathogenic nematodes are vital for survival and infectivity (Lee and Atkinson, 1976; Selvan et al., 1993b). The levels of food reserves in *Meloidogyne* spp. and *Tylenchulus semipenetrans,* for example, were correlated with their activity and infectivity (Van Gundy et al., 1967). When lipid reserves in *Globodera* spp. dropped below 65% of the initial lipid level, infectivity was negatively affected (Robinson et al., 1987). A decrease of lipid content below 10% (measured as stained area) in entomopathogenic nematodes also led to reduced mobility and infectivity (Vänninnen, 1990). Westerman and Stapel (1992) found that the proportion of infective juveniles with ample food reserves was positively correlated with the migration rate of a strain of *Heterorhabditis* sp. Depletion of energy reserves also is likely to be a major factor in restricting the shelf life of industrially produced entomopathogenic nematodes. It is important, therefore, to have an easy and reliable method for testing lipid levels of stored nematodes as an indication of quality, particularly their ability to find and infect hosts.

Lipids in nematodes are stored mainly as triglycerides and other neutral lipids (Lee and Atkinson, 1976). Measurements of neutral lipids can be relative or absolute. One method often used is to stain neutral lipids with Oil Red O (Croll, 1972); the density of staining can then be measured with a densitometer (Croll, 1972; Storey, 1983; Wright et al., 1989). More recently, Vänninnen (1990), Tiilikkala (1991), and Hendrikx et al. (1994) used image analysis to measure the area stained by Oil Red O. Christophers et al. (1996) developed a quick and simple way of assessing lipid content of Oil Red O-stained nematodes for *Meloidogyne* spp. using an index scale from 6 (fully stained) to 1 (no staining) by reference to a series of standard photographs. A modified lipid index scale (8 to 1) has been developed for *Steinernema* spp. (Patel et al., 1996). Lipid levels also can be determined chemically. Triglycerides, for instance, can be broken down enzymatically to glycerol and fatty acids (Stryer, 1988), and the amount of glycerol present then can be measured photometrically. Lipid fractions can be separated and analyzed for their constituent fatty acids with gas chroma-

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tography (Badhwar et al., 1995). Such methods allow the lipids to be quantified but require much larger numbers of nematodes. A disadvantage of staining nematodes with Oil Red O is that nematodes shrink during the staining process.

Westerman and Stapel (1992) and Jung (1991) used a semi-quantitative method on unstained nematodes by recording the percentage of nematodes considered to be light or dark in appearance. Reversat et al. (1980) used a related photographic image analysis technique. Juveniles of *Meloidogyne javanica* at three stages of starvation were stained with Sudan black and photographed, and the nematode images were cut out of the photographic prints with scissors. Silver was then removed from the paper with nitric acid and measured with atomic absorption spectrometry. Silver concentrations were proportional to lipid concentrations in nematodes measured colorimetrically with sulphosphovanillic acid reagent. In this paper we describe a quantitative method for measuring lipid reserves in nematodes with image analysis. Image analysis-based measurements of optical density (OD) were compared with Oil Red O staining of neutral lipids and enzymatically determined triglyceride levels.

Materials and Methods

Nematodes: The nematodes used were *Steinernema carpocapsae* All strain and *Heterorhabditis* isolates HF85 and UK211. Both *Heterorhabditis* isolates belong to the North West European (NWE) group (Smits et al., 1991). Nematodes were reared on larvae of the greater wax moth, *Galleria mellonella,* at 20 °C. After harvesting, the infective juveniles (IJ) were washed three times by sedimentation in tap water. Prior to staining or image analysis, nematodes were allowed to migrate through a sieve (35-μm pore size) to separate live from dead nematodes. Only living nematodes were processed for lipid and image analysis.

Method of image analysis: A "Magiscan" image analysis system, using GENIAS software (both from Applied Imaging, Sunderland, UK) was used to measure optical density, area, and length of the nematodes. Images were taken with a video camera (MTI, series 68, 625 scan lines) mounted on a Nikon Optiphot-2 microscope. Using a 10× objective, the length of a single nematode comprised about 75% of the screen width. The digital image consisted of 512 × 512 pixels with 64 gray levels. Light intensity reaching the camera was kept constant throughout all experiments: before each session, the background gray level was adjusted to 35 to compensate for aging of the light bulb or pollution of the optical system. Microscope settings (condenser, filters, and diaphragm) also were kept constant throughout all experiments.

Following digital image acquisition, one or more nematodes (objects) in each image were separated from the background by means of a computed optimal threshold level. To further distinguish the nematodes, all objects that still had non-thresholded spaces were filled, regardless of their gray level. To exclude debris and overlapping nematodes, objects that were bigger than 3.0×10^4 or smaller than 1.0×10^4 pixels were deleted. The remaining objects were the nematodes. Nematodes touching the edge of the field were discarded. The OD (sum of pixel values) and area (μm^2) of each nematode were measured and data expressed as either OD or OD per area (density per unit nematode area). Nematode length also was measured in some experiments. To measure length, a binary thinning operation was performed that reduced each nematode's image to a one-pixel wide line along its longitudinal axis. Measurements were performed on 30 to 40 randomly chosen nematodes per replicate.

Neutral lipids and OD per area: Neutral lipids in the nematodes were stained with Oil Red O according to a modified method of Croll (1972). Briefly, washed nematodes were concentrated in a glass tube and flooded with 70% (w/v) ethanol saturated with Oil Red O and incubated at 60°C for 20 minutes. The nematodes were allowed to settle, and excess stain was removed with a Pasteur pipette. A glycerol:water (50:50 v/v) solution was added (5 ml), and the nema-

todes were left to settle overnight at room temperature. The lipid content of stained nematodes was estimated by means of a neutral lipid index scale from 8 (fully stained) to 1 (no staining) and visually referenced to a series of standard photographs according to the method of Patel et al. (1996). Measurements were performed on 100 nematodes per replicate.

Two experiments relating neutral lipids to OD per area were conducted, employing *S. carpocapsae* and *Heterorhabditis,* respectively. Freshly harvested *S. carpocapsae* were concentrated to 3,000 IJ per petri dish (9-cm diam., ca. 120 IJ/ml tap water) and stored at 20 °C in the dark. After 0, 70, 89, 105, and 112 days storage, aliquots of nematodes from three replicate dishes were stained with Oil Red O and the lipid index was calculated. The remaining nematodes in the dishes were stored 2 to 8 weeks in the preservative TAF (2% v/v triethanolamine in 2.8% v/v formaldehyde solution (Southey, 1970)) for image analysis measurements. Freshly harvested *Heterorhabditis* isolates (UK211 and HF85) were concentrated to 4,000 IJ/ml tap water, stored in plastic food containers with snap lids (20 × 10 × 7 cm) at 20 °C in the dark, and shaken daily. There were three replicates per isolate. After 7, 21, and 35 days storage, aliquots of nematodes were stained with Oil Red O and the lipid index calculated while the remaining nematodes were stored (8 to 13 weeks) in TAF for subsequent image analysis measurements.

Triglycerides and OD: Freshly harvested *Heterorhabditis* sp. (HF85 and UK211) were concentrated to 4,000 IJ per petri dish (5-cm. diam., ca. 500 IJ/ml tap water) and stored at 20 °C in the dark. Image analysis measurements were performed on 0-, 7-, 14-, 21-, 35-, and 49- day-old IJ (40 nematodes × 3 replicates) after heat-killing. At the same time periods, nematodes from three other petri dishes were examined under a stereomicroscope to estimate the proportion alive. The latter nematodes (≤4,000) were then concentrated into 0.1 ml of tap water in Eppendorf tubes and stored at −20 °C for subsequent assessment of triglyceride levels. After thawing, nematodes were sonicated (Cyborg soniprobe, type 7532B, Middlesaxon, UK) using three 8-second bursts at level 6; nematodes were kept on ice during and after sonication. Triglycerides were extracted from sonicated nematodes with 2 ml chloroform: methanol solution (1:1), and the mixture was centrifuged at 8,000 rpm for 5 minutes. The chloroform phase containing the triglycerides was removed and dried by evaporation in air. Triglycerides were quantified with the use of an enzymatic triglyceride test (Sigma, GPO-Trinder).

Image analysis on living and dead nematodes: Image analysis measurements of heat-killed nematodes were compared with measurements on live nematodes. The nematodes used for this experiment were IJ of *Heterorhabditis* HF85 (which had been stored for 70 days at 5 °C in the dark) and 7-day-old IJ of *Heterorhabditis* UK211. Half of each batch of nematodes (3 replicates of ca. 1,000 nematodes per replicate) was concentrated in a watch glass and heat-killed by adding tap water at 80 °C. Directly after killing, slides were prepared, each containing ca. 60 nematodes in 17 µl water, and cover-slips were sealed immediately with nail varnish. Slides with live nematodes were prepared in a similar manner. Nematodes on slides prepared in this manner displayed increased activity, but after about 5 minutes their mobility was reduced again to a level that allowed image analysis.

Image analysis on TAF-stored nematodes: It is not always convenient to measure nematodes immediately. Therefore, the effect of storage in TAF on the OD and size of IJ was tested. Experiments were conducted with *S. carpocapsae* and the two *Heterorhabditis* isolates. Freshly harvested IJ of *S. carpocapsae* (All) and *Heterorhabditis* isolates UK211 and HF85 (three replicates of ca. 5,000 nematodes each) were heat-killed at day 0 and concentrated in an Eppendorf tube to which 1.5 ml TAF was added. The OD, area, and length of TAF-stored IJ were measured in TAF after 0, 3, 7, 14, 21, 29, and 35 days (*S. carpocapsae*) or after 0, 2, 7, 56, and 77 days (*Heterorhabditis*) of storage in Eppendorf tubes at 4 °C in the dark. The nematodes at day 0 were measured in water.

Statistics: If not mentioned otherwise, the statistical analyses performed between isolates were *t*-tests with a *P* value < 0.05. Within isolates over time, a Student-Newman-Keuls test ($P < 0.05$) was used.

Results

Neutral lipids and OD per area: The neutral lipid index (percentage body area stained with Oil Red O) was compared to OD per area (Figs. 1a and 2) rather than OD, because OD is unrelated to body size. The neutral lipid index was correlated with OD per area for both nematode species. Coefficients of determination were 0.97 for *S. carpocapsae* (Y = 0.025X + 0.31) and 0.89 for the *Heterorhabditis* isolates (HF85 and UK211) (Y = 0.028X + 0.33). There was a significant decrease in the area of *S. carpocapsae* over the experimental period but no significant change in length (Fig. 1b). The *Heterorhabditis* isolates did not change significantly in area over the course of the experiment.

Triglycerides and OD: Optical density measurements were correlated with triglyceride levels over the 49-day storage period at 20 °C for both *Heterorhabditis* isolates (Fig. 3). Co-

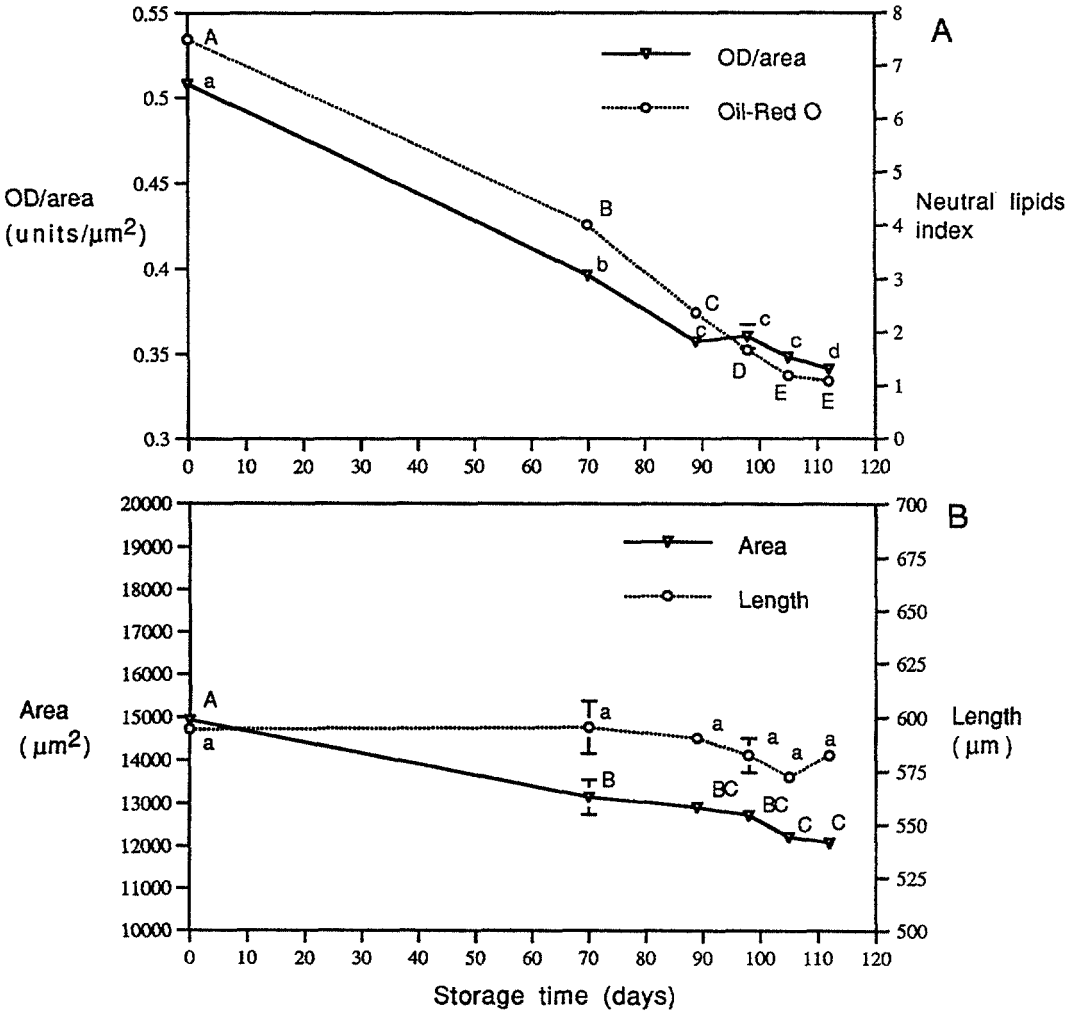

FIG. 1. Changes in infective juveniles of *Steinernema carpocapsae* over a 112-day storage period in water at 20 °C. A) Mean optical density per μm^2 of nematode (OD per area) and neutral lipid index values stained by Oil red O, and B) Area and length of infective juveniles. For each line, points followed by the same letter are not significantly different according to the Student-Newman-Keuls test ($P \geq 0.05$, bars = SE).

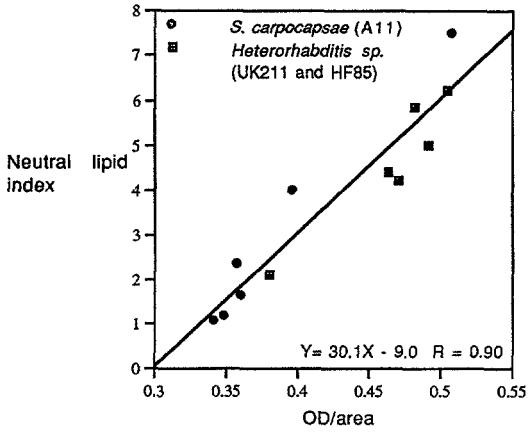

FIG. 2. Correlation between neutral lipid index and mean optical density per μm^2 of nematode image (OD per area) for infective juveniles of *Steinernema carpocapsae* and *Heterorhabditis* sp. during 112 and 35 days, respectively, of storage in water at 20 °C.

efficients of determination were 0.96 and 0.80 for HF85 and UK211, respectively. Both OD and triglycerides declined with time; however, while triglyceride levels continued to decrease throughout the 49-day observation period, there was no change in OD for either HF85 or UK211 between days 35 and 49. However, numbers of nematodes alive after 49-day storage at 20 °C were less than 30% (5% for HF85); therefore, triglyceride levels measured were less reliable.

Heterorhabditis isolate UK211 had higher OD values than HF85 at all times, with significant differences between the isolates in the first 21 days. There was no significant difference in triglycerides between the two isolates at any time point. Also, *Heterorhabditis* UK211 was significantly larger than HF85, both in area and length (HF85: 20,290 μm^2 and 798 μm; UK211: 22,050 μm^2 and 836 μm). There was no significant difference in the initial OD per area values for UK211 and HF85. However, OD per area decreased more quickly in HF85 than in UK211, with a significant difference between the isolates at week 1.

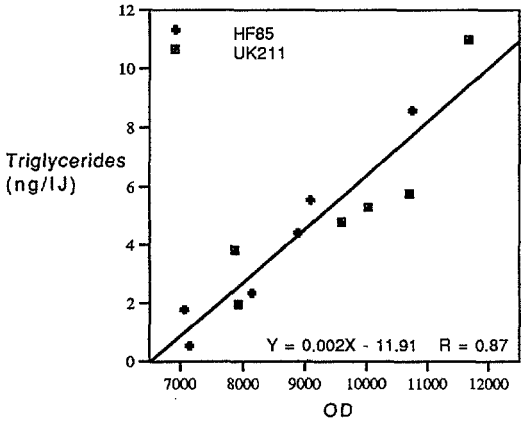

FIG. 3. Correlation between triglyceride content and total optical density (OD) of infective juveniles of *Heterorhabditis* sp. HF85 and UK211 over a 35-day storage period in water at 20 °C.

Image analysis on living and dead nematodes: Image analysis of living and fast-moving nematodes is time-consuming because of waiting for a suitable image. Acquired images of fast-moving nematodes were sometimes blurred due to movement and could not be used. Heat-killed nematodes were still and straight, both of which facilitated measurements. There was no significant difference in OD, area, or OD per area between living and heat-killed nematodes for OD, area, or OD per area. However, a significant but small difference in length was observed for both HF85 and UK211 (Table 1).

Image analysis on TAF-stored nematodes: Storage of both *S. carpocapsae* and *Heterorhabditis* IJ in TAF had no significant effect on either area or length. There was no significant change in OD or OD per area for *S. carpocapsae* over the 35-day storage period in TAF, though a non-significant decrease in OD (4%) and OD per area (8%) was observed within the first 14 days (Fig. 4a). The two *Heterorhabditis* isolates were grouped together because no difference between them was measured at each assessment day (*t*-test). The OD and OD per area of the combined *Heterorhabditis* isolates were significantly reduced (by 6% and 7%, respectively), on days 56 and 77 when compared with day 0 (Fig. 4b). Measurements in TAF had a negligible effect (<0.5%) on the background density.

DISCUSSION

Correlations between OD and both measures of neutral lipids (Oil Red O and tri-

TABLE 1. Image analysis measurements (mean ± SE) on live and heat-killed infective juveniles of *Heterorhabditis* sp. (isolates HF85 and UK211).

Isolate	OD[a] (units)	Area (μm^2)	OD/area (units/μm^2)	Length (μm)
HF85				
Live	9,001 ± 60a	20,302 ± 96a	0.442 ± 0.004a	760 ± 6a
Dead	9,351 ± 210a	20,872 ± 351a	0.447 ± 0.003a	785 ± 5b
UK211				
Live	10,538 ± 63a	20,866 ± 106a	0.504 ± 0.002a	775 ± 5a
Dead	10,804 ± 186a	21,263 ± 208a	0.507 ± 0.007a	796 ± 5b

Within each isolate and column, data followed by the same letter are not significantly different (*t*-test, $P < 0.05$). $n = 3$; each replicate consisted of 30 nematode measurements.

[a] Optical density. Cumulative pixel gray level values for all pixels in each nematode image.

glycerides) showed that OD can be used as an indicator of lipid reserves in the entomopathogenic nematodes examined.

Staining with Oil Red O clearly showed that a large part of the entomopathogenic nematode IJ is filled with neutral lipid globules that diminish with age. Unstained nematodes become less optically refractive and appear visibly more transparent when illuminated with transmitted light. The observed decline in OD is probably due largely to the depletion of neutral lipids, also confirmed by Reversat et al. (1980). However, carbohydrates, proteins, and other lipids also can be used as an energy source for nematodes. For example, glycogen is generally the most abundant energy reserve in the parasitic stages of animal-parasitic nematodes, which may be exposed to partially anaerobic environments (Atkinson, 1976; Badhwar, 1995). We have no data showing whether glycogen, carbohydrates, and proteins affect OD differently than neutral lipids do. If these energy sources affect OD similarly to neutral lipids, then OD could be more widely applicable for measuring energy reserves in nematodes than methods that measure triglycerides or total neutral lipids only.

By using OD per area, it is possible to compare lipid consumption of nematodes that differ in size. Although the two *Heterorhabditis* isolates are closely related (Griffin et al., 1994; Smits et al., 1991), UK211 is slightly bigger than HF85. This may account for the slightly greater OD and triglyceride content measured for UK211 at day 0. However, the OD per area of the two isolates was similar at day 0. The rate of decrease in OD per area for UK211 was less than for HF85, suggesting a lower rate of energy depletion in UK211. Atkinson (1976) has suggested a weight-dependent metabolism when comparing metabolic rate in different species of nematodes to account for differences in body size.

The decrease in area observed in *S. carpocapsae* during storage in water (Fig. 1b) could be due to the depletion of lipid and other energy reserves. A similar decline in area of *Heterorhabditis* sp. of the NWE group has been observed (data not shown), though the decrease observed in the present experiment was not significant. Selvan et al. (1993a) found that the water content of *S. carpocapsae, S. glaseri,* and *H. bacteriophora* increased by 14% to 16% toward the end of their shelf life; any uptake of water by *S. carpocapsae* in the present experiment was not sufficient to compensate for nematode shrinkage.

Heat-killing nematodes extends their body length. Nematode area, however, was not significantly affected in the present experiment. There also was no significant difference between heat-killed and live nematodes in OD and OD per area measurements. Thus, the image analysis-based method of determining lipid reserves appears suitable for use with heat-killed nematodes. Heat-killing is particularly appropriate for routine measurements of IJ of the *Heterorhabditis* NWE group because they are highly active and no time is lost waiting for a suitable image. Also, when taking measurements on live nematodes, experimenter

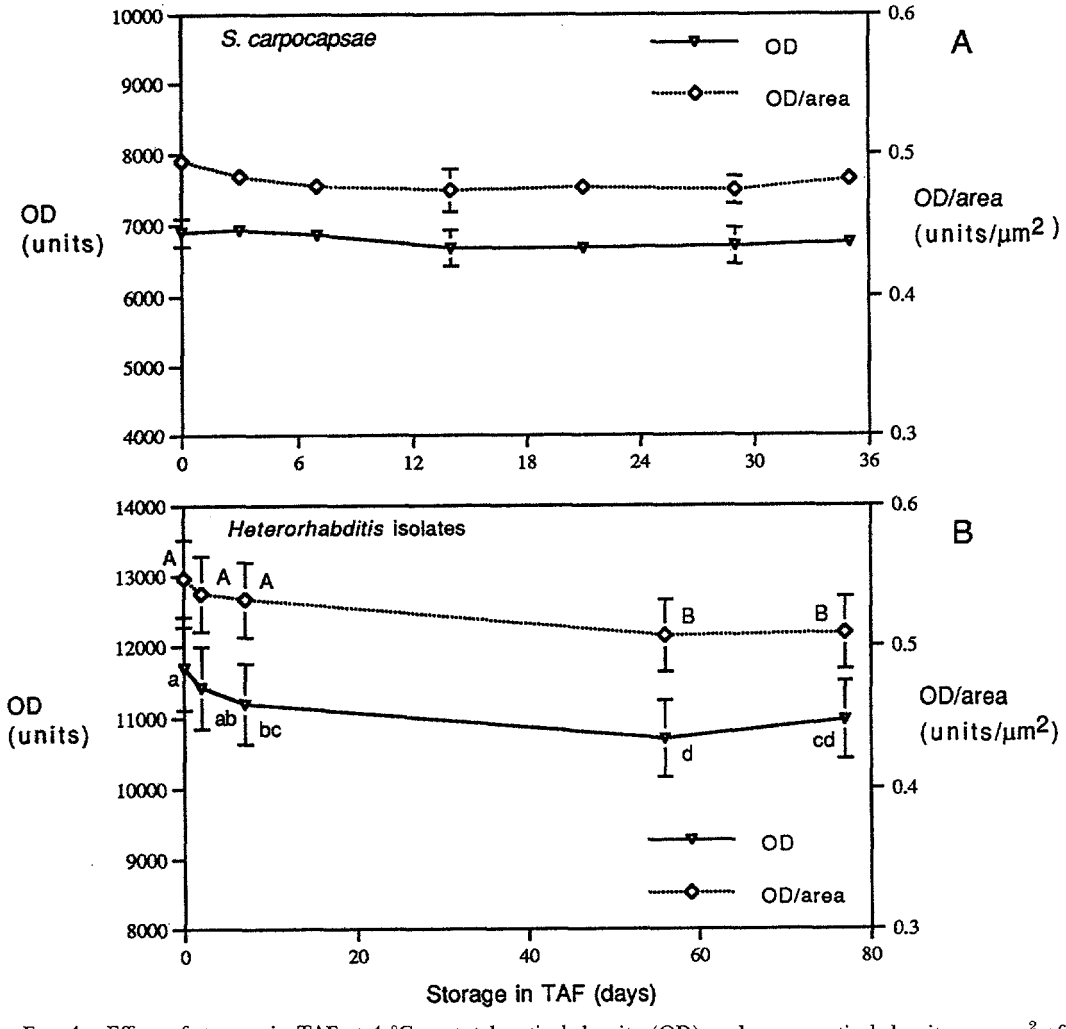

FIG. 4. Effect of storage in TAF at 4 °C on total optical density (OD) and mean optical density per μm^2 of nematode (OD per area) of infective juveniles of A) *Steinernema carpocapsae* (ALL) over a 35-day storage period, and B) *Heterorhabditis* sp. HF85 and UK211 over a 77-day storage period. Within an isolate, points followed by the same letter are not significantly different according to the Student-Newman-Keuls test ($P \geq 0.05$, bars = SE).

bias may favor slower-moving specimens. Heat-killing makes it possible to measure nematodes systematically. However, nondestructive repeated measurements on individual live nematodes can be useful in studies of starvation processes. Currently, we are optimizing such methods.

Storing IJ in the fixative TAF did not significantly alter length or area. The OD and OD per area were reduced for both nematode species, although this was not significant for *S. carpocapsae*. A maximal reduction of 8% compared to the measurements at day 0 was observed in the first 14 days of storage in TAF for *S. carpocapsae* and between days 7 and 56 of storage in TAF for the *Heterorhabditis* isolates. A preliminary experiment with an isolate of the Irish Group of *Heterorhabditis* showed a decrease in OD of about 10% within 7 days of fixing in TAF, but after 5 months no further decrease had occurred. These observations suggest that OD measurements of TAF-preserved nematodes should be made after a suitable period (probably about 2 weeks at 4 °C) to allow fixative-induced decreases in OD to stabilize. The decrease in OD per area is a result of the decrease in OD, as no changes in area

were observed. Staining with Oil Red O after fixing in TAF is also possible (Alphey, 1982).

An additional advantage of determining lipid reserves by image analysis of unstained nematodes is that it is possible to carry out repeated measures on individual living nematodes, preferably slow-moving specimens, over time. Also, since no extraction or staining procedures are needed, variance between operators over time is low. Moreover, length and area measurements are more accurate than after staining with Oil Red O, which causes shrinkage.

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