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Counting Nematodes with a Microplate Reader[1]

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Abstract: The feasibility of counting plant-parasitic nematodes in aqueous suspensions by measuring light transmittance through aqueous suspensions with an ELISA microplate reader was explored. Absorbance readings for eggs or vermiform stages of three species were linearly related ($R^2 > 0.99$) to concentrations between 0 and 10,000 nematodes/ml. Coefficients of variation ranged from 12–23%, depending on the species and developmental stage used. The method, therefore, was at least as accurate as direct counts of nematodes in aliquots on a microscope and more than 100 times as fast. The method should have direct application in research programs on plant resistance to nematodes, nematode population dynamics, and nematode behavior.

Key words: counting, *Ditylenchus phyllobius*, enumeration, *Meloidogyne incognita*, nematode, *Rotylenchulus reniformis*, technique.

Counting nematodes and their eggs at the microscope is one of the most time-consuming tasks in many kinds of nematological research. To facilitate counting nematodes, various counting chambers have been devised, and electronic metronomes have been built that keep count of audio signals given to the microscopist (2,4). To our knowledge, however, the only truly automatic, successful methods employ the measurement of light scattering by nematodes (i.e., nephelometry [12]), the measurement of light transmittance through nematode suspensions with a spectrophotometer (1,8), or electronic particle counting with an impedance bridge (3,7), as is done with a Coulter counter. None of these methods has gained wide acceptance. Image analysis systems also could be used for nematode counting but generally are prohibitively expensive.

Fiber-optic spectrophotometers, usually called microplate readers, were developed to read ELISA (enzyme linked immunosorbant assay) plates, are made by several companies, and are increasingly common instruments in biological research laboratories. Microplate readers are microprocessor based, require almost no training to use, and can read absorbance for 96 samples in less than 10 seconds. Also, computer software is available for immediate statistical analysis of results. Theoretically, these instruments could quantitate nematode density in aqueous suspensions. Using an EMAX Precision Microplate Reader (Molecular Devices Corp., Menlo Park, CA 94025), we examined absorbance readings for known concentrations of three species of plant-parasitic nematodes in water to determine the feasibility of counting nematodes with a microplate reader.

Materials and Methods

Eggs of *Meloidogyne incognita* were obtained by maceration of infected roots of *Hibiscus cannabinus* in NaOCl solution followed by sieve-separation and centrifugal flotation of the eggs (5). Fourth-stage juveniles of *Ditylenchus phyllobius* were isolated from foliar galls of *Solanum elaeagnifolium* by soaking desiccated galls in water, followed by vacuum filtration of nematodes from the water and a Baermann saucer clean-up (11). Mixed vermiform stages of *Rotylenchulus reniformis* were obtained from soil by direct Baermann funnel extraction (10). All stock suspensions were optically clear and essentially free of soil particles and organic debris. Nematode motility in suspensions of *D. phyllobius* and *R. reniformis* exceeded 98%.

Suspensions of each species were prepared separately at concentrations of 0, 2,000, 4,000, 6,000, 8,000, and 10,000 nematodes or eggs per ml water, and 200 µl of each suspension were placed in 10 replicate wells of a 96-well ELISA plate.

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Suspensions containing less than 10,000 nematodes/ml were prepared by direct dilution of a suspension with 10,000 nematodes/ml, and final concentrations were verified by counting nematodes within aliquots. Absorbance was read at 550, 570, 595, and 630 nm.

For each species, we also compared absorbances of live versus heat-killed nematodes, and we examined the effect of mixing the well contents just before reading by vibrating the plates on a Vortex shaker. (Some microplate readers have a built-in shaker for this purpose.) Absorbance of freshly extracted juveniles of *D. phyllobius* was compared with absorbance of juveniles starved for one week at 23 C.

Results

In each case, mixing the well contents reduced sample variation several fold, and a linear relationship ($R^2 > 0.988$) existed between absorbance units and nematode concentration over the range of concentrations tested (Fig. 1). For concentrations greater than 400 nematodes/well, coefficients of variation (CV) for each nematode species were essentially independent of concentration and wavelength. The average CV, after subtracting absorbance for the plate and water, ranged from 12% for vermiform *R. reniformis* to 23% for eggs of *M. incognita*. Thus, to measure the nematode concentration to within 15% at $P = 0.05$, three and nine replications were needed for *R. reniformis* vermiform stages and *M. incognita* eggs, respectively (6). Absorbance readings at 630 nm were always about 7% higher than readings at 570 nm; standard deviations, however, increased by the same amount, so no additional precision was obtained.

Viability had no measurable effect on the mean absorbance for any species. Heat killing eggs of *M. incognita* made them appear more uniformly refractive under the

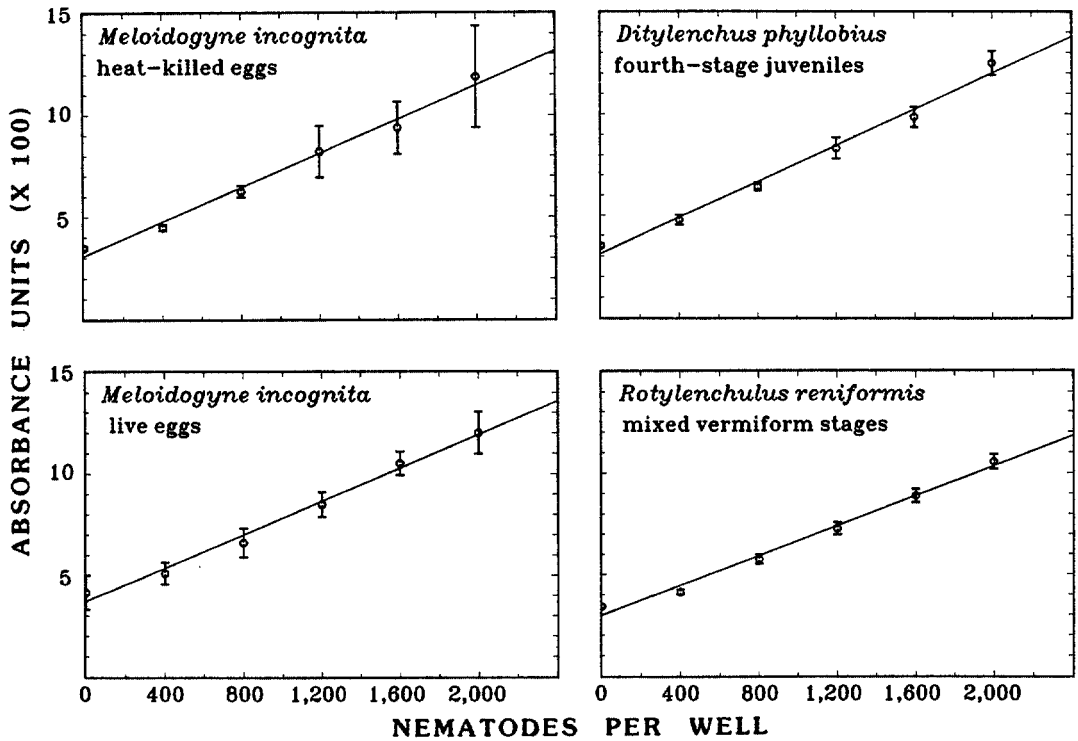

FIG. 1. Absorbance readings obtained at 550 nm with a 96-well microplate reader for aqueous suspensions of nematodes or nematode eggs. Suspensions were stirred just before measuring absorbance. Each datum is the mean of measurements from 10 replicate wells. Brackets indicate confidence intervals at $P = 0.05$. Regression lines were fit by least squares.

microscope, yet their total light absorbance was the same as that of live eggs. Viability did affect statistical variation. Absorbance readings for aliquots of heat-killed eggs were about twice as variable as those of live eggs. Juveniles of *D. phyllobius,* on the other hand, were highly motile and clumped into nematode wool faster than they could be read, necessitating heat-killing to reduce variability. Vermiform *R. reniformis* gave better data when alive, because when heat-killed they assumed a "C" shape and tended to hook together in clumps; when alive they stayed suspended separately after shaking.

Starved *D. phyllobius* juveniles that appeared almost transparent under the microscope produced absorbance readings about two-thirds those of unstarved juveniles at the same concentrations.

Discussion

Coefficients of variation that we typically measure for visual counts of nematodes in repeated aliquots from suspensions containing 50–1,000 nematodes/ml water are between 5% and 15%, depending on the species examined and the nematode concentration. This agrees with levels of variation reported by others and with the theoretical prediction that variance in nematode counts in repeated aliquots from a suspension should be linearly related to the mean count according to the Poisson distribution, with some deviation due to species and sampling technique (9). Therefore, variation in plate reader absorbances was a little higher than but certainly comparable to that obtainable from exact counts of nematodes within aliquots. Slightly higher variation obtained with a microplate reader would be easily compensated for by including more replications.

The absorbance values we obtained for 2,000 nematodes per well were less than 5% of the range of the instrument. Visual observations of nematodes within wells, similarly, indicated that only a small percentage of the total light transmission was blocked by the nematodes. Thus, it should be possible to estimate nematode densities reliably in much more concentrated suspensions than those that we examined. Of greater interest to us was the minimum number of nematodes that could be detected; this was on the order of 400 nematodes per well.

We conclude that use of a microplate reader would be an excellent substitute for direct microscopic observation and counting in many research applications, provided that certain precautions are taken. A series of standards should be counted visually for reference, only one species should be present, and nematodes in the suspension should not vary appreciably in size. The effect of size on estimates of nematode concentration by spectrophotometry has been examined for *Caenorhabditis elegans* (8), which increases in volume more than 10-fold during development. The age distribution within *C. elegans* suspensions markedly affected absorbance readings. The infective stages of many plant-parasitic nematodes, by comparison, are highly uniform in size; therefore, size variation may be unimportant.

The optical refractivity of vermiform plant-parasitic nematodes results largely from stored lipids, and lipid depletion could affect absorbance readings significantly. Starved juveniles of *D. phyllobius* produced absorbance readings two-thirds those of unstarved juveniles. Juveniles of *D. phyllobius* are exceptionally motile plant-parasitic nematodes and starve to death within 10 days at room temperature (11). Juveniles of root parasites typically starve much more slowly, and lipid depletion may be of relatively minor importance when using a microplate reader to measure their concentrations. The effect of starvation, however, should be examined for other species.

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