

Nematode specific gravity profiles and applications to flotation extraction and taxonomy

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Summary – A technique is described that refines the standard sugar flotation procedure used to isolate nematodes from their surroundings. By centrifuging nematodes in a number of increasing specific gravity solutions and plotting the fraction floating, the cumulative probability distribution of the population's specific gravity is generated. By assuming normality, the population mean, μ , and standard deviation, σ , are found by a nonlinear least squares procedure. These density parameters along with their error covariance matrix may be used as a taxonomic physical character. A chi-squared test is derived for comparing populations. Mean and standard deviation pairs (μ , σ) were found for the specific gravities of the adult stage of the plant parasites *Pratylenchus agilis* (1.068, 0.017), *P. scribneri* (1.073, 0.028), *P. penetrans* (1.058, 0.008) and the bacterial-feeder *Caenorhabditis elegans* (1.091, 0.016).

Résumé – Profils de densité spécifique chez les nématodes et applications à leur extraction par flottation et à leur taxinomie

– La technique exposée affine le procédé standard par flottation au sucre utilisée pour séparer les nématodes de leur milieu. La centrifugation des nématodes dans une série de solutions de densités spécifiques et la mise en diagramme de la valeur de la fraction surnageante permettent de connaître la répartition de la probabilité cumulée de la densité spécifique de la population en cause. La normalité étant supposée, la moyenne de la population, μ , et la déviation standard, σ , sont calculées par la méthode des moindres carrés non linéaires. Ces paramètres relatifs à la densité ainsi que leur matrice de co-variance d'erreur peuvent être utilisés en taxinomie comme caractère physique. Un test χ^2 en est dérivé pour comparer les populations entre elles. Des données en paires — moyenne (μ) et écart-type (σ) — ont été définies pour les densités des adultes des espèces phytoparasites *Pratylenchus agilis* (1,068; 0,017), *P. scribneri* (1,073; 0,028), *P. penetrans* (1,058; 0,008), ainsi que pour l'espèce bactérivore *Caenorhabditis elegans* (1,091; 0,016).

Keywords – *Caenorhabditis elegans*, chi-squared, density, distortion, osmotic, sugar flotation, *Pratylenchus*.

A standard 1 M sugar solution extracts all nematodes in an often dehydrated and distorted condition, reducing their shelf-life before identification and counting may be completed. This 'one-size-fits-all' solution has obscured real density differences in different groups of nematodes. This study outlines an accurate and reproducible approach to determine the density profile of any optimally cultured nematode group, including the mean and standard deviation of its population distribution.

There are seven chronological milestones in specific gravity¹ characterization of nematodes. Overgaard-Nielsen (1949) arrived at a value of 1.02 for *Mononchus papilatus* in a weight-volume calculation using a salt gradient.

Caveness and Jensen (1955) proposed 1 M sucrose solution (specific gravity 1.13) for flotation extraction and estimated nematode specific gravity to be between 1.05 and 1.06 (*Pratylenchus penetrans*, *Rhabditis* sp., *Diplogaster* sp. and *Longidorus sylphus*) based on flotation in different sugar solutions.

Andrássy (1956) estimated a number of unspecified free-living nematodes to have a specific gravity between 1.082 and 1.086 based on a flotation study using various salt and acid concentrations.

Thistlethwayte and Riedel (1969) quoted a range of 1.04 to 1.09 for nematode specific gravity from the literature, and found approximately half the number of *Ditylenchus dipsaci* floating in 1.05 as in 1.10 specific gravity sugar.

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¹ At 20°C, specific gravity and density in g/cc are numerically identical.

Zuckerman *et al.* (1972) measured relative specific gravity of young and aged adult *Caenorhabditis briggsae* by centrifuging microcapillaries with different specific gravity solutions of phthalates. Their data is examined later in this paper.

Vigliierchio and Yamashita (1983) compared five different flotation solutes with *Pratylenchus vulnus*, *Cricone-mella xenoplax*, *Xiphinema index* and *Meloidogyne incognita*. The range of specific gravity solutions, number of nematodes and mathematics were inadequate to characterize different nematode density profiles.

Vigliierchio and Yamashita (1983) presented graphically a number of cumulative population distribution curves for various nematodes floated in sucrose and non-sucrose solutions with the intent of identifying adequate sucrose replacements. No tables of actual data were presented, making examination by the methods of this present paper difficult.

Length, biochemical composition including percentage of water (Andrássy, 1956) and osmotic adaptability (Vigliierchio *et al.*, 1969) have been invoked to explain differences in nematode specific gravity independent of differences in solute density and osmotic characteristics (Vigliierchio & Yamashita, 1983). Reduced adaptability to osmotic stress (Bollinger & Willett, 1980) and lower levels of lipids may be important in the higher specific gravities reported in aged compared to young adult nematodes (Zuckerman *et al.*, 1972) and in plant parasitic trichodorids compared to tylenchids (Atkinson, 1985).

Osmotic stress is a known problem of sucrose solutions for flotation extraction. Osmotic pressure can produce noticeable distortion in nematodes by withdrawal of water within minutes of exposure to sufficiently dense sucrose solutions. Nevertheless, sugar is still a cost-effective method for routine extraction compared to non-osmotic non-water-miscible fluids such as phthalate esters (Danon & Marikovsky, 1964; Zuckerman *et al.*, 1972). Osmotic distortion can be reduced by determining the minimum density sucrose solution necessary to separate nematodes from soil, and in some cases to separate life stages of a single nematode from one another, or to separate mixed species. Results from the present study will be useful in the selection of appropriate densities.

In this paper a procedure is developed to quantify and compare specific gravity profiles within a single developmental stage for freshly extracted nematodes. The profiles are characterized by a population probability mean and standard deviation and their associated error covariance matrix. Knowledge of this profile will not only improve

centrifugation-separation procedures, but under standard age and nutritional conditions may serve taxonomically to uniquely characterize different plant-parasitic and free-living nematode species and developmental stages within a species.

Materials and methods

OVERVIEW

A cultured nematode stage is gently layered on top of a precisely prepared sucrose solution of specific gravity s_i and centrifuged. Let T_i (top), and B_i (bottom) represent the respective numbers of nematodes floating and sinking after centrifugation. Let $f_i = T_i / (T_i + B_i)$ be the fraction of nematodes floating. Upon reflection, it is evident that f_i records a point in the cumulative probability distribution for the population density. By calculating f_i using solutions of increasing specific gravity and graphing the pairs (s_i, f_i) , a cumulative probability distribution curve can be constructed for any nematode species or stage. Assuming normality, the pairs (s_i, f_i) can be fitted by a nonlinear least squares procedure to the cumulative normal probability distribution in which the nematode population mean μ , standard deviation σ , and their associated error covariance P , are extracted. Details of the estimation procedure and the importance of P are described in the appendix.

SELECTION OF SPECIES TO EXAMINE

The plant parasitic genus *Pratylenchus* was selected for study since all stages were available in culture; they are readily tissue-cultured and frozen, and intestinal reserves are not rapidly depleted after extraction from roots when kept in conditions of moderate oxygen at 15-20°C. The bacterial feeder *Caenorhabditis elegans* was chosen because of the ease of synchronizing and identifying life stages (Wood, 1988) as well as for comparison with *Caenorhabditis briggsae*, a close relative, for which partial specific gravity information exists (Zuckerman *et al.*, 1972). The adult stage was selected since it is the most taxonomically useful and is readily identified by a vulval slit under a dissecting microscope.

PREPARATION OF INOCULUM

For plant parasites, Gamborg's B5 tissue-cultured inoculum was prepared (Huettel & Rebois, 1985) with 2-day corn cv. Iowa Chief root explants grown at 28°C, and then Baermann funnel extracted in tap water (Southey,

1986). For the bacterial feeder *C. elegans*, age-synchronous cultures were prepared from bleached eggs and allowed to grow on *Escherichia coli* OP50-seeded NGM agar (Wood, 1988) at 20°C for 4 days for gravid adults.

Ideally, one would like to work with a single stage such as second stage juveniles or adults, but frequently several developmental stages will simultaneously be present in each centrifuge run. In this study, for ease of counting, a mixture of stages of a *Pratylenchus* species was enriched for adults by pipetting off smaller, more-slowly-sinking early stages from a just-filled culture dish under a dissecting microscope. It was verified that no adults had been removed. In the present study, no separation of stages was performed for *C. elegans* as the adult stage was easily distinguished at the time of counting. For maximal accuracy, as many nematodes as could reasonably be counted or staged was used. In this study the aim was for 100-400 individuals per ml of inoculum.

One drop of red dye was added per 20 ml of inoculum. The dye helps determine visually that the floating fraction of the solution has been completely removed, and stains any dead nematodes to be excluded from the count. Additionally, one drop of household concentrated liquid detergent, diluted to 10% in water, was added per 20 ml of inoculum. The detergent breaks any surface tension that may impede nematode motion between the inoculum and the specific gravity solution on which it is layered.

SELECTION OF CENTRIFUGATION TIME

Centrifugation hastens the time required for nematodes to float or sink and lessens their exposure to osmotic damage. Selecting the optimal length of centrifugation time involves a trade-off between the time required to produce a clean separation of floating vs non-floating nematodes and a sucrose-induced osmotic density increase over time. A third, more subtle, effect is that denser sucrose solutions are more viscous and under the same centrifugal force nematodes fall more slowly. As a compromise among the opposing effects, and after some experimentation, 4 to 6 min of centrifugation time at approximately 1000 g was selected.

PREPARATION OF FLOTATION SOLUTIONS

Commercial cane sugar (sucrose) was dried in an oven at 60°C for at least 6 h, and then placed in a desiccator if not used immediately. Sucrose and water weights to prepare the various solutions were taken from a standard table (Hodgman, 1963). The table entries were spaced in

units of Brix, the percent sucrose in the resulting solution. Although the exact corresponding solution specific gravity (S.G.) values were shown in the table, it was found more convenient to prepare and identify solutions and samples throughout their processing by their Brix number. These numbers varied from Brix = 10 corresponding to S.G. = 1.0400, to Brix = 32 corresponding to S.G. = 1.1386. The Brix numbers were converted to specific gravity during data analysis. Solutions were prepared a minimum of 6 h before using, and left to stand overnight at room temperature. It was observed that after the sucrose has apparently gone completely into solution, the solution density increased slightly over time. The dissolved sucrose appears in some way to equilibrate in solution. Routine verification of specific gravity was made using a refractometer, which measures the amount of refraction a given sucrose solution exerts on light. Refractometers provide indirect measurements which though not extremely accurate are very fast. Occasional very accurate direct measurements were made with a 50 ml pycnometer which cross-checked the refractometer values and confirmed the solutions were accurately prepared.

FLOTATION PROCEDURE

Prepared sucrose solutions of 11 ml were added to 15 ml centrifuge tubes. Six tubes of different specific gravity were processed at a time. One ml of well-mixed nematode inoculum was gently layered by pipette on top of each flotation solution. Tubes were spun in swinging buckets at 3000 RPM which in our centrifuge produced about 650 g at the tops of the centrifuge tubes and 1500 g at the bottoms. Using a plastic pipette with a relatively wide opening, 2 ml was gently removed from the tops of the tubes by sweeping slowly back and forth over the surface while taking up the pink floating layer. These nematodes were counted and recorded as "Top". Nematodes contained in the 10 ml of solution remaining were counted as "Bottom". Care was taken to dislodge and completely remove the bottom pellet which usually had a trace of dye but occasionally was clear.

Results

Flotation data for *Pratylenchus agilis*, *P. scribneri*, *P. penetrans*, and, *Caenorhabditis elegans* are recorded in Table 1, and the corresponding fits by least squares in Fig. 1.

Table 1. Top and bottom counts for four species of nematodes floated in various specific gravity solutions.

Brix	S.G.	<i>Pratylenchus agilis</i>		<i>Pratylenchus scribneri</i>		<i>Pratylenchus penetrans</i>		<i>Caenorhabditis elegans</i>	
		Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom
11	1.0441	6	151	13	69	10	295	0	120
12	1.0483	10	160	17	191	37	305	0	127
13	1.0525	44	163	38	113	81	261	0	135
14	1.0568	68	134	45	107	182	146	0	123
15	1.0610	66	90	76	130	196	96	0	148
16	1.0653	–	–	–	–	277	71	1	129
17	1.0697	105	91	77	70	263	50	14	81
18	1.0741	99	45	103	111	287	54	28	101
19	1.0785	173	51	177	136	278	25	–	–
20	1.0829	151	50	193	122	296	26	48	72
21	1.0874	166	35	202	81	300	17	59	68
22	1.0918	227	13	199	68	242	13	51	44
23	1.0963	220	12	208	64	252	10	90	40
24	1.1009	222	5	159	26	237	12	75	43
26	1.1102	238	2	–	–	–	–	106	20
28	1.1195	252	2	–	–	–	–	–	–

–, no observation.

Specific gravity values s_i and the corresponding $f_i = T_i / (T_i + B_i)$ are calculated and plotted as circled points. The cumulative normal curve (solid line) was fitted to the data using the technique outlined in the appendix. The error bars for the data were calculated according to Eq. 9 of the appendix with the standard deviation σ_d of a data point taken as $\sqrt{r_{Total}^2}$. The error bars extend $\pm 2\sigma_d$ from the corresponding data point and statistically should encompass the fit curve 95% of the time. The mean and standard deviation of the cumulative normal fit are given in the upper left corner of each figure. The associated error covariance matrices which are required to compare and combine profiles are as follows:

$$P_{agil.} = 10^{-6} \begin{bmatrix} 0.2955 & -0.0712 \\ -0.0712 & 0.3071 \end{bmatrix},$$

$$P_{scrib.} = 10^{-5} \begin{bmatrix} 0.0723 & -0.0494 \\ -0.0494 & 0.2589 \end{bmatrix},$$

$$P_{pene.} = 10^{-6} \begin{bmatrix} 0.1473 & -0.0461 \\ -0.0461 & 0.1896 \end{bmatrix},$$

$$P_{eleg.} = 10^{-6} \begin{bmatrix} 0.5173 & 0.1657 \\ 0.1657 & 0.5294 \end{bmatrix}.$$

The *P. penetrans* figure displays an interesting anomaly that leads to an investigation of error sources. At first

glance, it appears that a normal curve does not fit well. Careful consideration indicates potential problems at the high specific gravity data points. There are two known sources of error that have not been quantified in the appendix. The first is due to osmotic distortion causing nematode density to increase, and is evidenced by f_i not approaching unity. The second arises from incomplete pipetting of the top fraction after centrifugation and results in a double error: the count T_i of floating nematodes is diminished by some increment and the count B_i increased by the same increment. This type of error causes an occasional data point to fall significantly below the curve fit to all the data. The *P. penetrans* fit was recalculated with the suspect high specific gravity values removed. The resulting plot, shown in Fig. 2, is highly consistent with all remaining measured values. The fit values were $\mu = 1.0576$ and $\sigma = 0.0079$ with covariance error matrix

$$P_{pene.} = 10^{-4} \begin{bmatrix} 0.0753 & 0.0215 \\ 0.0215 & 0.1094 \end{bmatrix},$$

and are to be preferred over those of Fig. 1.

The results shown in Fig. 1 for *Pratylenchus agilis* are actually a replication of preliminary results three days earlier with lower nematode counts from the same inoculum. That experiment gave $\mu = 1.0675$, $\sigma = 0.0156$, and

$$P_{agil.} = 10^{-5} \begin{bmatrix} 0.1292 & -0.0530 \\ -0.0530 & 0.1582 \end{bmatrix}.$$

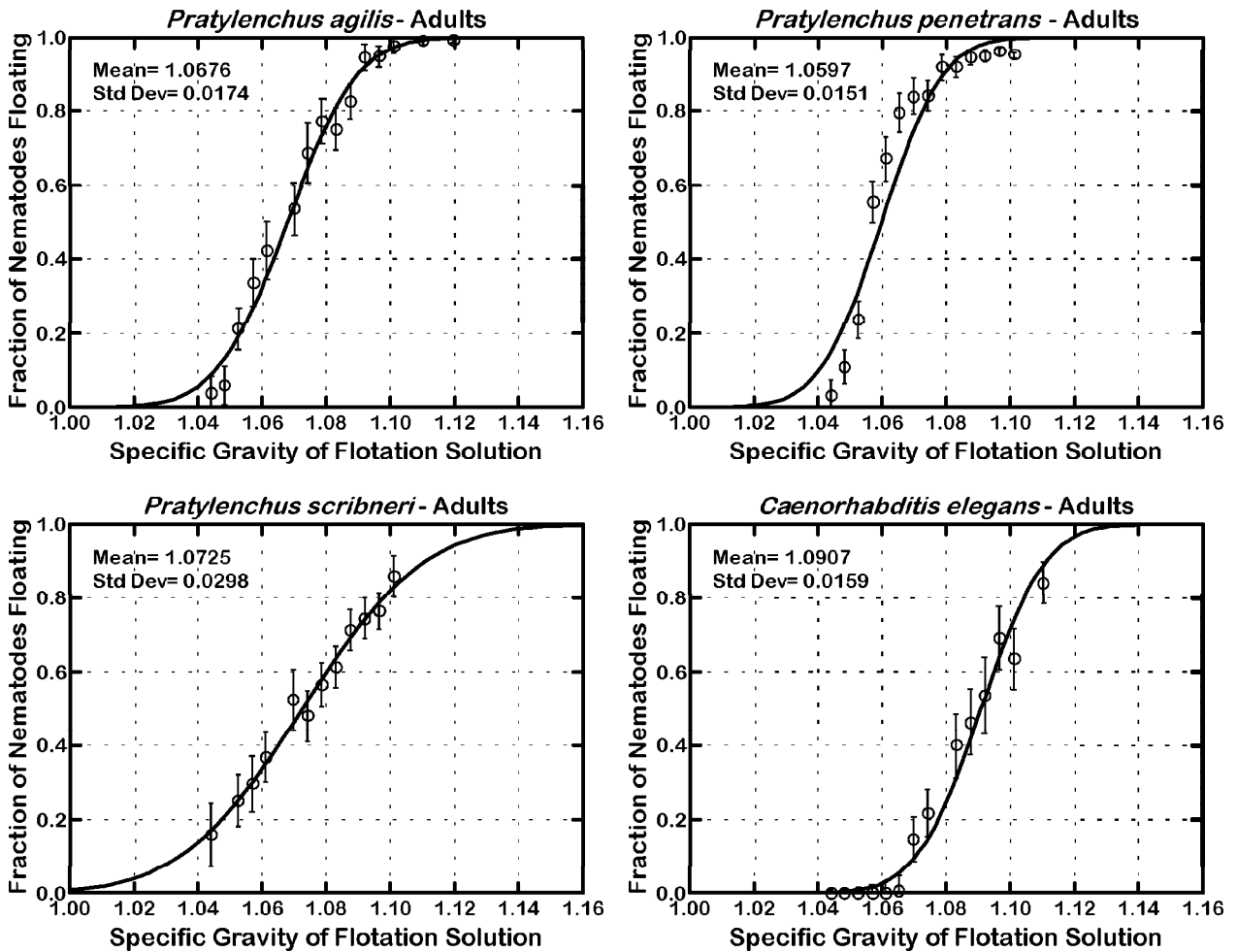


Fig. 1. Specific gravity profile of the adult stage of four nematodes. The mean and standard deviation of the best-fit cumulative normal distribution are indicated.

These results are consistent with those of Fig. 1, indicating the population density distribution is stable over a few days with the same inoculum (stored at 20°C). Consistency is demonstrated by using the statistical test Eq. 12 derived in the appendix for the hypothesis of no difference between profiles. A chi-square value of 2.05 for the difference in the density profiles was found. Using two degrees of freedom as indicated in the appendix, the commonly-used 5% rejection value for testing the hypothesis of equality is 5.99. Therefore, the hypothesis that the difference is zero is accepted.

Given that equality is accepted, the appendix result Eq. 14 was used to combine the two results into one. This gave the consolidated values, $\mu = 1.0675$, $\sigma = 0.0171$,

and

$$P_{agil.} = 10^{-6} \begin{bmatrix} 0.2395 & -0.0648 \\ -0.0648 & 0.2567 \end{bmatrix}.$$

Similarly, for *P. scribneri*, a replication with smaller numbers of nematodes was performed 7 months later than the experiment shown in Fig. 1. From this later experiment, the population parameters found were $\mu = 1.0725$, $\sigma = 0.0230$, and

$$P_{scrib.} = 10^{-5} \begin{bmatrix} 0.4118 & -0.2151 \\ -0.2151 & 0.8280 \end{bmatrix}.$$

The chi-square test was applied to compare the two *P. scribneri* and resulted in a value of 5.02, so again equality is accepted. The results were thus combined using ap-

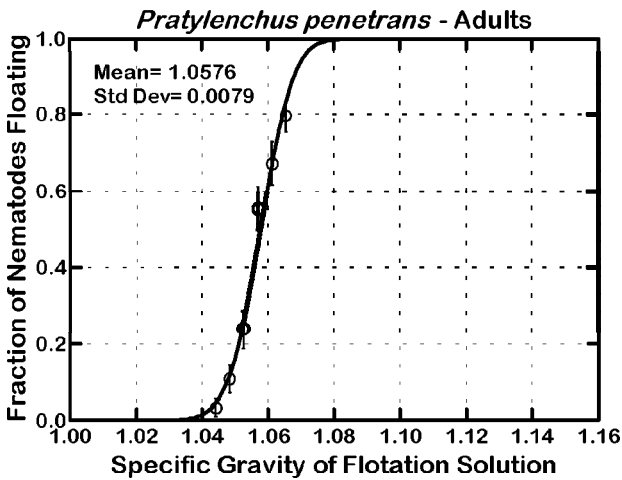


Fig. 2. *Pratylenchus penetrans* profile resulting from fit after deletion of points deemed erroneous at high specific gravities.

pendix Eq. 14 to give the consolidated values for *P. scribneri* of $\mu = 1.0726$, $\sigma = 0.0282$, and

$$P_{scrib.} = 10^{-5} \begin{bmatrix} 0.0614 & -0.0396 \\ -0.0396 & 0.1968 \end{bmatrix}.$$

By using data derived above and appendix Eq. 12, the three identically-cultured *Pratylenchus* species can be compared and the statistical uniqueness of their profiles demonstrated. For example, comparing *P. scribneri* and *P. penetrans* to one another gave a chi-squared value of 51 and to *P. agilis* values of 124 and 17 respectively. These are all to be compared as above to 5.99 as the 95% confidence rejection value. The hypothesis of equality of profiles is unequivocally rejected in all cases.

We attempted to apply this method to data from the literature. Only the study of Zuckerman *et al.* (1972) provided usable numbers. Because of the different objective of that paper, data were omitted that were of importance to the estimation procedure developed here. Nevertheless, it was of interest to attempt a fit to the limited data presented. Since only the percentages of nematodes falling to the centrifuge bottoms were given, it was necessary to reconstruct top and bottom counts for any of their specific gravity runs. The total number of nematodes counted was given, but the number of runs not clearly stated. It appeared that total counts varied between 15 and 30 per flotation run so that, for our analysis, counts in that range were created and apportioned between top and bottom to reproduce the percentages given in the paper.

The results of applying our analysis to this reconstructed data are seen in Fig. 3 and reveal some interesting features. The sparseness of the data and the wide error

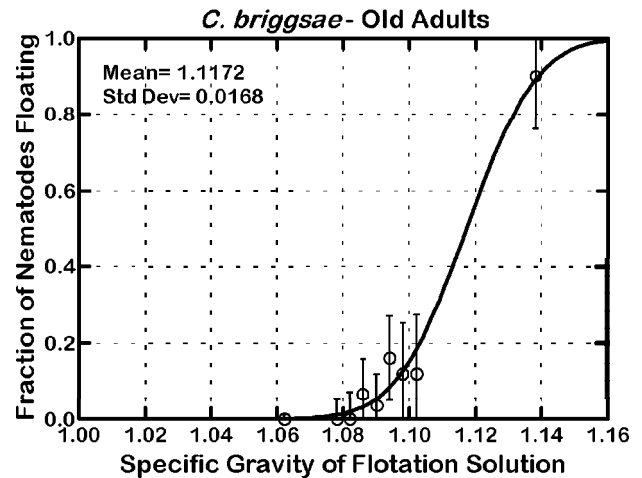
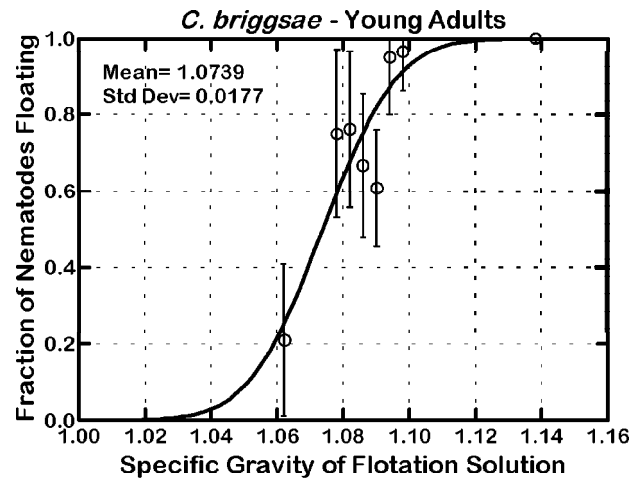


Fig. 3. Cumulative normal curve fit to *C. briggsae* flotation data from younger and older specimens.

bars due to the low count numbers are visible. Despite the wide error bars, at least one of the errors is larger than would be expected by chance, possibly due to poor suitability of phthalates as flotation media. Also interesting is the standard deviation. While the increase in the mean specific gravity over age was known and is evident, the standard deviation has remained approximately constant, an observation that may have taxonomic or developmental significance.

Discussion

APPLICATION TO TAXONOMY

The two replications described in the above section demonstrate the reproducibility needed if nematode den-

sity profile is to be useful as a taxonomic character. For one nematode, *P. agilis*, even though the same but slightly-aged inoculum had been used, the replicate produced statistically identical results. We note, however, that this time stability seen with adult *P. agilis*, a relatively long-lived parasite, should be considered fortuitous and might not be found with other plant parasites or with most bacterial feeders. The 7-month-separated, independently-produced inocula for the *P. scribneri* replications are of greater interest than the *P. agilis* replication done with identical-source inoculum. In producing statistically identical profiles, these replicates demonstrate that a consistent cultivation procedure, rigorously followed, will provide the reproducibility essential for taxonomic use.

Specific gravity taxonomic results can be compared and integrated with information derived from other sources. Recent evidence indicates that *Pratylenchus* can be divided into two separate molecular groups (Al-Banna *et al.*, 1997). One group, represented in this paper by *P. scribneri*, can be characterized by two lip annules, while a second which includes *P. penetrans* can be characterized by three. Although *P. agilis* was not included in the molecular analysis, it is closely related to *P. scribneri* morphologically (Handoo & Golden, 1989), and molecularly (unpublished observations). As seen in the figures, their specific gravity parameters are clearly distinct. In fact, all three *Pratylenchus* species could be distinguished (though not cleanly separated) based on their specific gravity profiles. More species will be needed to see whether a density pattern exists which reflects this recent molecular phylogeny of the genus and related taxa.

We do not speculate here on the origins or determinants of density differences. Our interest has been to outline the procedures, categorize the values, note that consistent differences do exist, and show how profiles can be compared and combined. With a reproducible method, hypotheses of density differences can be tested and variations within and between taxa examined. In general, the specific gravity means of *C. elegans* adults and of other tested rhabditid and cephalob bacterial feeders (unpublished results) are higher than those of any of the *Pratylenchus* adults. It is interesting to taxonomists that as a group the bacterial feeders may be denser than the plant feeders. Ultimately, this knowledge may help identify determinants of density.

APPLICATION TO FLOTATION SEPARATION

For a single nematode, if the distribution is known, a cleaner separation from soil can be made by choosing a flotation solution specific gravity for which 95% of the

population will float. This approach will also minimize osmotic damage. If the distributions of two nematode stages are sufficiently different, even under less-than-optimum field conditions they can be cleanly isolated by selection of an appropriate specific gravity flotation solution.

A potential application of the method of this paper is the production of tables for the specific gravity profiles (means, standard deviations, and error covariance matrices) for various nematodes in their several stages of development. These might be used in a number of interesting ways for taxonomic, developmental, or experimental greenhouse studies. They will be useful for sieved samples of a known nematode requiring further cleaning with the minimum amount of sugar to reduce distortion, or for separating mixed nematode samples to facilitate enumeration by automatic imaging systems. Since nematode eggs may similarly be categorized by density profile, it may be possible to separate eggs of different coexisting parasitic and bacterial-feeding nematodes that cannot easily be distinguished under a dissecting microscope.

ERROR SOURCES

Two sources of error were mentioned and discussed above: a) osmotic damage, and b) incomplete recovery of floating nematodes. The first is minimized by exposing the nematodes to sucrose for the minimum time required for separation. An attempt was made to model the second error as a fixed percentage of top counts that was estimated along with the parameters μ and σ . The attempt failed as no better value than 100% could be found.

Two sources of error are described in the appendix: c) counting error, and d) partitioning error. Both of these sources are well-understood and fully accountable by statistical modeling.

There are other sources of error, none of which are amenable to modeling. Examples are: e) incomplete mixing of inoculum before withdrawal for layering, f) using a method of collection or preparation that affects nematode density distribution, and g) misidentification of stage or species during counting.

For the estimation procedure described herein to be effective and the results valid, the presence or level of unmodeled errors must be minimized. A satisfactory estimation is evidenced by the two-sigma error bars of the data points encompassing the estimated curve 95% of the time.

We have seen that for a nematode stage the population mean, standard deviation, and error covariance can be found using a common flotation extraction procedure and

an appropriate mathematical fit to the distribution profile. These parameters provide not only a means of performing extractions more efficiently, but also of separating nematodes and nematode stages, and categorizing nematode populations for taxonomic purposes.

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Appendix. Mathematical analysis of data

LEAST SQUARES FIT TO CUMULATIVE NORMAL PROBABILITY DISTRIBUTION

It is assumed that a given population, say the adult stage of *P. penetrans*, follows the bell-shaped normal probability distribution

$$y(s) = \frac{1}{\sigma\sqrt{2\pi}} \exp \left[-\frac{1}{2} \left(\frac{s - \mu}{\sigma} \right)^2 \right] \quad (1)$$

for its specific gravity. In the above equation s represents specific gravity, μ the population mean specific gravity, and σ its standard deviation. We observe that a single specific gravity s_1

divides the area under the curve into two portions: a fraction f_1 less dense than s_1 and a fraction $(1 - f_1)$ more dense.

The area f_1 under the curve up to s_1 can be expressed as

$$f_1 = \int_{-\infty}^{s_1} y(s) ds = \frac{1}{\sigma\sqrt{2\pi}} \int_{-\infty}^{s_1} \exp\left[-\frac{1}{2}\left(\frac{s-\mu}{\sigma}\right)^2\right] ds. \quad (2)$$

In terms of a flotation process in which nematodes are layered on top of a specific gravity solution of density s_1 and centrifuged, a quantity T_1 will float and B_1 will sink. That is, $f_1 = T_1/(T_1 + B_1)$.

In terms of the cumulative unit normal probability distribution function,

$$\Phi(x) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^x \exp\left(-\frac{1}{2}t^2\right) dt, \quad (3)$$

and by the change of variable $x = (s - \mu)/\sigma$ we have $f_1 = \Phi[(s_1 - \mu)/\sigma]$. In general then, for a flotation solution of specific gravity s_i a floating fraction

$$f_i = \Phi[(s_i - \mu)/\sigma] \quad (4)$$

is expected. This is the basis for the estimation procedure. At various specific gravities s_i counts T_i and B_i are measured and $f_i = T_i/(T_i + B_i)$ calculated. A least squares estimate is performed by choosing μ and σ to minimize the sum of squares differences between the observed and expected floating fractions. Specifically, the loss function

$$L(\mu, \sigma) = \sum_{i=1}^n r_i^{-2} \left[\frac{T_i}{(T_i + B_i)} - \Phi\left(\frac{s_i - \mu}{\sigma}\right) \right]^2 \quad (5)$$

is minimized with respect to μ and σ .

The weight factor, the inverse variance r_i^{-2} , has been introduced in anticipation of the following discussion: for ordinary least squares, all measurements are assumed of equal value and the minimization is performed with $r = 1$. For a more precise estimate, weights are chosen according to the variance of expected errors in the observations f_i , i.e. $r_i^2 = E[(f_i - f_{i(true)})^2]$. The form and magnitude of these errors will be examined.

DEVELOPMENT OF DATA ERROR SOURCES AND MAGNITUDES

There are two sources of error in f_i that can reasonably be modeled mathematically. The first source is errors in the counting of its components T_i and B_i . The second is what we call partitioning error: a selection of N nematodes is randomly taken from a population with mean μ and standard deviation σ and divided by a specific gravity s into top and bottom numbers T and B . Assuming normality, for large N , T and B approach their expected population values but in general are never exact and for small N can differ considerably. This difference will be quantified statistically.

First consider the counting error: suppose that the observed top count T_i equals the true count $T + e$ where e is the error in T . Similarly, $B_i = B + d$. Then the error in f_i will be

$$\begin{aligned} Error &= \frac{T_i}{T_i + B_i} - \frac{T}{T + B} \\ &= \frac{T + e}{T + e + B + d} - \frac{T}{T + B} \\ &= \frac{T(1 + \frac{e}{T})}{N(1 + \frac{e+d}{N})} - \frac{T}{N} \end{aligned}$$

where $N = T + B$. Then to first order in e and d :

$$\begin{aligned} Error &\approx \frac{T}{N} \left(1 + \frac{e}{T} - \frac{e+d}{N} \right) - \frac{T}{N} \\ &= \frac{Be - Td}{N^2}. \end{aligned}$$

So that

$$r_{counting}^2 = E[Error^2] = \frac{B^2\epsilon^2 + T^2\delta^2}{N^4}, \quad (6)$$

where ϵ^2 and δ^2 are the mean squared errors in the top and bottom counts respectively. These numbers can be determined empirically by a small number of recounts on samples of various sizes and will in some way be related to the total counts T or B . If it is assumed that ϵ and δ are some fixed fraction of the number of counts, say $\epsilon = \alpha T$ and $\delta = \alpha B$, then

$$r_{counting}^2 = \frac{2\alpha^2 B^2 T^2}{N^4}, \quad (7)$$

and in terms of f

$$r_{counting}^2 = 2\alpha^2 f^2 (1 - f)^2. \quad (8)$$

This form of the data error counting variance was used for this study with $\alpha = 0.02$. Note also the following limiting cases:

$$\begin{aligned} r &\simeq \sqrt{2}\alpha \frac{B}{T} \quad \text{for } B \text{ small compared to } T, \\ r &\simeq \sqrt{2}\alpha \frac{T}{B} \quad \text{for } T \text{ small compared to } B. \end{aligned}$$

Thus, the counting error contribution to the variance approaches zero as either the top or bottom count approaches zero.

Consider now the partitioning error in f_i . This was determined empirically by generating a two-dimensional table, $r_{partitioning}^2 = r_p^2(i, j)$, where the i, j element is generated by the procedure below using N_i , the number of nematodes, and u_j , the deviation from zero in a unit normal standard distribution. We note that f and u are uniquely related one to one.

For any given combination of values (N_i, u_j) , a large number of values f_i were randomly generated by choosing exactly N_i samples up to 100000 times from the unit normal distribution. For each sample, the numbers T , (counts less than u_j), B , (counts greater than u_j) and resulting f were calculated. Over

the ensemble of f values generated for each pair (N_i, u_j) , a mean μ_f and variance σ_f^2 were calculated. The value for r_p^2 at the table point (N_i, u_j) was set to σ_f^2 . In this manner, the entire matrix $r_p^2(N_i, u_j)$ was generated. No further use was made of μ_f other than to verify that it was in fact equal to f_i which can be independently and exactly calculated from the cumulative unit normal distribution. The spacing between adjacent N_i values was five for N_i under 50 and 50 for the balance up to 2000 counts. The spacing for the u_j was 0.25 over the range $(-6.0, 6.0)$.

Subsequently, when a partitioning variance $r_p^2(N_q, u_q)$ was required for any particular combination of N_q and $u_q = (s_q - \mu)/\sigma$, the appropriate value was found by two-dimensional interpolation into the table $r_p^2(N_i, u_j)$.

The total variance resulting from the counting and partitioning sources is simply the sum, *i.e.*

$$r_{i(\text{total})}^2 = r_{i(\text{counting})}^2 + r_{i(\text{partitioning})}^2. \quad (9)$$

The least squares estimation loss function Eq. 5 can be used with any least squares solver package or custom programmed. The custom route was chosen using an extended Kalman filter iteratively since the variance weight functions r_i^2 arise naturally in the Kalman approach and the parameter error covariance matrix is directly available for later use in comparing and combining estimates. All procedures were written and graphs created using the MATLAB programming language.

COMPARISON OF DISTRIBUTION PROFILES

The covariance matrix for the error of estimated parameters that results from the Kalman procedure can be used to make comparisons between different nematodes or populations. Suppose that for one population we obtain in vector-matrix format the estimated parameter:

$$\mathbf{x} = \begin{bmatrix} \mu_x \\ \sigma_x \end{bmatrix}, \text{ and its } 2 \times 2 \text{ error covariance matrix } P_x.$$

Let the corresponding values for a different nematode or population be called \mathbf{y} and P_y . Suppose one wishes to know if \mathbf{x} and \mathbf{y} are statistically equal. In Zelen, 1962, (credited to Lancaster, 1954) the following theorem is presented: (In the presentation following, the superscript T represents the matrix transpose.)

Theorem 1 Let $\mathbf{w}^T = (w_1, w_2, \dots, w_n)$ follow a normal distribution with $E(\mathbf{w}) = \boldsymbol{\mu}$ and $\text{var}(\text{covariance}) \mathbf{w} = D(\boldsymbol{\sigma}^2) = D(\sigma_1^2, \sigma_2^2, \dots, \sigma_n^2)$, a diagonal matrix. Then, the quantity

$$\chi^2 = (\mathbf{w} - \boldsymbol{\mu})^T D^{-1}(\boldsymbol{\sigma}^2) (\mathbf{w} - \boldsymbol{\mu}) \quad (10)$$

$$= \sum_{i=1}^n \frac{(w_i - \mu_i)^2}{\sigma_i^2}$$

follows the chi-square (χ^2) distribution with n degrees of freedom.

Consider now $\mathbf{z} = \mathbf{x} - \mathbf{y}$. Following standard rules, \mathbf{z} will have covariance $Q = P_x + P_y$ about its mean. Premultiply \mathbf{z} by U , an orthogonal matrix, *i.e.* $U^{-1} = U^T$. Then $\mathbf{w} = U\mathbf{z}$ has error covariance UQU^T . Because Q is a covariance matrix and thus symmetric, a U can always be found such that $UQU^T = D$, a diagonal matrix (Bellman, 1970). The diagonal elements of D are positive by virtue of Q being a proper covariance matrix (no combination of elements 100% correlated). Now apply Eq. 10 with $\mathbf{w} = U\mathbf{z}$, $D = UQU^T$, and $\boldsymbol{\mu} = \mathbf{0}$ (testing for equality between \mathbf{x} and \mathbf{y}). Note that by virtue of U being orthogonal, $D^{-1} = UQ^{-1}U^T$, so that

$$\begin{aligned} \chi^2 &= \mathbf{w}^T D^{-1} \mathbf{w} \\ &= (U\mathbf{z})^T (UQ^{-1}U^T) (U\mathbf{z}) \end{aligned}$$

which simplifies to

$$\chi^2 = \mathbf{z}^T Q^{-1} \mathbf{z}. \quad (11)$$

Note that the orthogonal matrix U need never be explicitly derived.

In terms of original variables then, under the hypothesis that the difference between \mathbf{x} and \mathbf{y} is zero, the quantity

$$\chi^2 = (\mathbf{x} - \mathbf{y})^T (P_x + P_y)^{-1} (\mathbf{x} - \mathbf{y}) \quad (12)$$

is distributed as chi-square with two degrees of freedom. Eq. 12 can be used with standard chi-square tables to test the hypothesis that the profiles \mathbf{x} and \mathbf{y} are equal.

Suppose that the chi-square test for equality of \mathbf{x} and \mathbf{y} is passed. Then the data sets can be combined to produce a composite profile \mathbf{a} . Analogously to Eq. 5, a value for \mathbf{a} and its error covariance can be found by minimizing the loss function

$$L(\mathbf{a}) = (\mathbf{x} - \mathbf{a})^T P_x^{-1} (\mathbf{x} - \mathbf{a}) + (\mathbf{y} - \mathbf{a})^T P_y^{-1} (\mathbf{y} - \mathbf{a}) \quad (13)$$

with respect to \mathbf{a} . The result is

$$\mathbf{a} = (P_x^{-1} + P_y^{-1})^{-1} (P_x^{-1} \mathbf{x} + P_y^{-1} \mathbf{y}), \quad (14)$$

and with some manipulation one can obtain its corresponding error covariance matrix:

$$P_a = (P_x^{-1} + P_y^{-1})^{-1}. \quad (15)$$

The above equations are readily extended for consolidating multiple estimates.