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AGE RELATED CHANGES IN THE CHEMICAL OXYGEN DEMAND OF SECOND STAGE JUVENILES OF *MELOIDOGYNE JAVANICA* AND *HETERODERA ORYZAE*

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The chemical oxygen demand (COD) of juveniles of increasing ages of *Meloidogyne javanica* and *Heterodera oryzae* was measured by oxidation with a hot solution of dichromate in sulphuric acid. In freshly hatched juveniles the COD equalled 35.3 nl O_2/juvenile for *M. javanica* and 33.8 nl O_2/juvenile for *H. oryzae*. The COD decreased more rapidly with age in *M. javanica* (-72.5% after 4 weeks) than in *H. oryzae* (-45% after 5 weeks). Results are compared with data from respirometry and direct chemical determinations.

Metabolism of starving soil nematodes can be determined by respirometry: volumetric (Nielsen, 1949; Rohde, 1960; Sembdner *et al.*, 1961; Wallace & Greet, 1964; Van Gundy *et al.*, 1967; Bhatt & Rohde, 1970; Reversat, 1980a, 1981a), polarographic (Atkinson, 1973) and chemical (Bair, 1955). Other methods include time related estimations of dry weight (Van Gundy *et al.*, 1967; Reversat, 1980a, 1981b), chemicals (Chitwood, 1951; Van Gundy *et al.*, 1967; Cooper & Van Gundy, 1970; Ogunfowora, 1979; Reversat, 1980a, 1981a & b) and body content (Van Gundy *et al.*, 1967; Reversat *et al.*, 1980). Recently, Nicholas & Stewart (1978) suggested estimation of the calorific value of nematodes. Another possible method is the estimation of the chemical oxygen demand (COD), that is the oxygen required for the complete oxidation by means of an oxidizing chemical of the organic matter in the nematode. This can be achieved simply and inexpensively by wet oxidation with a hot solution of dichromate in sulphuric acid. This method oxidizes not only carbon (Allison, 1965) but also other elements contained in organic matter (Ivlev, 1934; Dobbs & Williams, 1963). This paper describes the method, its efficiency for various organic compounds and its application to the study of starved juveniles of *Meloidogyne javanica* Treub and *Heterodera oryzae* Luc & Berdon. Results are compared with those from respirometry and direct chemical determinations.

MATERIALS AND METHODS

General

Samples of juveniles of *M. javanica* and *H. oryzae* were from those prepared for chemical determinations in previous work (Reversat, 1980a, 1981b). Freshly hatched juveniles were stored in 4 mM pH 7 sodium phosphate buffer

at 28°C in the dark and every week, motile juveniles were distributed in a series of centrifuge tubes (Reversat, 1976, 1980b). Tubes of the same series contained similar numbers of juveniles (± 30,000), estimated by counting aliquots from three tubes. Chemical determinations were made with juveniles from the remaining tubes (Reversat, 1980a, 1981b). Three tubes of each species and each age were stored at -25°C for 2 days, their contents lyophilized and the tubes re-stored at the same temperature.

To determine the relationship between the COD and the number of juveniles/sample, six series of three samples were prepared containing increasing numbers of freshly hatched juveniles of *H. oryzae* from 4,900/sample for the first series to 27,200/sample for the last. The rate of oxygen uptake by juveniles of *M. javanica* at increasing ages was also determined, using glass stoppered cartesian divers (Reversat, 1975, 1977).

COD determination

The method was adapted from Ryding & Forsberg (1977).

Reagents

(a) Potassium dichromate, $K_2Cr_2O_7$, 0.5 N: $K_2Cr_2O_7$ (p.a.) was dried at 105° for 2 h.; 24.52 g was dissolved in de-ionized water to 1,000 ml. (b) Concentrated sulphuric acid, H_2SO_4 (p.a., 95-97%) containing 13.3 g of silver sulphate (Ag_2SO_4, purum) per l. (c) Ferrous ammonium sulphate, $Fe(NH_4)_2(SO_4)_2$, 0.06 N: 23.53 g of $Fe(NH_4)_2(SO_4)_2$, $6H_2O$ was dissolved in about 500 ml of de-ionized water, 50 ml of concentrated sulphuric acid was added and the solution made up to 1,000 ml with de-ionized water. (d) Potassium permanganate, $KMnO_4$, 0.01 N: for 0.25 N stock solution, 7.902 g of $KMnO_4$ (p.a.) was dissolved in de-ionized water to 1,000 ml; a titration solution (0.01 N) of $KMnO_4$ was prepared from the stock solution just before use and standardized as follows: m mg of $Fe(NH_4)_2(SO_4)_2$. $6H_2O$ (in the range of 180 mg) was placed in a beaker with 100 ml of de-ionized water and 2 ml of concentrated sulphuric acid and titrated with 0.01 N $KMnO_4$ until the colour changed from blue-green to light pink (Vt ml of solution). The actual strength of the solution was calculated as $t = m/392\ V_t$.

Procedure

(a) To a tube containing lyophilized juveniles were added 1 ml of 0.5 N $K_2Cr_2O_7$ and 3 ml of concentrated H_2SO_4 with Ag_2SO_4 and the content mixed vigorously. (b) A blank tube was prepared in the same manner. (c) Tubes were closed with a glass marble and heated for 2 h. in a boiling water bath and juveniles were completely dissolved. (d) Tube contents were poured into 500 ml beakers rinsing with 100 ml of de-ionized water and stirred magnetically. (e) 10 ml of 0.06 N $Fe(NH_4)_2(SO_4)_2$ was added to the beakers: the colour of the contents changed from orange to bluegreen. (f) The solutions were titrated with 0.01 N M $KMnO_4$ until the colour changed to light pink.

Calculation

The COD, expressed in nanoliter of oxygen per juvenile (nl/juv.) was calculated as: $COD = (V_s - V_b) \times t \times 56.035 \times 10^5 \times N^{-1}$ where V_s is the volume (ml) of 0.01 N $KMnO_4$ used for sample, V_b is the volume (ml) of 0.01 N $KMnO_4$ used for blank, t is the actual normality of 0.01 N $KMnO_4$ and N is the number of juveniles in the sample (The constant 56.035×10^5 is the volume of oxygen, in nl, theoretically liberated when 1 ml of 1 N $KMnO_4$ is decomposed by sulphuric acid).

RESULTS

Rate of oxygen uptake

The initial rate of oxygen uptake by juveniles of *M. javanica* was 120 pl/juv./h.. Afterwards the rate decreased to 12 pl/juv./h at the end of the 3rd week of storage (Fig. 1). In the 4th week, juveniles could not be introduced into the divers by settlement (Reversat, 1975) and no measurement were made.

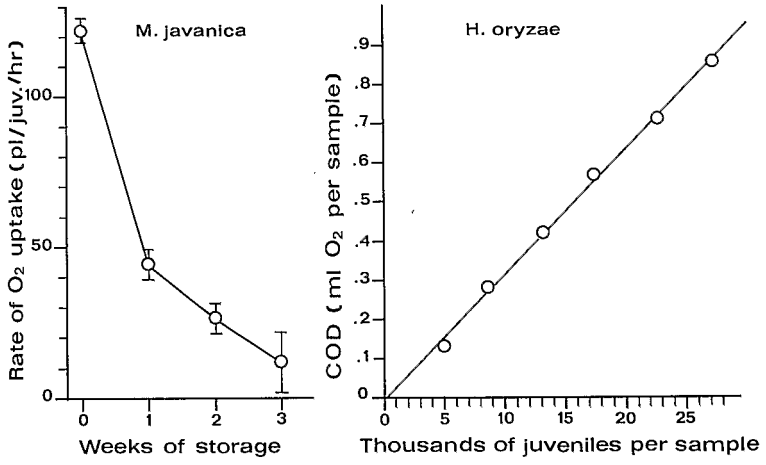

Fig. 1. The effect of storage time on the rate of oxygen uptake by juveniles of *Meloidogyne javanica*. Each point is the mean of eight replicates and the vertical line equals the confidence interval at 95%.

Fig. 2. Relationship between the chemical oxygen demand (ml O_2/sample) and the number of freshly hatched juveniles of *Heterodera oryzae* per sample. Each point is the mean of three replicates and the standard deviation equals less than 3% of the mean.

COD of organic compounds

Solutions of glucose (2 mg/ml), sodium lauryl sulphate (1.4 mg/ml) and pentanol (1 mg/ml) were prepared and distributed in test tubes (0.5 ml/tube). To each tube was added 0.5 ml of 1 N $K_2Cr_2O_7$ and the procedure described above was followed. Results are given in Table I.

TABLE I

The chemical oxygen demand of some organic compounds expressed as ml O_2/mg of substance

Substance	Formula	† Measured COD	Calculated COD C only	Calculated COD C, H & O
Glucose	$C_6H_{12}O_6$	0.75	0.747	0.747
Sodium lauryl sulphate	$C_{12}H_{25}$-SO_4Na	1.41	0.933	1.420
Pentanol	$C_5H_{10}O$	1.88	1.273	1.906

† Means of three replicates (S.D. < 1% mean) N.B.: Values in the last two columns calculated from: $C_nH_pO_q + (n + p/4 - q/2)\ O_2 \rightarrow n\ CO_2 + p/2\ H_2O$. When considering C only, p = q = O. $-SO_4Na$ assumed to be unaffected.

Relationship between COD and the number of juveniles

Fig. 2 gives the relationship between COD and the number of juveniles/sample. The correlation coefficient (r) was 0.99 and the regression coefficient (b) 32.1 nl/juv..

COD changes in starved juveniles

Initial values of COD were 35.3 nl/juv. for *M. javanica* and 33.8 nl/juv. for *H. oryzae*. Afterwards COD decreased steadily (Fig. 3) and this was more pronounced in *M. javanica* (-72.5% after 4 wks) than in *H. oryzae* (-45% after 5 wks).

DISCUSSION

Oxidation of the organic matter

Table I shows that, under the conditions described, the organic matter underwent a stoechiometric oxidation. Aliphatic chains of pentanol and sodium lauryl sulphate are models for fatty acids in lipids which account for most of the reserves consumed by these nematodes and were probably the most difficult substances to oxidize. The great efficiency of this procedure is due to: (a) The presence of Ag_2SO_4 as a catalyst (Dobbs & Williams, 1963), (b) The excess (3/1) of concentrated H_2SO_4 to aqueous solution, (c) The application of external heat (100°C) for 2 h., (d) The small COD of the sample (maximum 1 ml O_2) related to COD capacity of 1 ml of 0.5 N $K_2Cr_2O_7$ (2.8 ml O_2).

The measured COD of a sample was strictly related to the number of juveniles in the sample (Fig. 2) and results were reproducible since the standard deviation of three replicates was less than 3% of the mean. Moreover, provided 0.5 N $K_2Cr_2O_7$ and 0.06 N $Fe(NH_4)_2(SO_4)_2$ were added in equal

quantities to each tube, only the 0.01 N $KMnO_4$ solution for titration had to be standardized (See the constant for calculation of COD). Ionized halogens (Cl^-, Br^-) must be eliminated since they exhibit a certain chemical oxygen demand (Dobbs & Williams, 1963). The small amount of sodium phosphate buffer (20 µl of a 4 mM solution) lyophilized with juveniles did not affect the determination of COD.

Age related changes of COD

COD of freshly hatched juveniles of *H. oryzae* varied (32.1 nl/juv. in Fig. 2 versus 33.8 nl/juv. in Fig. 3), depending on the time of the year they were recovered from the rearing. This has been already observed for the dry weight (Reversat, 1975, 1976, 1980a). Probably the quantities of chemicals stored during oogenesis and remaining after embryogenesis of juveniles are dependent upon some environmental factors which are poorly controled during the rearing.

The observed COD corresponding to juveniles of different ages (Fig. 3) can be compared with theoretical COD values calculated from (a) The chemical composition of juveniles of increasing age (Reversat, 1980a, 1981b) and (b) the specific coefficients for oxidation of lipids, carbohydrates and proteins (Table II). Fig. 3 gives theoretical COD values for nematode lipids, carbohydrates and proteins and their sum for each age. The theoretical COD values were close to those measured (Fig. 3).

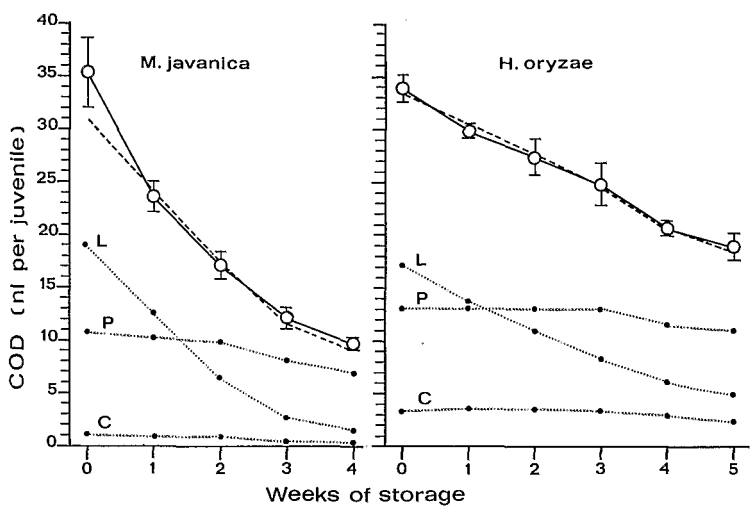

Fig. 3. The effect of storage time on the chemical oxygen demand of juveniles of *Meloidogyne javanica* and *Heterodera oryzae*. o——o measured COD, each point is the mean of three replicates and the vertical line equals the confidence at 95%; ●·····● calculated COD for lipids L, carbohydrates C, and proteins P; ---- sum of calculated COD for lipids, carbohydrates and proteins.

The decrease in measured COD during the first 3 wks of storage of *M. javanica* juveniles, 23.2 nl, agreed well with the volume of oxygen consumed by the juveniles during the same period, 23.1 nl (Calculated by integrating the respiration curve; Fig. 1). Thus, COD changes with age represent a measurement of respiration provided catabolism of reserves leads mainly to CO_2 and H_2O. This seems to contradict the results of Wang & Bergeson (1978), who found an appreciable excretion of amino-acids and carbohydrates in *M. javanica* juveniles. Some additional data on CO_2 output of these juveniles are needed.

TABLE II

Coefficients of chemical determinations for calculation of chemical oxygen demand and calorific value. Data from Polonowski et al. *(1973); in parentheses are relative values of these coefficients assuming that for carbohydrates = 1.*

	Lipids	Proteins	Carbohydrates
Chemical oxygen demand ml O_2/g	2,019 (2.44)	966 (1.16)	829 (1.00)
Calorific value K cal./g	9.46 (2.26)	4.44 (1.06)	4.18 (1.00)

Comparison of methods for metabolism determinations in starved nematodes

Determination of the calorific value of nematodes with the microbomb calorimeter requires samples of at the least 10 mg of dry nematodes (Nicholas & Stewart, 1978), equivalent to half a million *M. javanica* juveniles. Such numbers of plant parasitic nematodes are difficult to obtain when several

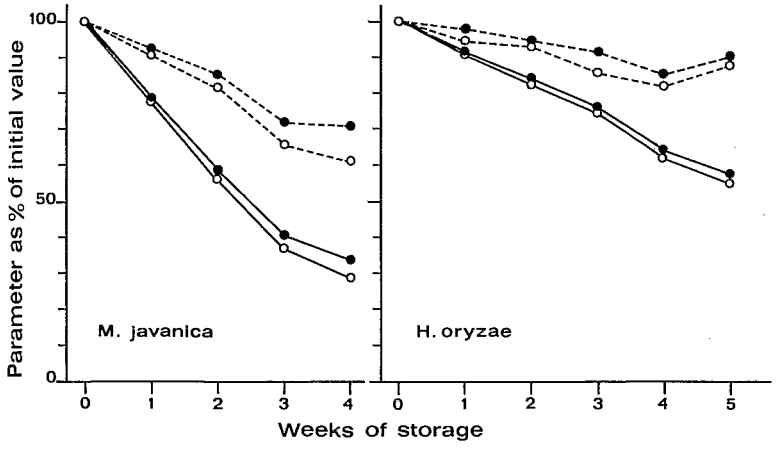

Fig. 4. The effect of storage time on changes in chemical oxygen demand (○) and calorific value (●) expressed on individual basis (———) and on dry weight basis (----) in juveniles of *Meloidogyne javanica* and *Heterodera oryzae*. COD and calorific values calculated from chemical determinations.

replications are needed during age-related studies over several weeks. COD determination requires only 30,000 juveniles/sample (0.7 mg dry weight).

In addition, the relative rates of decrease of the calorific value and the chemical oxygen demand can be calculated for juveniles of *M. javanica* and *H. oryzae* from dry weight and chemical determinations (Reversat, 1980a, 1981b) and specific coefficients (Table II). Results are expressed on a dry weight and an individual basis (Fig. 4).

The rate of decrease is faster for the COD than for the calorific value, mainly because lipids are the principal food reserve and these have a greater COD coefficient than a calorific value coefficient (2.44 versus 2.26, Table II).

The rate of decrease is also faster on an individual rather than on a dry weight basis, because the dry weight decreases with age. However, results can be expressed on an individual basis only when all individuals are of the same stage and age, as in this work on 2-nd stage juveniles of Heteroderidae. When animals are obtained by mass rearing as are microbivorous and mycophagous nematodes (Cooper & Van Gundy, 1970; Nicholas & Stewart, 1978), developmental stages are mixed and results must be expressed on a dry weight basis.

RÉSUMÉ

Variations, en fonction de l'âge, de la demande chimique d'oxygène chez les juvéniles du second stade de Meloidogyne javanica *et d'*Heterodera oryzae.

La demande chimique d'oxygène (DCO) de juvéniles d'âges croissants de *Meloidogyne javanica* et d'*Heterodera oryzae* est mesurée par oxydation à chaud par le sulfochromique. Chez les animaux fraîchement éclos, la DCO est de 35,3 nl par juvénile de *M. javanica* et de 33,8 nl par juvénile d'*H. oryzae*. Par la suite, la DCO décroit plus rapidement chez *M. javanica* (-72,5% après 4 semaines) que chez *H. oryzae* (-45% après 5 semaines). Les résultats sont comparés avec les chiffres obtenus par respirométrie et par dosages chimiques.

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