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Comparison of demographic (life- and fecundity table analysis) and biochemical (ATP and AEC) characteristics as sublethal pollution indices in the marine nematode *Monhystera disjuncta*

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Abstract

Sublethal effects of cadmium and nickel on demographic characteristics and adenylate metabolism of *Monhystera disjuncta*, a marine free-living nematode, were studied during chronic exposure in culture conditions. Mortality is a rather insensitive criterium to predict the environmental impact of pollutants. MEC (minimal effect concentration) values based on development rate and daily egg production were the most sensitive criteria : values were up to two orders of magnitude less than the corresponding LC50 (96h). EC50's, effective concentrations resulting in a 50% inhibitory effect on either the intrinsic rate of natural increase (r_m) or on net-fecundity (R_o) were, on the one hand, less sensitive than MEC's based on development and egg production, but on the other hand more than one order of magnitude less than LC50 values. Significant decreases of ATP content were observed at concentrations considerably less than the LC50'S. However, compared with the demographic characteristics studied, this criterion is less sensitive. It is argued that neither ATP concentrations, nor AEC measurements can give ecological relevant information about detrimental effects caused by long-term exposure to sublethal concentrations of the metals tested.

1.

INTRODUCTION

Up to now, the most widely used groups of marine invertebrates for bioassay tests are bivalves, crustaceans and polychaetes. Undoubtely, the economic importance of crustaceans and bivalves was the main reason for their selection.

Despite their ecological importance (Heip *et al.*,1979 ; Warwick & Price,1979 ; Heip *et al.*, 1985), marine free-living nematodes have only recently been used as test organisms in a few studies (Bogaert *et al.*, 1984 ; Howell, 1984 ; Tietjen, 1984 ; Vranken *et al.*, 1984a, 1985, 1986). The aim of the present study was to research the sublethal effects of cadmium and nickel on the free-living marine nematode *Monhystera disjuncta* in laboratory conditions.

Monhystera disjuncta is previously used as test organism in several bioassays, by Vranken and co-workers. Vranken *et al.*,(1984) found that the juvenile stage was the most sensitive life-stage, when exposed to three different mercury compounds. Short-term acute tests with cadmium as toxicant showed that LC50 values are very time-dependent and that MEC (minimal effect concentrations) values, based either on mortality or on a developmental assay in which success in attaining the adult stage was tested, are probably ecologically more meaningful than LC50 values (Vranken *et al.*,1985). Finally, a comparison between mortality, developmental rate and fecundity as toxicity-indices was made (Vranken *et al.*,1986). Based on a large data-base (seven heavy metals, pentachlorophenol and γ hexachlorocyclohexane were tested), fecundity turned out to be the most sensitive criterion, though, MEC values remained substantially high.

In this study , we tested for sub-lethal changes in demographic and biochemical characteristics. The demographic characteristics studied are mortality as a function of age, generation time and fecundity. From these figures we calculated the intrinsic rate of natural increase (r_m) and the net-reproductivity (R_o). Several authors proposed to determine EC50 (r_m) values, the concentration which has a 50% inhibitory effect on population growth (Hummon & Hummon, 1975 ; Sabatini & marcotte, 1983). They believe that such EC50 values are more reliable criteria than LC50 values, to determine so-called treshold-concentrations with regard to safe-guarding communities of organisms in the environment. The exact determination of these parameters is tedious since at each level of intoxication, complete life and fecundity tables (l_x and m_x) have to be constructed. In our study we tested how the EC50 (r_m) relates to the LC50 (96h) and the so-called MEC value (Vranken *et al.*,1985). The latter variables are much easier to de-

determine, but it is generally feared that lethal concentrations in short-term experiments) may seriously underestimate the influence of pollutants on a population.

Adenylate energy charge as a measure of pollution stress

In the late 1960s, Atkinson & Walton (1967) proposed the adenylate energy charge (AEC) as a means of expressing the metabolic energy status of an organism. It is given by :

$$AEC = (ATP + \tfrac{1}{2} ADP) / (ATP + ADP + AMP)$$

and varies between 0 and 1. The observation that its value decreases in response to stress, irrespective of the type of stress, led Ivanovici & Wiebe (1981) to propose AEC as a general biochemical index of sublethal stress : in optimal conditions, AEC ranges between 0.8 - 0.9, values between 0.5 - 0.7 point to suboptimal but still viable conditions whereas at values below 0.5 viability is lost (Ivanovici, 1980).

In several bio-assay studies, AEC determinations were used to assess "sublethal" effects of man-made pollutants on marine organisms (Bakke & Skjoldal, 1979 ; Zaroogian *et al.*, 1982 ; Neuhoff, 1983 ; Haya *et al.*, 1983). A major drawback, however, is the fact that no information is available in multicellular organisms on how a decrease of AEC is related to ecological relevant parameters as growth, reproduction, etc... (Livingstone, 1985). Without such information, the predictive power of AEC as an early warning indicator of unfavarouble environmental conditions is limited. If a decrease of AEC is correlated with for example low growth rates and/or impaired reproduction, than AEC would represent one of the most sensitive biochemical indices of stress available at present.

Another drawback of AEC measurements concerns the reliability of the methodology used. Verschraegen *et al.*(1985) described a reliable assay for ATP, ADP and AMP in two polychaetes (*Nereis diversicolor* and *Nephtys* sp.). In this study we tried to adapt this method. The main problem was the very small biomass of the nematodes (± 0.5 µg adult freshweight for *Monhystera disjuncta* against a mean of about 0.3 g for the two polychaetes.

ATP-concentrations as indicator for pollution stress

Due to these problems, we mainly concentrated on the determination of the ATP-concentrations in the nematodes. ATP-concentrations were determined in different life-stages of the nematodes under Ni as well as under Cd intoxication. The objective was to establish a possible correlation between changes in ATP-concentrations in an early stage of chronic exposure experiments and

changes in demographic characteristics as net-reproductivity R_o and intrinsic rate of natural increase r_m. This would considerably reduce time necessary to evaluate sublethal effects.

MATERIAL AND METHODS

Testspecies - culturetechniques

Monhystera disjuncta was sampled in the Suice dock of Ostend, a marine lagoon near the Belgian coast. *M.disjuncta* is a marine bacterivorous nematode with a cosmopolitan distribution. Adults have a mean length of ± 0.85 mm. The procedures for isolation and cultivation are described at full lenght by Vranken *et al.*(1984a,b) and Vranken *et al.*(1985). Stockcultures were maintained on 0.5% bacto-agar (Difco) plates with a mixed bacterial culture as food. The experiments were conducted in completely controlled monoxenic cultures. A monospecific bacterial isolate (belonging to the *alteromonas haloplanktis* rRNA branch) was added as food in a ring-formed excavation in agar-plates (see Vranken *et al.*, 1985). In order to avoid pH-fluctuations, Tris buffer (5mM) was added to the culture medium (Vranken *et al.*, 1986).

Toxicity tests

Nickel and cadmium were added as $NiCl_2.6H_2O$ (Merck) and $CdCl_2.2\tfrac{1}{2}H_2O$ (Baker Chemicals BV Holland), both of analytical grade. The final concentrations tested ranged between 0.5 and 10 mg/l Cd and 1 and 35 mg/l Ni. for each concentration, 3 replicates were studied. All cultures were kept in the dark at a constant temperature (17°C) and salinity (30%).

I. Demographic characteristics

The demographic characteristics studied are mortality as a function of age, generation time and fecundity. For each concentration, 10 gravid females were allowed to deposit eggs during 24h (48h for 35 mg/l Ni). Development was daily followed to calculate minimal generation time T_{min}, egg mortality, juvenile and total preadult mortality. The minimum generation time T_{min} is estimated as the period between identical stages of successive generations - this is almost equal to the development time (Vranken & Heip, 1983). Criteria for death were inactivity and the lack of movement even after prodding with the tip of a needle.

When females became adult (after a period T_{min}), eggproduction of 5 adult ♀♀ together with 3 ♂♂ was counted every 2 days for each test con-

centration. Every 4 days the adults were transferred to a new culture. To test for adult survival, observations were done every 2 or 3 days on 40 ♂♂ and 40 ♀♀ per concentration. Every 6 days, surviving adults were transferred to fresh cultures to distinguish between parents and offspring. Dead organisms were eleminated from the cultures.

Calculation of the demographic parameters :

The intrinsic rate of natural increase r_m is calculated with the Euler - Lotka equation.

$$\sum_{x=0}^{\text{max age}} e^{-r_m} l_x m_x = 1$$

x = pivotal age, age of the females in the age-interval (X,X+1)

l_x = age-specific survival rate, probability to survive from the egg-stage onwards until age x

m_x = age-specific fecundity, number of female offspring produced per female alive in the age interval (X,X+1)

The age-specific fecundity m_x is estimated from the egg-counts as $m_x = Ne_x \cdot p$ where Ne_x is the number of eggs produced by a female of the parental generation with age x and p is the proportion of females in the adult population.

The net-reproductivity R_o, the multiplication rate per generation, is calculated as

$$\sum_{x=0}^{\text{max age}} U_x = \sum_{x=0}^{\text{max age}} l_x m_x$$

U_x = net-fecundity, the realized number of female offspring per female of the preceeding generation, with the latter in the age-interval (X,X+1)

The mean generation time T is estimated as $T = (\ln R_o)/r_m$

II. Biochemical characteristics

The tests were executed simultaneously with the demographic assay. The organisms were harvested from two of the three replicates of each test-concentration studied. Procedures to determine ATP, ADP and AMP were based on the method used for *Nereis diversicolor* and *Nephtys* sp. in previous studies (Verschraegen *et al.*, 1985). This method is based on the firefly bioluminescence reaction. The (ATP dependent) light emission of the luciferin-luciferase substrate-enzyme complex was measured with the integration method. A Constant Light Signal (CLS) reagent was used as

bioluminescence reagent. For a detailed description of the reagents and equipment used, see Verschraegen *et al.*, 1985.

a. Extraction procedures

Preliminary experiments revealed that the results improved when the nematodes in the extracts were fractionated by sonication. It appeared that by doing so, the variance between the results decreased considerably. A sonicator (Brown, Labsonic 1510) with a needle probe (Ø : 4mm) was used to generate waves of 20 kHz (power 100W) during 60 sec. To avoid warming up of the extracts, the test tubes were placed in an ice-bath. Furthermore, extracts had to be diluted (with Tris-HCl buffer : Tris 0.02M, pH 7.75) as much as possible to reduce possible interfering factors (Karl & LaRock, 1975) in ATP measurements. Prolongation of the extraction time from 1 min to 10 min did not significantly affect the results.

Comparison of 7 different extracting agents

For each technique, 4 or 5 replicate extractions were made in Eppendorf Test Tubes, using adult nematodes from stockcultures. The media used were ice-cooled, except otherwise mentioned. Extracts were stored at -20°C until determination of the adenylate concentrations.

EXTR 1 : TCA : 10 nematodes were transferred into 50 µl TCA extraction medium (0.5M trichloroacetic acid Cl_3CCOOH and 0.25 Na_2HPO_4). After neutralization with 50 µl NaOH (0.5N) and dilution to 1 ml with Tris-HCl buffer (Tris 0.02M ; pH 7.75), the extracts were stored at -20°C. Immediate before the ATP determination, extracts were sonicated during 60 sec.

EXTR 2 : H_2SO_4 : Extraction as in EXTR 1, but with H_2SO_4 (0.5N) instead of TCA extraction medium.

EXTR 3 : Formic acid : 10 individuals were transferred in 50 µl 10% formic acid (HCOOH). Extracts were lyophilised (Chriss, Delta IIs) to remove acids, and diluted to 1 ml with Tris-HCl.

EXTR 4 : PCA : Extracts consisted of 10 nematodes in 50 µl 6% PCA (perchloric acid). Upon neutralisation with 25 µl K_2CO_3 (5N), the extracts were centrifuged. The supernatans was diluted to 1 ml with TrisHCl

EXTR 5 : Boiling Tris : 10 nematodes were transferred into 10 µl artificial seawater (Dietrich & Kalle, 1957). 490 µl boiling Tris-HCl was added ; after 30 sec extracts were cooled-down, sonicated and stored at -20°C.

EXTR 6 : Boiling ethanol : same procedure as in EXTR 5 but with boiling ethanol instead of Tris-HCl, and with 10-fold dilution just prior to ATP-measurement.

EXTR 7 : NRB/NRS (Lumac) : The extracting medium was a mixture of 25 µl NRB and 25 µl NRS (NRB is an extractant for bacteria, NRS for somatic cells ; composition is not mentioned by the manufacturer).

950 µl Hepes buffer (4(2hydroethyl) 1piperazin - ethansulfon acid) was added.

b. Determination of ATP

Extracts were thawed on ice ; for each extract, light emission is measured of :

1) 100 µl extract + 100 µl Tris + 200 µl CLS
2) as in 1) but 100 µl internal standard (10^{-8}M ATP) is added in stead of Tris
3) Blank : 200 µl Tris + 200 µl CLS

Extracts for the actual assay were made in TCA extraction medium (EXTR 1) but with 30 nematodes per extract. For each testconcentration, 4 or 5 replicate extractions were made. ATP was measured in juveniles of about 3d old and in nematodes of about 8.5d old - this is when females became adult in the blank.

c. Determination af ATP , ADP , AMP

Extractions were performed according to EXTR 1 (TCA) but with 100 nematodes per extract. Preliminary experiments revealed that an extra amount of ATP, ADP and AMP had to be added to enhance the transformation of ADP and AMP into ATP.

The procedure is summarized in fig 1. The incubation mixture consisted of 50 µl extract and 25 µl Tris (containing, where necessary, the internal standard), added to 100 µl TrisA (Tris buffer + Mg^{++} and K^{+}), Tris B (Tris A + Phosphenolpyruvate, pyruvate kinase and co-factors Mg^{++} and K^{+}, for transformation of ADP into ATP) or Tris C (Tris B + myokinase to transform ADP and AMP into ATP) for the determination of ATP, ADP or AMP respectively. To each mixture, 25 µl ATP, ADP or AMP (each 1 $.10^{-7}$M) was added, according to which nucleotide was determined. To determine AMP, an extra amount of 25 µl ATP (5.10^{-7}M) was added. For ADP and AMP measurements, the mixture was incubated for 30 min at 30°C, 10 µl TCA and 5 µl pepsin were added, and incubation continued for 60 min at 35°C. After neutralization with 10 µl NaOH (0.5N), the solution is diluted to 300 µl with Tris-HCl buffer.
For each nucleotide, 3 measurements were done : one with and one without an internal standard, and one blank.

d. Determination of freshweight

ATP-content is expressed on freshweight-base. For each concentration, maximal length and width of 25 fixed individuals (4% formalin ; 80°C) was measured. Freshweight is calculated with Andrassy's formula (Andrassy, 1956) :

$$\frac{ab^2}{1600000} = \text{freshweight (ug)}$$

a = maximal length (um) ; b = maximal width (um)

RESULTS

I. Demographic characteristics

Minimum generation time

Tables 1 and 2 show that minimum generation times increased with increasing concentrations of Cd and Ni. This was very clear at 2.5 mg/l Cd and 15 mg/l Ni. A Games & Howell test (Sokal & Rohlf, 1981) revealed a MEC value (minimal effect concentration) of 1 mg/l (P=0.05) for both Cd and Ni.
In the whole experiment, males developed a little faster than females. For Cd, the sex ratio (measured as the percentage females in the adult population) increased with increasing concentration (except for 0.5 mg/l Cd) while for the Ni-assay, an opposite trend was observed.

Mortality as a function of age

For both Cd and Ni, mortality during the egg stage increased only slowly with increasing concentrations (table 1 & 2). The increase was very steep between 25 and 35 mg/l Ni, the latter concentration causing 100% mortality.

Concerning the juvenile mortality, a G-test (Sokal & Rohlf, 1981) showed that juvenile mortality was significantly influenced by the amount of metal added ($P<0.001$: $G_{H/q}$ =1186 for Cd ; $G_{H/q}$ = 509 for Ni). The MEC values (P=0.05) are 2.5 mg/l for Cd and 5 mg/l Ni.

The preadult mortality, which compiles both egg- and juvenile mortality, showed about the same pattern as the juvenile mortality.

The adult survival of the females is represented in fig. 2. The mean adult female longevity in both the Ni and Cd assay was not significantly different (P=0.05) from the control (Games & Howell test : Sokal & Rohlf, 1981).

Fecundity

Total eggproduction decreased with increasing Cd and Ni concentrations ; the mean cumulative eggproduction per female alive is a linear function of time (fig. 3). All regressions were significant ; the parameters of the regressions are given in table 3.

The slope b represents the mean daily egg production of a female alive. It dropped significantly (in comparison to the control) at 1 mg/l for Cd and 2.5 mg/l Ni ($P<0.001$) ; these values are minimal effect concentrations for this criterion.

Other demographic characteristics R_o; r_m; T

From the results above, several demographic parameters were calculated (table 4). Both the net-reproductivity R_o and the intrinsic rate of natural increase r_m were clearly depressed by metal intoxication, mean generation time T was prolonged compared to the control (table 4). The values calculated for 0.5 mg/l Cd are exceptional due to the very low sex ratio (44.2% females against 81% for 1 mg/l Cd) observed.

For both metals, EC50(R_o) and EC50(r_m) were calculated. These are concentrations at which R_o, respectively r_m, are depressed with 50% compared to the control. For Cd there was no clear correlation between R_o (r_m) and the concentration of the metal. This is due to the aberrant value of R_o (r_m) at 0.5 mg/l Cd (fig. 4a). Linear interpolation between the control and 2.5 mg/l Cd gave :

$EC50(r_m)$ = 1.37 mg/l Cd

$EC50(R_o)$ = 0.64 mg/l Cd

An exponential correlation was found between r_m (R_o) and Ni concentration (table 5, fig. 4b). From the regressions, EC50 values were calculated as

$EC50(r_m)$ = 12.29 mg/l Ni

$EC50(R_o)$ = 3.48 mg/l Ni

II. Biochemical characteristics

Extraction procedures test

Results were expressed on freshweight base and are represented in table 6. A Bartlett's chi-square test showed that variances were heterogeneous. Therefore, the results were analysed with the Games & Howell test. This revealed that there were no significant differences between the four acidic methods (P=0.05). EXTR 1 gave the highest ATP-yield. All non-

acidic extraction techniques (EXTR 5,6,7) were significantly different from EXTR 1 and 2. From this group, results with EXTR 5 approximated these with formic acid (EXTR 4). EXTR 6 and 7 gave the lowest ATP-yield.

ATP concentration in the bio-assay

The ATP-content of 3d old juveniles decreased allometrically with metal concentration (fig. 6 and 7). A similar relationship was found between freshweight and Cd (Ni) concentration (fig. 6 and 7).

Analysis of variance showed that the weight-specific ATP content was significantly influenced by Cd (F_s = 4.717 ; $0.001<P<0.01$) and by Ni (F_s = 6.395 ; $0.001<P<0.01$). Comparison limits (P=0.05) were calculated with the Gabriël-test (Sokal & Rohlf, 1981) and given in fig 4. Values for 10 mg/l Cd were (marginally) significantly different of values at 0.5 and 2.5 mg/l Cd. For Ni, a (marginal) significant difference existed between 15 mg/l Ni and 1 mg/l Ni. Of more importance is that none of the measured values differed significantly from the values in the control (P=0.05).

For 8.5d old organisms, Cd and Ni again affected the weight-specific ATP content measured significantly (F_s = 43.988 , $0.001<P<0.01$ for Cd and F_s = 64.674 , $P<0.001$ for Ni). Comparison limits (Gabriël test, P=0.05) showed a significant decrease compared to the control at the highest concentrations of both metals tested (fig. 5) At the lowest concentration, this difference was not significant except at 1 mg/l and 2.5 mg/l Cd. At these concentrations, significantly higher values (P=0.05) were calculated.

Adenylate Energy Charge

ADP and/or AMP were not always measurable. A few results are given in table 7. The AEC was higher in "healthy" organisms than in starved organisms (After 5 to 6 days starvation, the nematodes were barely alive).

DISCUSSION

Several studies, field studies as well as laboratory experiments, show that nematodes commonly exhibit relative high resistance to pollutants. LC50 values recorded after intoxication with inorganic and organic xenobiotics are regularly among the highest values noted for other taxa, or even higher.

Vranken *et al.*(1986) studied the acute toxicity of seven heavy metals, PCP and γHCH on *Monhystera disjuncta*. For Ni and Cd, they found for the J2 juvenile stage a LC50 (96h) of 103 mg/l Ni and 37 mg/l Cd. Others (Haight *et al.*,1982)mentioned 50% mortality in the J2 juvenile stage of *Panagrellus silusiae* after 48h intoxication with 15.1 mg/l Cd and 105 mg/l Ni. In a mixed population of *Panagrellus* and *Rhabditis* the LC50(48h) value ranged between 35 and 40 mg/l Cd (Feldmesser & Rebois, 1965). In this study, no significant difference was found neither in adult survival, nor in mean adult longevity of *M. disjuncta*, at the different Cd and Ni concentrations studied. In the juvenile stage, which is more sensitive than the adult stage (Vranken *et al.*, 1984a), mortality increased significantly at 2.5 mg/l Cd and 5 mg/l Ni.

In table 8, a summary of the effects of Cd and Ni on the demographic criteria studied is given. Obviously, the LC50 (96h) values reported by Vranken *et al.*(1986) are much higher (almost one to two orders of magnitude) than all demographic citeria studied here. Also, the minimal effect concentration (MEC) for mortality during the juvenile stage, although less than the LC50, is higher than MEC's based on T_{min}, daily egg production, net reproductivity (R_o) and population growth (r_m).

When compared with the LC50 values, development rate and daily egg production are the most sensitive criteria. In the Cd-assay, this difference amounts to a factor 37. In the Ni-assay, treshold levels as measured by the daily eggproduction and development rate are 41 and 103 times less when compared with the LC50.
Similar results are reported in the literature. Reish & Carr (1978) and Petrich & Reish (1979) found a significant reduction of fecundity in polychaetes, exposed to a variety of heavy metals at levels almost two orders of magnitude less than the corresponding LC50(96h). For the nematode *Panagrellus redivivus*, Samoiloff *et al.*(1980) reported a difference of three orders of magnitude between the Cd level suppressing fecundity and the MEC as measured by juvenile mortality. Furthermore they showed that growth inhibition in this species is a more sensitive toxicity index than mortality. For *Diplolaimella* spec 1, Vranken & Heip (in press) found differences of two orders of magnitude (Cu & Pb) and 1.5 (Hg) between development time and the corresponding LC50. Other observations are however in variance with these findings. Vranken *et al.*(1984a) found no effect on the development rate of some specimens of *Monhystera disjuncta*, whereas for most individuals the mercury concentration tested was lethal. Haight *et al.*(1982) needed concentrations of 100 mg/l for e.g.

Cd to stop growth of *Panagrellus silusiae* whereas at 15 mg/l Cd, 50% of the J2 juvenile stage died.

A comparison with the results reported by Vranken *et al.*(1986) revealed that, although the same test organism and an identical culture technique was used, our MEC values, based on juvenile mortality, development rate and fecundity, are consistently less.This can be explained by 1) differences in exposure time used in the experimental design : Vranken and co-workers studied these criteria during a pre-set period of time (96h) whereas in this study physiological standards (development time and total lifespan) were used as time duration of the experiment, and by 2) a higher susceptibility of the smallest juveniles as freshly hatched juveniles (2.5 - 3d old) were excluded from their experiments (they started with 4.5d old juveniles).

In the Cd-assay, there was a steep decrease of both the intrinsic rate of natural increase (r_m) and the net-reproductivity (R_o) with increasing concentrations. In the nickel-assay, the decrease of both parameters is less pronounced. R_o can be considered as the most sensitive life history parameter. EC50's based on R_o are 1.64 mg/l Cd and 3.48 mg/l Ni. At these concentrations the production of female progeny drops with 50% when compared to the control. EC50 values based on r_m are 2.37 mg/l for Cd and 12.29 mg/l for Ni. Consequently the effective concentrations based on r_m and R_o are higher than MEC's based on development time and fecundity.

Biochemical characteristics in pollution studies

For a particular biochemical response to be acceptable as an index of biological effect, it must fulfill two important criteria (Livingstone, 1985) : 1. The measurable change in biochemical processes must result from, or be a response to, a change in the environmental conditions. 2. It must be possible to demonstrate that the change in biochemical process(es) will have, either direct or indirect, a detrimental effect on growth, reproduction or survival of the organism. Concerning the use of the adenylate energy charge in multicellullar organisms, only the first criterium is fulfilled implying that its use is limited.

Up to now, only a few papers have reported on the ATP content of marine nematodes. Only Ernst (1970) and Goercke & Ernst (1975) made measurements to study the relationship ATP - biomass.
Expressed in percentage of the total amount of organic carbon, Ernst (1970) found 2.3% for *Panagrellus redivivus*, while for *Anoplostoma*

viviparum and *Adoncholaimus thalassophygas* values ranged from 0.9% to 1.3% (Goercke & Ernst, 1975). In this study, the mean ATP content in *Monhystera disjuncta* was 2.4% to 3.6% of the total amount of organic carbon (calculated with an approximate conversion factor $C_{org} = 1/12$ freshweight).

The above mentioned authors used boiling Tris buffer as extracting fluid. Our experience is, however, that the cuticle of *Monhystera disjuncta* remained intact after boiling, probably resulting in less homogeneous and less stable extracts. Our results improved when the nematodes were fractionated by sonication. Nevertheless, the ATP-yield remained below the values obtained with the four acid extraction procedures tested. TCA was selected as extracting fluid as it gave the highest ATP-yield, although it was not significantly different from the three other acid extractions.

ATP as a measure of pollution stress in bio assay tests

The objective was to establish a possible correlation between changes in ATP concentrations in an early stage of the experiment and changes in demographic characteristics.

In juveniles (3d old), no significant alteration of weight-specific ATP content was observed at each level of Cd and Ni intoxication compared to the control. Yet, at this stage of the experiment, the impairement of growth and development could be observed even at the lowest concentrations tested. In a later stage of the experiment (8.5d old organisms), ATP content decreased significantly at the highest metal concentrations. At lower concentrations, at which significant effects on R_o and r_m were measured, the ATP concentration retained the same level as in the control (fig. 4 & 5), except at 1 and 2.5 mg/l Cd. At these concentrations, a (only marginally) significantly higher weight-specific ATP content was calculated. We believe, however, that these two values are erroneous and that the mean weight-specific ATP content should remain constant up to 2.5 mg/l Cd inclusive, for two reasons. Firstly, it is very unlikely that ATP concentrations would increase when organims live in stressful conditions. Changes of ATP concentration as a consequence of harmful conditions are more than once reported, but the change is always in the reverse direction. Secondly, the mean ATP content per individual, which is based on the measurement in 120 to 150 nematodes per concentration, remained constant up to and inclusive 2.5 mg/l Cd. It is possible that

the aberrations are a consequence of an inadequate biomass determination. In fact, the determination of the mean body mass was based on its measurement in only 25 individuals, which exhibited a very steep exponential growth at the time of the observations (8.5d). The variance of the body weight after 8.5 days was relatively high (and higher than at higher metal concentrations where growth stopped almost completely - results not shown) so that it became difficult to pick the nematodes at random from the culture, possibly leading to erroneous estimates. Such errors would not occur when body mass and ATP were measured in the same organism. In this study, this was not possible as *Monhystera disjuncta* is too small (± 0.5 µg freshweight per adult).

Summarizing we can say that : within the range of metal concentrations tested, the ATP turn-over remained constant in an early stage (juveniles), and also at the lowest Cd and Ni concentrations in a later stage of the chronic exposure experiment. 2) developmental inhibition, delayed and impaired reproduction already occured at metal concentrations below those affecting the ATP concentration.

A possible explanation is that with increasing metal concentration and probably with increasing exposure time, an increasing amount of energy (potentially available in the adenylate system) is used in processes related to adaptive responses such as avoidance reactions and active detoxification. As a consequence, less energy will be available for growth and reproduction. For Cd, the level at which growth ceased completely was observed at 5 mg/l Cd (the organims are moribund, won't survive till adulthood and consequently won't reproduce) and for Ni it is above 15 mg/l Ni. The situation in the Ni-assay seems to be different from in the Cd-assay as at the highest concentrations (5 and 15 mg/l Ni) the organisms still grew and reproduced despite the significant decrease of the mean weight-specific ATP content measured in 8.5d old organims. We believe however, that the ATP content in reproducing adults (although less reproducing) may have remained constant and that the decrease may have been a reflection of the proportion moribund animals (see the increased juvenile mortality) at the time of (at random) sampling.

AEC as a measure of pollution stress in bio-assays with nematodes

We encountered many problems in determining the AEC in *Monhystera disjuncta*, using the same method as previously described for two polychaetes (Verscraegen *et al.*, 1985). The major problem was, again, the

low biomass of the nematode and consequently the very low concentrations of the adenylates. Even with 100 nematodes per extract (which were manipulated one by one with a needle), transformation appeared to be too low to be measurable. In all different adaptations of the method tried, AMP was the least reliable factor. In the long run, the procedure became sufficiently complex, and still results were not always consistent. In spite of it, we determined AEC's in starved nematodes and in organisms from old neglected stock cultures (old nematodes in overcrowded and hypersaline conditions). Results showed that AEC in *Monhystera disjuncta* actually drops with increasing stress.

Haya & Waiwood (1983) summarized four possible ways in which the adenylate energy metabolism can be altered during sublethal intoxication with xenobiotics. These are : 1) AEC decreases due to an alteration in relative proportions of the adenine nucleotides while the level of total adenylates remains constant. 2) AEC remains constant while the level of total adenylates decreases. 3) AEC and total adenylates decrease. 4) Total adenylates and AEC remain constant, but precursors or endproducts of adenylate energy metabolism are altered.
Applied to our own data, this means that if a decrease of AEC could have been measured, this would only have been possible in organisms of 8.5d old, at the highest metal concentrations tested (at 5 and 10 mg/l Cd and possibly but not likely at 5 and 15 mg/l Ni). Indeed, if AEC were decreased at concentrations where the ATP concentration is constant, this would imply that, the more the individuals were stressed, the larger their total adenylate pool would be. Such a respons type has never been observed (Haya & Waiwood, 1983).

In conclusion, we think that neither ATP concentration or AEC measurements can give an ecological relevant idea about harmful effects caused by long-term exposure to sublethal concentrations of heavy metals. Both criteria are less sensitive than the demographic characteristics studied. These findings strengthen our previous idea that the usefulness of AEC as an index in pollution monitoring in the field is questionable (Verschraegen *et al.*, 1985). The hypothesis was that the maintenance of a stable population is impossible when the individuals constantly have low AEC's, so that in polluted stations only pollutant-resistant species will be found with normal AEC's.

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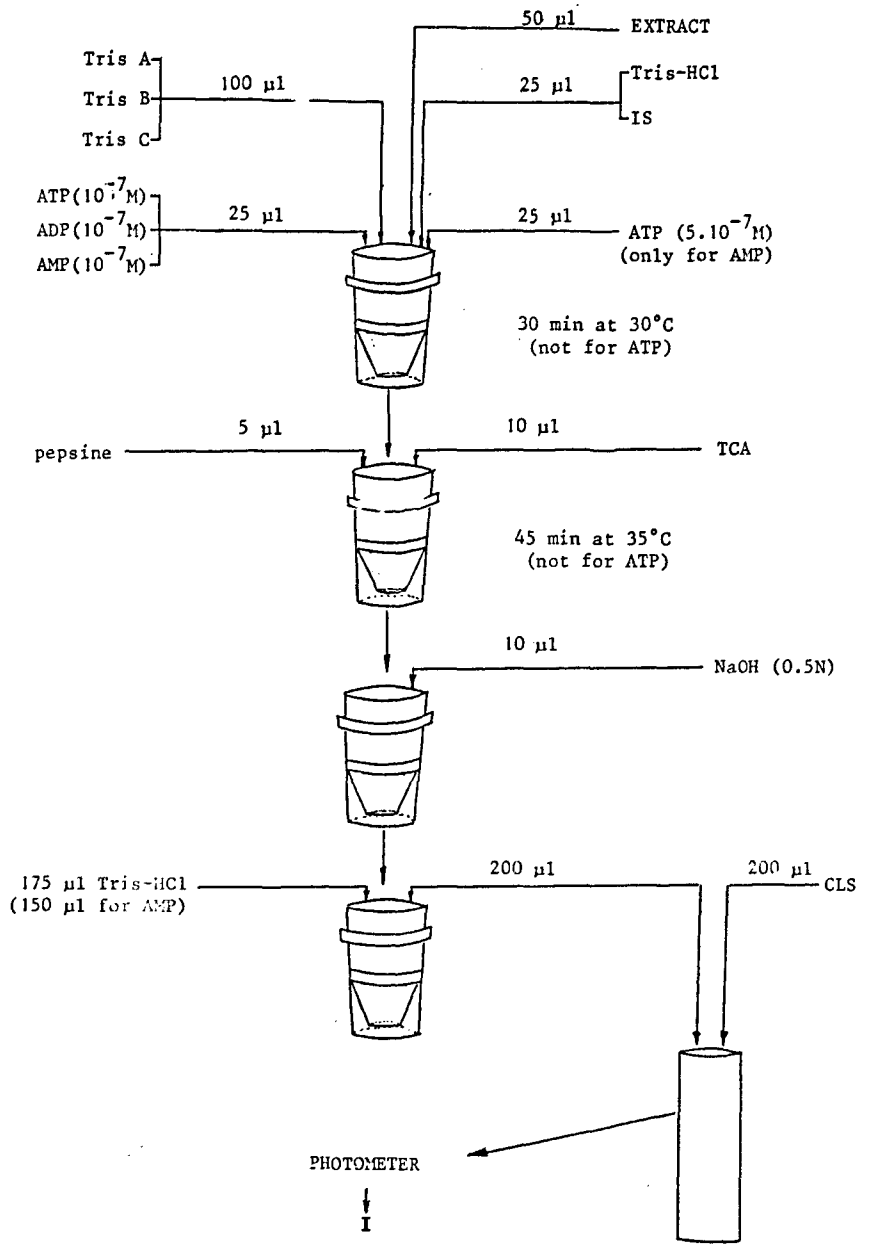
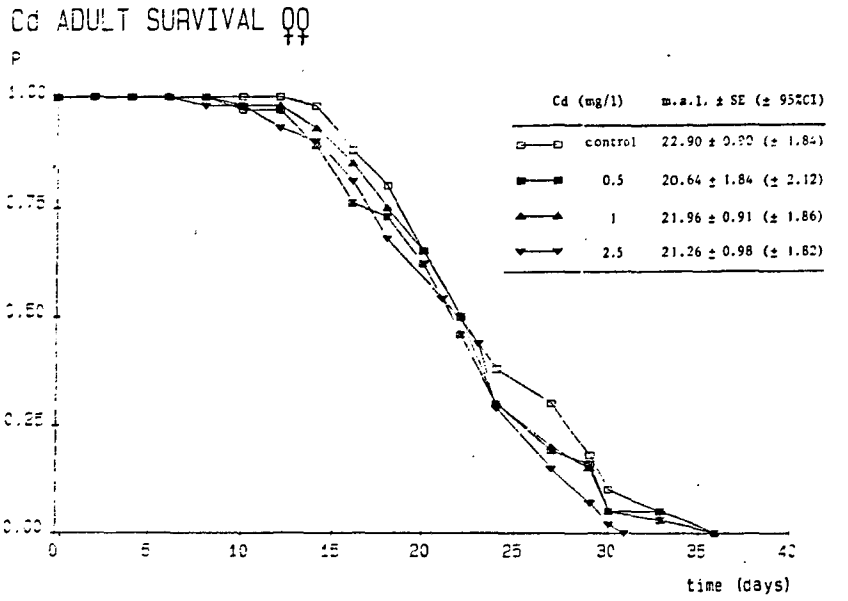


Fig. 1 : Protocol for ATP-, ADP-, and AMP-determination.
IS = internal standard



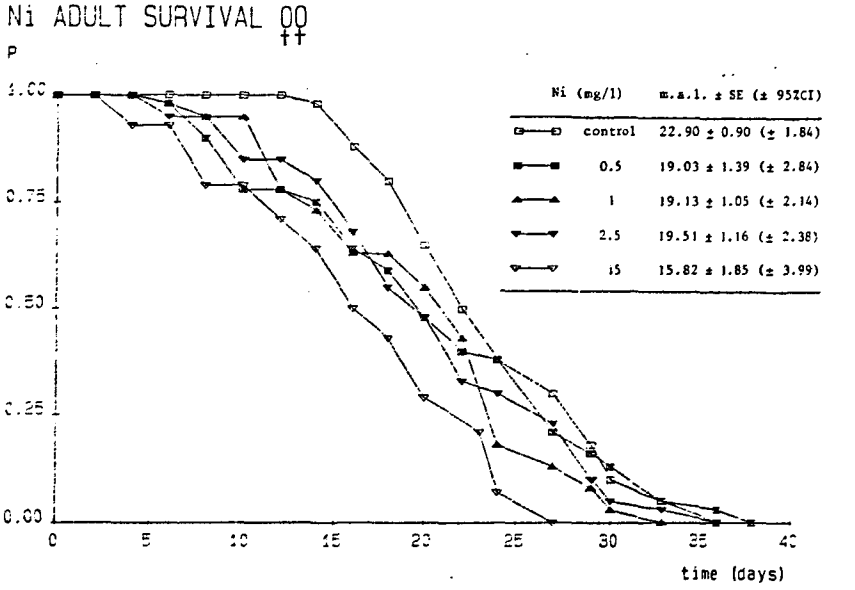


Fig. 2 : Female adult survival at different Cd (upper) and Ni (lower) concentrations. P is survival in proportions, m.a.l. is the mean adult longevity (in days), SE is standard error. 95%CI between brackets.

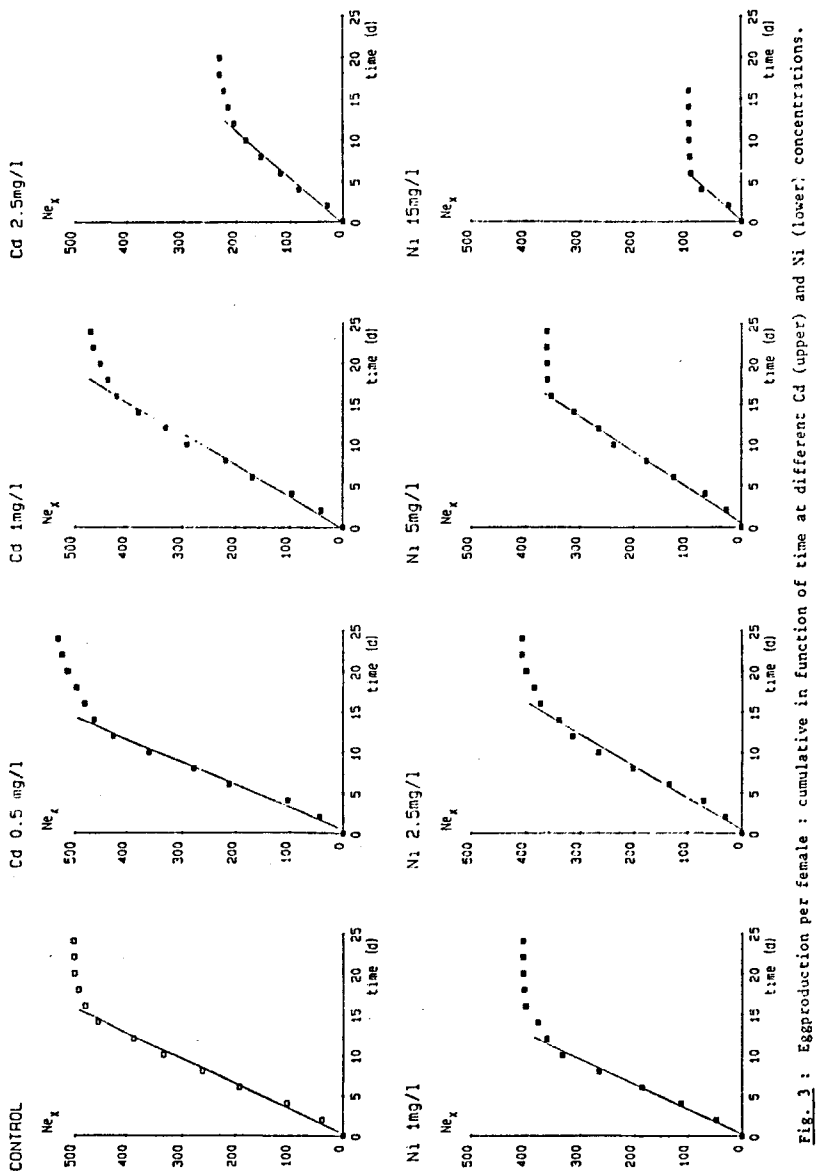


Fig. 3 : Eggproduction per female : cumulative in function of time at different Cd (upper) and Ni (lower) concentrations.

Fig 4a

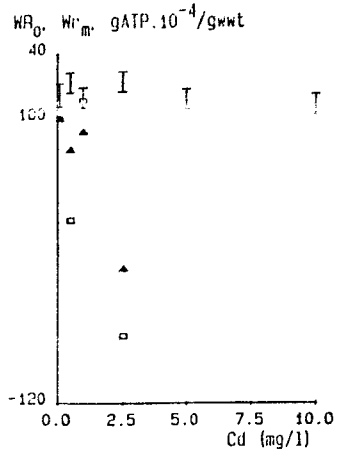


Fig 4b

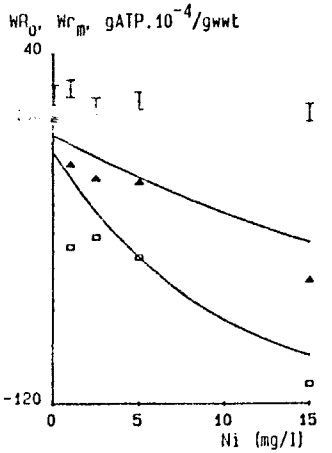


Fig. 4 : Comparison of demographic parameters and weight-specific ATP content of juvenile (3d old) at different Cd (fig. 4a) and Ni (fig. 4b) concentrations (mg/l). Ordinate : W, fitness relative to the the control; ▲ r_m intrinsic rate of natural increase ; ▫ R_o net-reproductivity ; · mean ATP content (in g ATP 10^{-4}) per g freshweight with comparison limits (P=0.05 ; Gabriël test).

Fig 5a

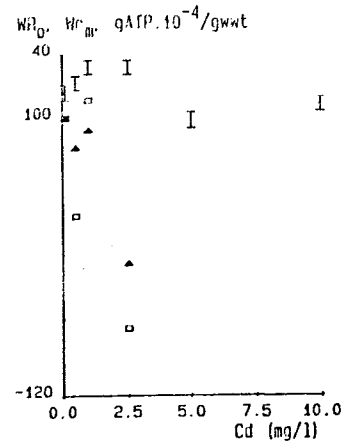


Fig 5b

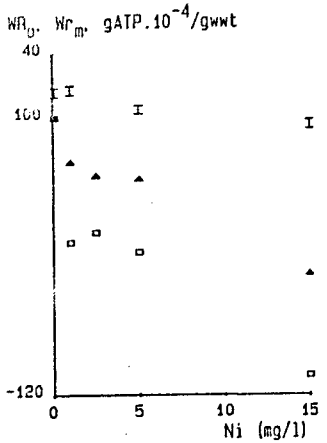


Fig. 5 : Comparison of demographic parameters (r_m, R_o) and weight-specific ATP content in nematodes of 6.5 d old at different Cd (fig. 5a) and Ni (fig. 5b) concentrations (mg/l). Abbreviations and symbols as in fig. 4.

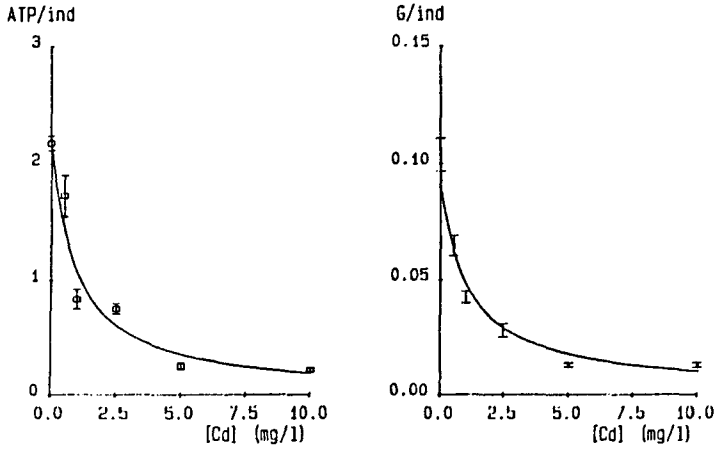


Fig. 6 : Cadmium : correlation between ATP content per juvenile nematode (in g ATP 10^{-10}) and Cd concentration (left) and between mean fresh weight perjuvenile nematode (in ug) and Cd concentration (right).

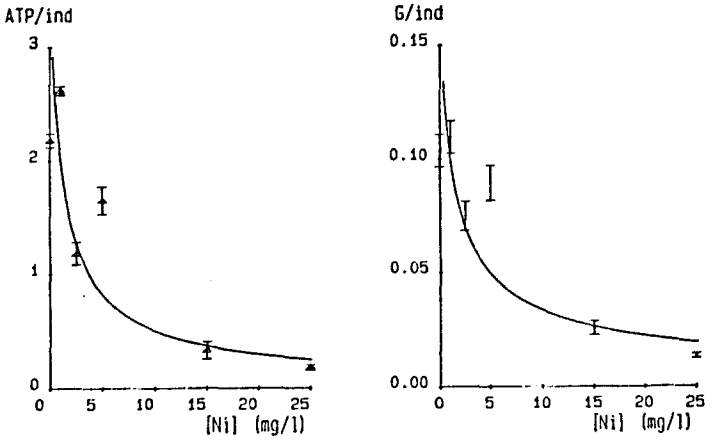


Fig. 7 : Nickel : correlation between ATP content per juvenile nematode (in g ATP 10^{-10}) and Ni concentration (left) and between mean fresh weight per nematode (in ug) and Ni concentration (right).

CADMIUM	control	0.5 mg/l	1 mg/l	2.5 mg/l	5 mg/l	10 mg/l
Ne	233	223	331	304	206	176
e(+)	6.01	6.28	6.04	6.25	7.30	14.18
j(+)	2.28	6.22	5.14	61.40	100	100
p(+)	8.15	12.10	10.80	63.80	100	100
N ♀♀	143	102	245	92	-	-
T_{min} ♀♀	8.14 ± 0.086 (± 0.173)	8.59 ± 0.105 (± 0.209)	8.86 ± 0.088 (± 0.176)	16.05 ± 9.234 (± 0.467)	-	-
N ♂♂	71	129	56	11	-	-
T_{min} ♂♂	8.01 ± 0.099 (± 0.199)	7.98 ± 0.065 (± 0.129)	8.84 ± 0.192 (± 0.384	13.73 ± 0.407 (±0.906)	-	-
sex ratio	66.8	44.2	81	89	-	-

Table 1 : CADMIUM : Minimum generation time T_{min} ± standard error (with 95% CI between brackets), egg mortality e(+), juvenile mortality j(+) and preadult mortality p(+) at different Cd concentrations (mg/l). Ne is the number of eggs studied, N ♀♀ (♂♂) the number of adults and sex is the proportion females in the adult population.

NICKEL	control	1 mg/l	2.5 mg/l	5 mg/l	15 mg/l	25 mg/l	35 mg/l
Ne	233	412	359	233	125	110	34*
e(+)	6.01	6.79	7.52	9.01	9.60	10	100
j(+)	2.28	6.51	3.92	11.32	33.63	100	100
p(+)	8.15	12.86	11.10	19.30	40	100	100
N ♀♀	143	185	184	111	29	-	-
T_{min} ♀♀	8.14 ± 0.036 (± 0.173)	9.61 ± 0.121 (± 0.243)	9.67 ± 0.129 (± 0.258)	9.49 ± 0.173 (± 0.347)	13.62 ± 0.291 (± 0.597)	-	-
N ♂♂	71	173	149	76	46	-	-
T_{min} ♂♂	8.01 ± 0.039 (± 0.199)	8.68 ± 0.091 (± 0.182)	8.84 ± 0.113 (± 0.226)	8.83 ± 0.174 (± 0.348)	12.22 ± 0.093 (± 0.188)	-	-
sex ratio	66.8	52	55	60	39	-	-

Table 2 : NICKEL : life-history features studied at different Ni concentrations. Abbreviations as in table 1. * after 46h.

CADMIUM (mg/l)	a (± 95% CI)	b (± 99% CI)	r^2	F_s	n
control	-8.14 (± 24.40)	32.30 (± 3.79)	0.992	888	9
0.5	-14.02 (± 28.89)	35.77 (± 5.23)	0.991	33	8
1	4.80 (± 24.62)	25.92 (± 3.35)	0.988	67	10
2.5	4.28 (± 16.61)	17.54 (± 3.62)	0.987	38	7
NICKEL (mg/l)	**a (± 95% CI)**	**b (± 99% CI)**	**r^2**	**F_s**	**n**
control	-8.14 (± 24.40)	32.30 (± 3.79)	0.992	888	9
1	-6.71 (± 25.23)	32.28 (± 5.49)	0.991	562	7
2.5	-10.42 (± 25.14)	25.36 (± 3.91)	0.987	516	9
5	-10.64 (± 14.49)	23.03 (± 2.25)	0.995	1281	9
15	-1.22 (± 31.97)	16.37 (± 19.1)	0.973	72*	4

Table 3 : Egg production (cumulative) per female, at different Cd (Ni) concentrations (mg/l) : parameters of the regression $\sum_{x=0}^{max\ age} Ne_x = a + b.time\ (d)$ with b the mean daily eggproduction per female.
n is the number of observations, r^2 the coëfficient of determination, F_s the F-statistic of the linear regression ($P<0.001$; * $0.01<P<0.05$)

		R_o	$r_m(d^{-1})$	T (d)
CADMIUM (mg/l)	control	302	0.422	13.53
	0.5	194	0.376	12.48
	1	321	0.403	14.32
	2.5	72	0.199	21.40
NICKEL (mg/l)	control	302	0.422	13.53
	1	165	0.354	14.43
	2.5	176	0.333	15.52
	5	154	0.328	15.36
	15	21	0.182	16.85

Table 4 : Demographic parameters at different Cd (Ni) concentrations (mg/l) R_o is the net-reproductivity, r_m is the intrinsic rate of natural increase (per day) and T the mean generation time (in days).

NICKEL	a	b	r^2	F_s	n
R_o	267	-0.164	0.953	61.1	5
r_m	0.398	-0.052	0.965	81.5	5

Table 5 : Nickel : R_o (respectively r_m) as a function of Ni concentration (mg/l). Parameters of the regression $Y=ae^{b[Ni]}$ with a and b constants, $Y=R_o$ (respect. $Y=r_m$) and [Ni] in mg/l. Abbreviations as in table 3., $0.001<P<0.01$

Extraction procedure	n	ATP/gwwt ± SE
EXTR 1 (TCA)	5	26.771 ± 1.785
EXTR 2 (H_2SO_4)	5	22.782 ± 1.255
EXTR 3 (PCA)	5	19.599 ± 1.415
EXTR 4 (Formic acid)	4	12.115 ± 3.170
EXTR 5 (NRB/NRS)	5	11.303 ± 0.589
EXTR 6 (Tris 100°C)	4	3.559 ± 0.295
EXTR 7 (Ethanol ±80°C)	3	0.334 ± 0.143

Table 6 : Extraction procedures test : mean ATP content (in g ATP 10^{-4}) per g wet weight ± standard error. n is the number of replicate extractions

stress induction	repl. 1	repl. 2
control	0.86	AMP not measurable
overcrowded and hypersaline conditions	0.70	--
1 day starvation	0.72	0.47
3 days starvation	0.47	AMP n.m.
4 days starvation	0.49	AMP n.m.

Table 7 : Artificial stress induction : AEC in old nematodes (in overcrowded and hypersaline conditions) and in starved organisms.

CRITERIUM	Cd	Ni
LC50 (96h)*	37	103
MEC J(+)	2.5	5
MEC T_{min}	1	1
MEC Ne_x	1	2.5
MEC m.a.l.	-	-
EC50 (R_o)	1.64	3.48
EC50 (r_m)	2.37	12.29

Table 8 : Compiled table of demographic criteria studied in *Monhystera disjuncta* under Cd (Ni) intoxication. MEC = minimal effect concentration, EC = effective concentration with 50% inhibitory effect, J(+) = juvenile mortality, T_{min} = minimal generation time, Ne_x = mean daily egg production, m.a.l. = mean adult longevity, R_o = net-fecundity, r_m = intrinsic rate of natural increase. * = data from Vranken *et al.*, 1986. concentrations in mg/l.