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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*.

K. Verschraegen, G. Vranken, D. Van Gansbeke, C. Heip and A. Boffé
Marine Biology Section, Zoology Institute, State University of Gent, Belgium.

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 criterion 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_0*) 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.

INTRODUCTION

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

Despite their ecological importance (Heil et al., 1979; Warwick & Price, 1979; Heil et al., 1985), marine free-living nematodes have only recently been used as test organisms in a few studies (Hegner 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 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_0*). 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 threshold-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_9 and *R_0*) 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:

\[
\text{AEC} = \frac{\text{ATP} + \frac{1}{2} \text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}
\]

and varies between 0 and 1. The observation that its value decreases in response to stress, irrespective of the type of stress, led Ivanovic & 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 (Ivanovic, 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 unfavourable environmental conditions is limited. If a decrease of AEC is correlated with for example low growth rates and/or impaired reproduction, then 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. Verschaeren et al. (1985) described a reliable assay for ATP, ADP and AMP in two polychaetes (Nereis diversicolor and Nephys sp.). In this study we tried to adapt this method. The main problem was the very small biomass of the nematodes (± 0.5 mg; adult fresh weight 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 R₀ and intrinsic rate of natural increase rᵢ. This would considerably reduce time necessary to evaluate sublethal effects.

**MATERIAL AND METHODS**

**Testspecies – culturtechniques**

*M. disjuncta* was sampled in the Suice dock of Ostend, a marine lagoon near the Belgian coast. *M. disjuncta* is a marine bacteriivorous nematode with a cosmopolitan distribution. Adults have a mean length of ± 0.85 mm. The procedures for isolation and cultivation are described at full length 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 *Alphaproteobacteria* group) 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 NiCl₂.6H₂O (Merck) and CdCl₂.2½H₂O (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%).

1. **Demographic characteristics**

The demographic characteristics studied were 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 Tₘᵢₙ, egg mortality, juvenile and total preadult mortality. The minimum generation time Tₘᵢₙ 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 Tₘᵢₙ), egg production of 5 adult qq together with 3 d♂ 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 eliminated from the cultures.

Calculation of the demographic parameters:
The intrinsic rate of natural increase \( r_p \) is calculated with the Euler-
Lotka equation:
\[
\max_{x=0} \sum_{x=0}^{\text{max age}} e^{-r_p} t_x \cdot n_x = 1
\]
\( x \) = 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 \( x \)
\( n_x \) = age-specific fecundity, number of female offspring produced per
female alive in the age interval \((X,X+1)\).
The age-specific fecundity \( n_x \) is estimated from the egg-counts as \( n_x = N_{x+1} \cdot p \)
where \( N_{x+1} \) 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 \( R_0 \), the multiplication rate per generation, is cal-
culated as
\[
\max_{x=0} \sum_{x=0}^{\text{max age}} v_x = \max_{x=0} \sum_{x=0}^{\text{max age}} l_x \cdot n_x
\]
\( v_x \) = net-fecundity, the realized number of female offspring per female
of the preceding generation, with the latter in the age-interval
\((X,X+1)\).
The mean generation time \( T \) is estimated as \( T = (\ln R_0) / r_p \)

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 *Nematode diversicolor* and *Nereis sp.* in previous stu-
dies (Verschuuren 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 integra-
tion method. A Constant Light Signal (CLS) reagent was used as

bioluminescence reagent. For a detailed description of the reagents and
equipment used, see Verschuuren 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 Trix-HCl buffer : Trix 0.02M, pH 7.75) as
much as possible to reduce possible interfering factors (Karl & LaRock, 1973)
in ATP measurements. Frolongation 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 me-
dium (0.5N trichloroacetic acid Cl₃CCOOH and 0.25 Na₂HPO₄). After
neutralization with 50 µl NaOH (0.5N) and dilution to 1 ml with Trix-HCl buffer
(Trix 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: H₂SO₄ : Extraction as in EXTR 1, but with H₂SO₄ (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 (Chrisk, Delta 11a) to remove acids, and diluted to 1 ml with Trix-HCl.

EXTR 4: PCA : Extracts consisted of 10 nematodes in 50 µl 6X PCA (perchloric
acid). Upon neutralization with 25 µl K₂CO₃ (5N), the extracts
were centrifuged. The supernatants was diluted to 1 ml with Trix-HCl

EXTR 5: Boiling Trix : 10 nematodes were transferred into 10 µl artificial
seawater (Dietrich & Kalle, 1957). 490 µl boiling Trix-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 e-
thanol instead of Trix-HCl, and with 10-fold dilution. Just prior
to ATP-measurement.

EXTR 7: NRS/NRS (Lumac) : The extracting medium was a mixture of 25 µl
NRS and 25 µl NRS (NRS is an extractant for bacteria, NRS for soma-
tic cells ; composition is not mentioned by the manufacturer).
950 µl Hepes buffer (4(2-hydroethyl)piperazin - 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 (10⁻⁸M 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 test concentration, 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 of 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 + Mg²⁺ and K¹), Tris B (Tris A + Phosphoenolpyruvate, pyruvate kinase and co-factors Mg²⁺ and K¹, 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 1.10⁻⁷M) nucleotide was added, according to which was determined. To determine AMP, an extra amount of 25 µl ATP (5.10⁻⁷M) 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 fresh weight

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):

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

\[ a = \text{maximal length (um)}; b = \text{maximal width (um)} \]

**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; G² =1186 for Cd; G² = 509 for Ni). The MEC values (P=0.05) are 2.5 mg/l for Cd and 5 mg/l Ni.

The prediapause mortality, which comprises 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).
Total egg production decreased with increasing Cd and Ni concentrations; the mean cumulative egg production 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.

From the results above, several demographic parameters were calculated (table 4). Both the net-reproductivity R₀ and the intrinsic rate of natural increase rₘ 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(R₀) and EC50(rₘ) were calculated. These are concentrations at which R₀, respectively rₘ, are depressed by 50% compared to the control. For Cd there was no clear correlation between R₀ (rₘ) and the concentration of the metal. This is due to the aberrant value of R₀ (rₘ) at 0.5 mg/l Cd (fig. 4a). Linear interpolation between the control and 2.5 mg/l Cd gave:

\[
\text{EC50(rₘ)} = 1.37 \text{ mg/l Cd} \\
\text{EC50(R₀)} = 0.64 \text{ mg/l Cd}
\]

An exponential correlation was found between rₘ (R₀) and Ni concentration (table 5, fig. 4b). From the regressions, EC50 values were calculated as

\[
\text{EC50(rₘ)} = 12.29 \text{ mg/l Ni} \\
\text{EC50(R₀)} = 3.48 \text{ mg/l Ni}
\]

### II. Biochemical characteristics

#### Extraction procedures test

Results were expressed on fresh weight 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 those 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 fresh weight and Cd (Ni) concentration (fig. 6 and 7).

Analysis of variance showed that the weight-specific ATP content was significantly influenced by Cd (Fₛ = 4.717; 0.001<P<0.01) and by Ni (Fₛ = 6.393; 0.001<P<0.01). Comparison limits (P=0.05) were calculated with the Gabriel 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 (Fₛ = 43.988, 0.001<P<0.01 for Cd and Fₛ = 64.674, P<0.001 for Ni). Comparison limits (Gabriel 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

ATP 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 HCH 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 siliusiae 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 (Feldmeier & Rebbo, 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 criteria 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 ($R_e$) and population growth ($R_g$).

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, threshold 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 rediivus, 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 Diplotelaimella 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 siliusiae 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 ($R_e$) with increasing concentrations. In the nickel-assay, the decrease of both parameters is less pronounced. $R_e$ can be considered as the most sensitive life history parameter. EC50’s based on $R_e$ 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 $R_e$ 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 multicellular 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 (1973) 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 rediivus, while for Anoplostoma
viviparum and Adoncloaimus 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$_{org} = \frac{1}{12}$ fresh-weight).

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 sonicitation. 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 impairment 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$_g$ 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/1 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/1 Cd inclusive, for two reasons. Firstly, it is very unlikely that ATP concentrations would increase when organisms 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/1 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 (0.5d). 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 turnover 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 occurred 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/1 Cd (the organisms are moribund, won't survive till adulthood and consequently won't reproduce) and for Ni it is above 15 mg/1 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/1 Ni) the organisms still grow and reproduced despite the significant decrease of the mean weight-specific ATP content measured in 8.5d old organisms. 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 (Verscroeven 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, ATP 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 hypersalinic conditions). Results showed that AEC in Monhystera dilacunxa actually drops with increasing stress.

Haya & Waiood (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 response type has never been observed (Haya & Waiood, 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., 1935). 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. 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 control; rm, intrinsic rate of natural increase; R0, net-reproductivity; mean ATP content (in g ATP $10^{-4}$) per g freshweight with comparison limits (P=0.05; Gabriel test).

Fig. 5: Comparison of demographic parameters ($r_m$, $R_0$) and weight-specific ATP content in nauplii of 5.5 d old at different Cd (fig. 5a) and Ni (fig. 5b) concentrations (mg/L). Abbreviations and symbols as in fig. 4.
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, $N_{ff}$ ($\delta\delta$) the number of adults and sex is the proportion females in the adult population.
### Table 3: Egg production (cumulative) per female, at different Cd (Ni) concentrations (mg/l) : parameters of the regression $N_t = a + b \cdot \text{time}$ (d) with $b$ the mean daily eggproduction per female.

<table>
<thead>
<tr>
<th>Concentration (mg/l)</th>
<th>a (± 95% CI)</th>
<th>b (± 99% CI)</th>
<th>$r^2$</th>
<th>$F_s$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>-8.14 (± 24.40)</td>
<td>32.30 (± 3.79)</td>
<td>0.992</td>
<td>888</td>
<td>9</td>
</tr>
<tr>
<td>0.5</td>
<td>-14.02 (± 28.89)</td>
<td>33.77 (± 5.23)</td>
<td>0.991</td>
<td>33</td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td>4.80 (± 24.62)</td>
<td>25.92 (± 3.35)</td>
<td>0.988</td>
<td>67</td>
<td>10</td>
</tr>
<tr>
<td>2.5</td>
<td>4.28 (± 16.61)</td>
<td>17.54 (± 3.62)</td>
<td>0.987</td>
<td>38</td>
<td>7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (mg/l)</th>
<th>a (± 95% CI)</th>
<th>b (± 99% CI)</th>
<th>$r^2$</th>
<th>$F_s$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>-8.14 (± 24.40)</td>
<td>32.30 (± 3.79)</td>
<td>0.992</td>
<td>888</td>
<td>9</td>
</tr>
<tr>
<td>1</td>
<td>-6.71 (± 25.23)</td>
<td>31.97 (± 5.49)</td>
<td>0.991</td>
<td>562</td>
<td>7</td>
</tr>
<tr>
<td>2.5</td>
<td>-10.42 (± 14.49)</td>
<td>23.03 (± 2.25)</td>
<td>0.987</td>
<td>516</td>
<td>9</td>
</tr>
<tr>
<td>15</td>
<td>-1.22 (± 31.97)</td>
<td>16.37 (± 1.91)</td>
<td>0.973</td>
<td>72</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2: NICKEL: life-history features studied at different Cd concentrations. Abbreviations as in Table 1, * after 25b.

<table>
<thead>
<tr>
<th>Concentration (mg/l)</th>
<th>$R_0$</th>
<th>$r_m (d^{-1})$</th>
<th>T (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>302</td>
<td>0.422</td>
<td>13.53</td>
</tr>
<tr>
<td>0.5</td>
<td>194</td>
<td>0.376</td>
<td>12.48</td>
</tr>
<tr>
<td>1</td>
<td>321</td>
<td>0.403</td>
<td>14.32</td>
</tr>
<tr>
<td>2.5</td>
<td>72</td>
<td>0.199</td>
<td>21.40</td>
</tr>
<tr>
<td>Nickel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>302</td>
<td>0.422</td>
<td>13.53</td>
</tr>
<tr>
<td>1</td>
<td>165</td>
<td>0.354</td>
<td>14.43</td>
</tr>
<tr>
<td>2.5</td>
<td>176</td>
<td>0.333</td>
<td>15.52</td>
</tr>
<tr>
<td>5</td>
<td>154</td>
<td>0.328</td>
<td>15.36</td>
</tr>
<tr>
<td>15</td>
<td>21</td>
<td>0.182</td>
<td>16.85</td>
</tr>
</tbody>
</table>

Table 4: Demographic parameters at different Cd (Ni) concentrations (mg/l) $R_0$ is the net-reproductivity, $r_m$ is the intrinsic rate of natural increase (per day) and T the mean generation time (in days).
Table 5: Nickel: $R_0$ (respectively $r_m$) as a function of Ni concentration (mg/l). Parameters of the regression $Y=ab^x[Ni]$ with $a$ and $b$ constants, $Y=R_0$ (respectively $Y=r_m$) and [Ni] in mg/l. Abbreviations as in table 3.

$$
\begin{array}{cccccc}
\text{Ni} & a & b & r^2 & F_g & n \\
R_0 & 267 & -0.164 & 0.953 & 61.1 & 5 \\
r_m & 0.398 & 0.032 & 0.965 & 81.5 & 5 \\
\end{array}
$$

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.

<table>
<thead>
<tr>
<th>Extraction procedure</th>
<th>n</th>
<th>ATP/g wet ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXTR 1 (TCA)</td>
<td>5</td>
<td>26.771 ± 1.785</td>
</tr>
<tr>
<td>EXTR 2 (H$_2$SO$_4$)</td>
<td>5</td>
<td>22.782 ± 1.255</td>
</tr>
<tr>
<td>EXTR 3 (PCA)</td>
<td>5</td>
<td>19.599 ± 1.415</td>
</tr>
<tr>
<td>EXTR 4 (Formic acid)</td>
<td>4</td>
<td>12.115 ± 3.170</td>
</tr>
<tr>
<td>EXTR 5 (NRB/NRS)</td>
<td>5</td>
<td>11.303 ± 0.589</td>
</tr>
<tr>
<td>EXTR 6 (Tris 100°C)</td>
<td>4</td>
<td>3.559 ± 0.295</td>
</tr>
<tr>
<td>EXTR 7 (Ethanol 80°C)</td>
<td>3</td>
<td>0.334 ± 0.143</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>stress induction</th>
<th>repl. 1</th>
<th>repl. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.86</td>
<td>AMP not measurable</td>
</tr>
<tr>
<td>overcrowded and hypersaline conditions</td>
<td>0.70</td>
<td>--</td>
</tr>
<tr>
<td>1 day starvation</td>
<td>0.72</td>
<td>0.47</td>
</tr>
<tr>
<td>3 days starvation</td>
<td>0.47</td>
<td>AMP n.m.</td>
</tr>
<tr>
<td>4 days starvation</td>
<td>0.49</td>
<td>AMP n.m.</td>
</tr>
</tbody>
</table>

Table 8: Compiled table of demographic criteria studied in *N. dicyfi* under Cd (Ni) intoxication. MEC = minimal effect concentration, EC = effective concentration with 50% inhibitory effect, J(+) = juvenile mortality, $T_{min}$ = minimal generation time, $N_e$ = mean daily egg production, m.a.l. = mean adult longevity, $R_0$ = net fecundity, $r_m$ = intrinsic rate of natural increase. * data from Vranken et al., 1986.

$$
\begin{array}{ccc}
\text{CRITERION} & \text{Cd} & \text{Ni} \\
\text{LC50 (96h)} & 37 & 103 \\
\text{MEC-J(+)} & 2.5 & 5 \\
\text{MEC $T_{min}$} & 1 & 1 \\
\text{MEC $N_e$} & 1 & 2.5 \\
\text{MEC m.a.l.} & - & - \\
\text{EC50 ($R_0$)} & 1.64 & 3.48 \\
\text{EC50 ($r_m$)} & 2.37 & 12.29 \\
\end{array}
$$