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An Ecotoxicological Test Utilizing Heart Rate
in <u>Daphnia</u> <u>magna</u> and <u>D</u>. <u>pulex</u> with
Comparisons to Traditional Testing Methods

Douglas Charles Craig

A Thesis

in

The Department

of

Biology

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ABSTRACT

An ecotoxicological test utilizing heart rate in <u>Daphnia magna</u> and <u>D</u>. <u>pulex</u> with comparisons to traditional testing methods

Douglas C. Craig

The effects of aluminum sulfate, sodium chloride and cadmium chloride on the heart rate of <u>Daphnia magna</u> and <u>D</u>. <u>pulex</u> are reported and compared with results obtained using traditional ecotoxicological testing procedures (median lethal concentration and life history traits). Results indicate that all toxicants tested significantly reduced heart rate in both species. Linear regressions of concentration against heart rate were significant, indicating a concentration-dependent relationship. There were no differences in effect on heart rate between species. The incipient concentration causing a 10% reduction in heart rate (IC10) was 3.51 mg/L for aluminum, 785-925 mg/L for sodium and 0.12-0.15 mg/L for cadmium. In all cases, the IC10 was either equal to or less than LC50 estimates. However, had the organisms been subjected to IC10 concentrations for longer time periods, it is probable that mortalities would have occurred. Observation of life history traits may be more sensitive at estimating toxic stress, but less consistent than the heart rate bioassay. The results obtained in this preliminary investigation warrant further study.

ACKNOWLEDGMENTS

DEDICATION

This thesis, and all of the work that went into it, is dedicated to my wife Sima.

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INTRODUCTION

Background

In the field of aquatic toxicology, there is probably no individual bioassay as widely used as the *median lethal concentration* (LC50) (Stephen and Mount, 1973; Sprague, 1976; Buikema et al., 1979; Locke, 1990). In such a test, several concentrations of a toxicant are prepared, usually in a logarithmic ascending order (Environment Canada, 1990). Three or more organisms are tested at each concentration and observed for mortality after a specified period of time (usually 24 or 96 hours). Once the time has elapsed, the number of individuals that have died in each concentration is tabulated, and the cumulative mortality is plotted against concentration (Casarett, 1992). Since untransformed mortality percentages result in a poorly defined sigmoid curve, it is common practice to use the probit transformation. The probit transformation is simply the normal equivalent deviate (N.E.D.) plus 5 (Finney, 1971). When mortality data are transformed into probits, the relationship between concentration and mortality is linear and the median lethal concentration can be calculated.

Although mathematically sound regression equations can be obtained from probit analysis, the usefulness of the median effective concentration (MEC) is currently being questioned (Krewski et al., 1991). One problem with acute lethality tests is their inherent lack of robustness: they are sensitive to a few atypical observations. As a result of the inherent variability, the LC50 is

often only used to define a toxic concentration to within an order of magnitude (Amdur et al., 1991), meaning that LC50 estimates can often vary considerably (Appendix 1). A second problem with acute lethality tests is that they use relatively high concentrations of toxicant (DiDelupis and Rotondo, 1988), and therefore may not be practical for interpreting potential effects at environmentally relevant concentrations. Knowledge of the lethal concentration is certainly important, but it is only the beginning in understanding the toxic effect of chemicals.

In the pharmaceutical industry, acute and/or chronic toxicity studies are only preliminary range-finding tests. Once these tests are complete, much more revealing investigations, involving measurements of cardiac output, venous and arterial blood pressure, blood temperature, weight loss/gain and clinical signs, are conducted to determine potential harmful effects at sublethal doses (IBEX Study Reports IH001, IH002, 1994). Additional tests may investigate immunogenicity, and mutigenicity (IBEX Study Reports IH011, IH012, 1994; IH029-2, IH030-2, 1995).

Ecotoxicology would benefit by borrowing some of the testing procedures traditionally embraced by the pharmaceutical industry. Further investigation of how potentially toxic substances affect organisms may allow us to understand how a species would cope, thus survive, in the natural environment. For example, reduced co-ordination due to neurotoxicity, although potentially having little or no effect on LC50 results, could be a life threatening condition in a pond or lake.

Potential Alternatives to LC50

One type of test that is regaining popularity utilises life history trait information (Havel & Talbott, 1994; Bodar et al., 1988). In this type of test, treatment cohort(s) and a control cohort are followed through a life cycle, and life history traits (i.e. number of neonates, neonate length) are measured. This type of test, which can last for several generations for organisms as small as zooplankton, yields a great deal more information than LC50 tests. However, there are several problems with such tests.

Life history traits, even in the absence of any toxicant, often differ slightly between different populations of a species. Munzinger et al (1991) compared the sensitivity of three populations of Daphnia magna under chronic heavy metal stress, and found that survival, reproduction and body length corresponded to the population of origin. On one hand, this result might be interpreted as demonstrating adaptations in metal tolerance, since differences in the predictor variables give the impression that there were differences in stress perceived by each group. Alternatively, it could be argued that the "tolerant" population had simply evolved in response to other unidentified environmental variables such that it normally produced more neonates and was naturally larger. Indeed, their results suggest the latter, since the "tolerant" population produced significantly more neonates and was consistently larger under identical control conditions (Munzinger et al., 1991).

A second problem with using life history traits to measure tolerance is the difficulty in choosing a treatment dose. Bodar et al. (1988), in a study investigating the effect of cadmium on the reproductive strategy of Daphnia magna, showed that number of neonates per female first increased, peaked, then decreased as the dose of cadmium approached and exceeded 1 ppb, meaning that the toxic effect wasn't apparent until the concentration reached 1ppb. Furthermore, Munzinger et al. (1991) showed significant mortality at 5 ppb cadmium in chronic studies. The nearly similar toxicant levels between the chronic lethal concentrations and those causing changes in life history traits indicates that the window of choice in selecting concentrations can be quite limiting.

Other novel approaches in measuring toxicant effect include the observation of behavioural and physiological parameters in the presence and absence of a toxicant Some examples of behavioural characteristics that have been studied in the past include: differences in phototactic response between control and treatment conditions in Daphnia magna (Flickinger et al., 1982), D. magna, Artemia salina and Aedes aegypti (DiDelupis & Rotondo., 1988; Simonet et al., 1978); effects on filtration rate in Daphnia pulex and D. magna (Flickinger et al., 1982; Kring & O'Brien, 1976); and avoidance behaviour in rainbow trout (Pedder & Maly, 1985). One of the main problems with using behavioural endpoints in ecotoxicological tests is the high degree of variability. Physiological variables, such as respiration (Kettle et al., 1980; Arner et al., 1993); ion flux (Havas et al., 1984; Potts & Fryer, 1979), and heart

rate (Klugh & Miller, 1926; Alcaraz et al., 1994), although used less frequently, may provide more appropriate information on the toxic effects of exogenous substances to biota than behavioural and acute lethality tests. Of the many physiological variables that could theoretically provide useful indices of toxic stress, heart rate is one that can easily be measured in organisms as small as zooplankton.

Previous studies involving the observation of the crustacean heart have shown a close relationship between the availability of oxygen and heart rate (Wilkens 1993; Albert & Ellington, 1985; Taylor, 1982; Butler et al., 1978). The relationship is such that as the availability of oxygen decreases (towards hypoxia), there is a corresponding decrease in heart rate. Wilkens (1993) observed that as the PO_2 fell below approximately 13.3 kPa, heart rate decreased in a linear fashion with further decreases in PO_2.

The toxic effect of metals to aquatic organisms has often been attributed, at least in part, to impaired respiratory function due to metal binding to the gas-exchange sites (Cusimano et al., 1986; Carpenter, 1927; Gardner and Yevich, 1970; Eisler, 1971; Havas and Likens, 1985; Havas, 1986). Carpenter (1927) reported excess mucous production on the gills of fish upon exposure to metals or acids. Oxygen deficiency, or coagulation film anoxia, is thought to occur as a result of this mucous. Other investigators believe that metal-binding to the gills causes direct necrotic damage to the gill tissues, disrupting gaseous exchange (Cusimano et al., 1986; Mount and Stephen, 1967). In zooplankton, Havas (1986) and Havens (1990) reported that in acid and

aluminum sensitive species of cladocerans such as <u>Daphnia magna</u>, <u>D</u>. <u>galeata</u> <u>mendotae</u> and <u>D</u>. <u>retrocurva</u>, aluminum binds to the maxillary glands, which are sites of ion and gas-exchange in these species.

Due to correlations between heart rate and oxygen availability, and observations of respiratory disruption due to toxicant exposure, it seems reasonable that heart rate would decrease upon exposure to metals. If the hypothesis is correct, and heart rate is decreased upon exposure to toxicants at sublethal concentrations, assays employing heart rate as an endpoint may provide more insight into how toxicants affect zooplankton. For instance, reduced oxygen uptake may reduce swimming speeds, increasing susceptibility to predation, and movement to oxygen rich water near the surface (more light) may increase predation. In addition, reduced oxygen uptake would most likely result in a lowered rate of development, which in turn could adversely affect the probability of reaching maturity.

Objectives:

The objectives of the present study are three-fold. The first objective is to establish, under laboratory conditions, the effect, if any, of high concentrations of aluminum sulfate, sodium chloride and cadmium chloride on the heart rate of Daphnia magna and D. pulex. If there is an effect, the second objective is to determine if heart rate is affected in a consistent, concentration-dependent manner. The last objective is to compare the sensitivity and consistency of traditional ecotoxicological tests (life history traits and LC50) with the test utilising heart rate for detecting toxic stress between these two closely related species of Daphnia.

The investigation was divided into 4 sections. The first part was a preliminary investigation of if, and how, the heart rates of Daphnia magna and D. pulex are altered by exposure to aluminum. Two exposure concentrations, a control and a treatment, were tested. A third concentration was tested using only D. magna for regression comparisons in later sections of this thesis. Consistency of response was investigated between heart rate effects and traditional endpoints of chronic toxicity tests (i.e., affect on neonate length, number of neonates produced, and reproductive output), both within and between the two species.

The second part of the investigation concentrated on the development of a concentration-response curve for the effect of sodium chloride on heart rate. In addition, a comparison with the traditional *median lethal concentration*, or LC50, was conducted.

The third part of the investigation further examined the concentration-response curve, and compared the relative sensitivities of D. magna and D. pulex to cadmium, as measured with both the heart rate observations and LC50 test.

The last section of this thesis examined between-toxicant differences in dose-response regressions of aluminum sulphate, sodium chloride and cadmium chloride on heart rate.

METHODS

Daphnia magna and D. pulex as Test Organisms

Daphnia magna (Straus, 1820) and D. pulex (Leydig, 1860) were chosen as the test organisms due to their high rate of reproduction, short life cycle, ease of handling, and transparency, which allowed easy observation of the heart. Daphnia magna is among the largest of the cladoceran species, with the females averaging approximately 5 mm at maturity (Ward and Whipple, 1959). Daphnia pulex is a smaller species, with the adult female commonly found to be approximately 1.3-2.2 mm. Furthermore, Daphnia spp. are commonly used in toxicity tests (Environment Canada, 1990), there is a great deal of reference literature on their responses to toxic metals, and they are among the most sensitive species used in aquatic toxicology studies (Buikema et al., 1979).

In addition to being ideal test organisms, cladocerans are an ecologically important link in freshwater lakes. Examinations of the stomach contents of young fish rarely show less than 10% cladoceran by volume and often approaching up to 95% (Pennak, 1978). Aside from rapid streams and grossly polluted waters, cladocerans are found practically everywhere (Pennak, 1978). The diet of cladocerans is varied and includes algae, protazoa, bacteria and organic detritus (Ward and Whipple, 1959).

Culturing Techniques

Daphnia magna and D. pulex were obtained from laboratory cultures
originally acquired from Grand Truite Rouge (46°27"30′ N; 74°15"15′ W) and
Barbotte #3 (46°5"00′ N; 73°52"15′ W), respectively. Each culture was
maintained in separate 16 L aquaria in dechlorinated Montreal City tap water
(see Table 1 for water characteristics). Both cultures were fed a food
suspension (7mL/L) twice weekly. The food suspension was a combination of
yeast, digested trout chow and cerophyll (YTC) (Appendix 2), prepared
according to the EPS guideline for daphnid culture (Environment Canada,
1992). In addition, 500 mL of algae culture (Spirulina caldaria and Chlorella
vulgaris) as well as 250 mL of yeast suspension were added to each aquaria
once weekly. The daphnia were periodically harvested in order to keep the
population in exponential growth.

Table 1. Characteristics of dechlorinated Montreal city tap water.

Parameter	Value[1]
pH	6.95 (20°C)
Alkalinity	8.60 mg/l (CaCO$_3$)
Calcium	100 mg/l (CaCO$_3$)
Total Hardness	130 mg/l (CaCO$_3$)
Conductance	300 uohms/cm
Color	0 STD
Turbidity	0 FTU (formazin)

---

1 From Attar (1980)

Part 1: Comparison of Response in Heart Rate and Life History Traits to an Elevated Concentration of Aluminum Sulphate.

Heart Rate Observation

Before the experiment, approximately 75 to 100 daphnids were randomly removed from the culture aquaria. Control and treatment solutions were prepared in 400 mL glass beakers. The treatment solutions were 1.25 mg and 6.0 mg/L aluminum, and were prepared by separately weighing 15.0 and 72.1 mgs of aluminum sulphate ($Al_2(SO_4)_3\bullet17H_2O$) on an electronic balance and dissolving each in 1L of dechlorinated tap water in 1L volumetric flasks. The pH was adjusted to 6.0 with the addition of dilute sulphuric acid (A.C.S. Reagent grade, Aldrich Chemical) and sodium hydroxide (A.C.S. Reagent grade, Aldrich Chemical). The solution was then transferred to a 4 litre plastic container. The control solution was simply pH adjusted dechlorinated tap water. Exposure to the control and treatment solutions was accomplished by randomly placing 30 to 40 daphnids in each of the control and treatment solutions for a 2 hour time period. After the exposure periods, between 5-10 daphnids were removed from the beakers and placed into the well of a plastic depression slide containing approximately 1 mL of the appropriate exposure solution (Figure 1). A thin layer of Dow Corning silicon gel was applied to the lid which was then affixed to the slide. The daphnids quickly became immobilised in the silicon, usually with the second antennae. Other than the restricted movement of the large antennae, the daphnids would carry on the

Figure 1: Illustration of depression slide used to contain the

daphnids for microscopic evaluation of heart rate.

30.0mm

3.0mm
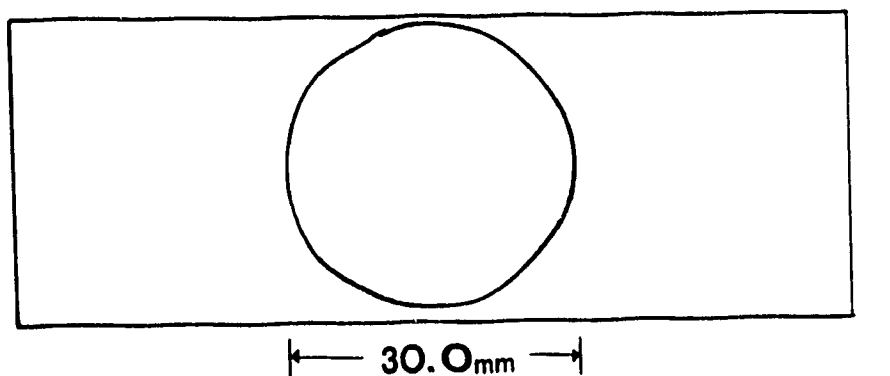
normal rhythmic beating movements with their thoracic appendages. After a five minute acclimatisation period, measurements of heart rate were taken.

Heart rate was obtained by placing the depression slide on a Wild M40-82791 microscope and focusing on the heart (Figure 2). The beating motion was monitored with a Hitachi CCTV video camera and recorded on a Mitsubishi HS-U48 VCR. The tape was then played back at 1/10 speed and counts were made during a 5 second time frame (50 seconds real time). Since not all daphnids from each trial stuck to the silicon in a manner that facilitated observation, this procedure was repeated several times.

Life History Traits

One day before the beginning of the sublethal chronic test, about 100 gravid females, between 12 and 16 days of age, were isolated and placed into two separate 3 L glass jars. The next day, all of the young were pooled and 54 individuals (<24 hours old) were randomly chosen and placed into fifty-four 30 mL polypropylene containers containing 25 mL of dechlorinated Montreal City tap water and YTC at an equivalent concentration of 7 mL/L. To half of these containers, 1,250 ug/L aluminum, in the form of aluminum sulphate (15 mg aluminum sulfate/L of distilled water), had been added. The concentration of the stock solution was confirmed colorimetrically (Hach DR-100). The pH was maintained at 6.0, and a photoperiod of 12 hours light:12 hours dark was maintained at a temperature of 20°C (± 2°C). During the test,

Figure 2: Microscopic view of a "typical" daphnid illustrating the position and relative size of the heart (H). The magnification is approximately 35X.

H
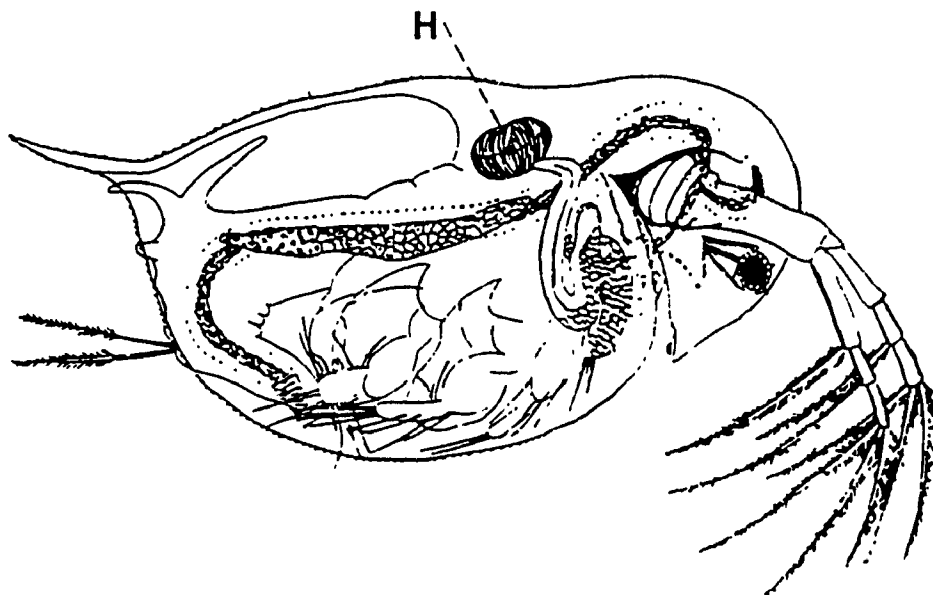
the daphnids were fed YTC (7 mL/L) twice weekly and algae (7mL/L) once weekly. The individuals were observed once daily for brood production. When neonates were observed in a container, the date was recorded, they were counted and removed. Three neonates were randomly chosen from each brood and lengths (tip of head to base of spine) were determined (Figure 3) under a Wild (N40-82791) microscope connected to a Hitachi 14 inch monitor. To obtain an estimate of the average mass of the young produced in each brood, the following formulae were used:

Species	Formula	Reference	
D. magna	Ln W=1.67 + 2.69(Ln L)	Bottrel et al. (1976)	(1)
D. pulex	Ln W=1.50 + 2.77(Ln L)	Dumont et al. (1975)	(2)

where W is the mass of the neonate in micrograms, and L is the neonate length (Figure 3). The experiment was continued for 14 days, representing appoximately one third of the average life span. After the 14 days, the adults were killed by addition of formalin and measured for length (accurate to 0.05 mm) under a Wild binocular dissecting microscope with an ocular micrometer.

Figure 3: An illustration of a 'typical' Daphnid showing where measurements were made.
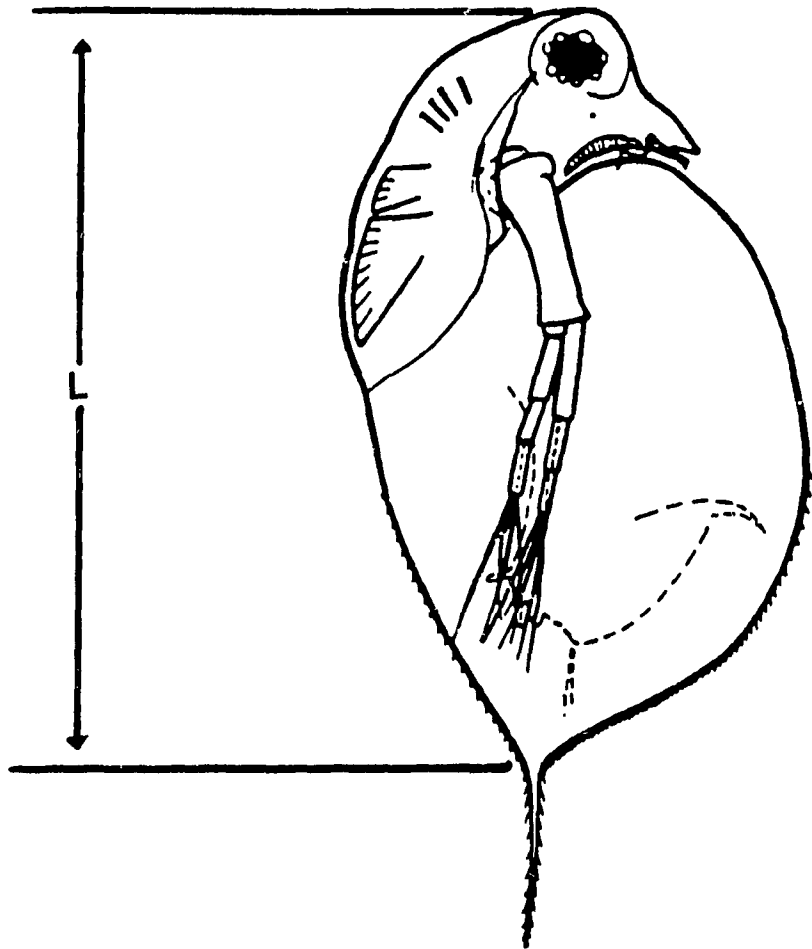
Analyses

Effects of aluminum sulphate on heart rate and life history traits between the control and treatment were compared using two-way analysis of variance (ANOVA). All data were checked for normality using the Kolmogorov-Smirnov test.

Since median lethal concentration utilises the 50th percentile of a population's response as a value to compare relative toxicity, a similar "effect" parameter was necessary for comparing relative effects on heart rate. The index calculated is defined as the increase in toxicant concentration, relative to the control, necessary to reduce heart rate by 10% of the control heart rate (IC10).

Part 2: Comparison of Heart Rate Observations with Acute Lethality Test

Results Using Sodium Chloride as the Toxicant.

Heart Rate Observations

The same procedures as described in Part 1 were employed to determine the effect of sodium chloride on heart rate. The treatment concentrations for sodium chloride were 3500 and 6000 mg/L, and were prepared by measuring 3.5 and 6.0 grams of table salt (Sifto®, iodized free running), respectively, on a digital balance and thoroughly mixing each amount in 1 litre of dilution water in 1 litre volumetric flasks. The concentration of NaCl in the control water was 330 mg/L, as reported by the Water Quality Department of the City of Montreal (P. Melanson, personal communication 1995). Since sodium chloride is non-volatile and very soluble, the nominal concentrations were considered accurate.

Acute Lethality Test

Twenty four hours before each test, approximately 50 gravid females were removed from the stock cultures and placed into 100 mL glass beakers containing dechlorinated Montreal City tap water and 7 mL/L yeast solution. The stocking density was approximately 4 adults/beaker.

Nine concentrations of sodium chloride (100, 500, 1000, 2000, 2500, 3000,

3500, 4000 and 5000 mg/L) were prepared by mixing the required mass of table

salt with the dilution water. For each of the three replicates, ten 50 mL glass

beakers were filled with 40 mL of each concentration plus a control

containing the dilution water.

On the day of the test, all of the neonates that were produced overnight

were pooled and 12 were randomly assigned to each test beaker. The

photoperiod was maintained at 12 hours light:12 hours dark at a temperature

of 20±2°C. Twenty-four and 48 hours after the initiation of the test the

Daphnia were observed for mortality and the dead were counted and

removed. Death was defined as the lack of movement of the antennae and

internal organs.

Analyses

Regression equations for heart rate vs. concentration were calculated

using 'standardised-to-control' heart rate values vs. Ln concentration (mg/L).

Analysis of covariance (ANCOVA) was employed to determine if there

was a significant difference between the slopes of the two regression lines (D.

magna and D. pulex), and whether their was any significant "species" effect

with respect to the effect of sodium chloride on heart rate. The IC10 estimates

were calculated from the standardised D. magna and D. pulex concentration-

response regression lines.

For the acute lethality test, dose-response regressions were constructed by

converting the percent mortalities to probits using transformation tables from

Finney (1971), and plotting these values against Ln concentration. LC50

values were calculated using the Maximum Likelihood Estimation (MLE)

methods (Finney 1971, Eirkson et al 1987).

Part 3. Comparison of Heart Rate Observations with Acute Lethality
Observations Using Cadmium as the Toxicant.

Heart Rate Observations

The same procedures as previously described were employed to
determine the effect of cadmium on heart rate. The treatment concentrations
were 4.93, 9.85, 19.7 and 39.4 mg/L, and were prepared by diluting 5, 10, 20 and
40 mL of a 2 g/L stock solution of cadmium chloride ($CdCl_2 \bullet 2_{1/2}H_2O$) in 1 L of
dilution water. Since cadmium chloride is non-volatile and very soluble, the
nominal concentrations were considered accurate.

Acute Lethality Test

Due to unacceptably high mortalities (>10%) in preliminary tests, this
portion of the study was conducted externally at Analex Laboratories. The
procedures employed at Analex are those outlined by Environment Canada
for performing acute lethality tests to Daphnia magna (Environment Canada,
1990).

Briefly, seven treatment concentrations of cadmium (1.6, 3.1, 6.2, 12.3,
24.6, 49.3 and 98.5 mg/L) and a control were used. There were three replicates
of 4 individuals for each concentration. Daphnia were placed in 25x200 mm
borosilicate tubes. Twenty four and 48 hours after the initiation of the test,

Table 2.   Characteristics of dilution water used at Analex Inc. Laboratories

Parameter	Value
pH	8.2
Conductance	700 uohms/cm
Dissolved Oxygen	5.5 (mg/L)
Hardness	98 ± 10 (mg $CaCO_3$/L)
Alkalinity	168 (mg $CaCO_3$/L)

the <u>Daphnia</u> were observed for mortality and the dead were counted and removed. Death was defined as the lack of movement of the antennae and internal organs. The water characteristics of the dilution water used at Analex Inc. are listed in Table 2.

Analyses

Effects of cadmium chloride on heart rate and acute lethality were analysed according to the procedures outlined in Part 2.

Part 4: A Comparison of Toxicity Ranking Order of Aluminum Sulfate,

Sodium Chloride and Cadmium Chloride According to Heart Rate

Observations

An analysis of covariance, which included heart rate observations after

treatment with all three toxins, was conducted in order to determine the

ranking order of toxic effect, and whether there were significant differences in

the slopes of the regression lines among toxins.

Regression analysis with probit values to determine the ranking order of

toxic effect was not necessary since Maximum Likelihood Estimation

provides the LC50 values with 95% confidence intervals.

RESULTS

Part 1: Comparison of Response in Heart Rate and Life History Traits to an

Elevated Concentration of Aluminum Sulphate.

The response of both D. magna and D. pulex to treatment with

aluminum sulphate at 6.0 mg/L was a significant ($F_{1,76}$=30.9; p<0.001, Figure 4;

Table 3) decrease in heart rate. There was no significant interaction ($F_{1,76}$=1.27;

p=0.263) between treatment and species, however, there was a borderline

significant species effect ($F_{1,76}$=3.98; p=0.05), with the heart rate of D. magna

being affected to a greater degree (lower standardised heart rate).

The regression of 'standardised heart rate' vs. Ln concentration (control,

1.25 and 6.0 mg/L) with D. magna was significant ($F_{1,76}$=7.24; p<0.01; r=0.297),

and had the form of:

Percent HR = 92.1- (1.67)Ln concentration

Neonate length was lower in individuals treated with aluminum

sulphate ($F_{1,105}$=35.7; p<0.001, Figure 5; Table 3). As expected, the neonates of

D. magna were significantly ($F_{1,105}$=679.8; p<0.001) larger than those of D.

pulex. There was no significant interaction ($F_{1,105}$=2.4; p=0.126) between

treatment and species.

Figure 4. Bar graph of heart rates of both <u>Daphnia</u> <u>magna</u> and <u>D</u>. <u>pulex</u> at control and treatment concentration (6.0 mg/L) of aluminum. The data are presented as mean ± standard deviation.

Table 3: Mean and Standard Deviations of Heart Rate and Life History
Parameters.

Daphnia magna				
	Control		Treatment	
Parameter	Mean	Std. Dev.	Mean	Std. Dev.
Heart Rate (beats/5 sec)	35	5	28	4
Neonate Length (μm)	828.9	31.7	783.4	42.5
Neonate Number (total)	35	32	27	10
Reproductive Output (μg)	112.7	31.7	74.1	27.9
Daphnia pulex				
	Control		Treatment	
Parameter	Mean	Std. Dev.	Mean	Std. Dev.
Heart Rate (beats/5 sec)	37	3	33	3
Neonate Length (μm)	665.2	20.4	638.8	22.2
Neonate Number (total)	38	11	52	11
Reproductive Output (μg)	55.1	16.5	67.9	15.7

Figure 5. Bar graph of neonate lengths of both <u>Daphnia magna</u> and

<u>D</u>. <u>pulex</u> at control and treatment concentration (6.0 mg/L) of

aluminum. The data are presented as mean ± standard

deviation.

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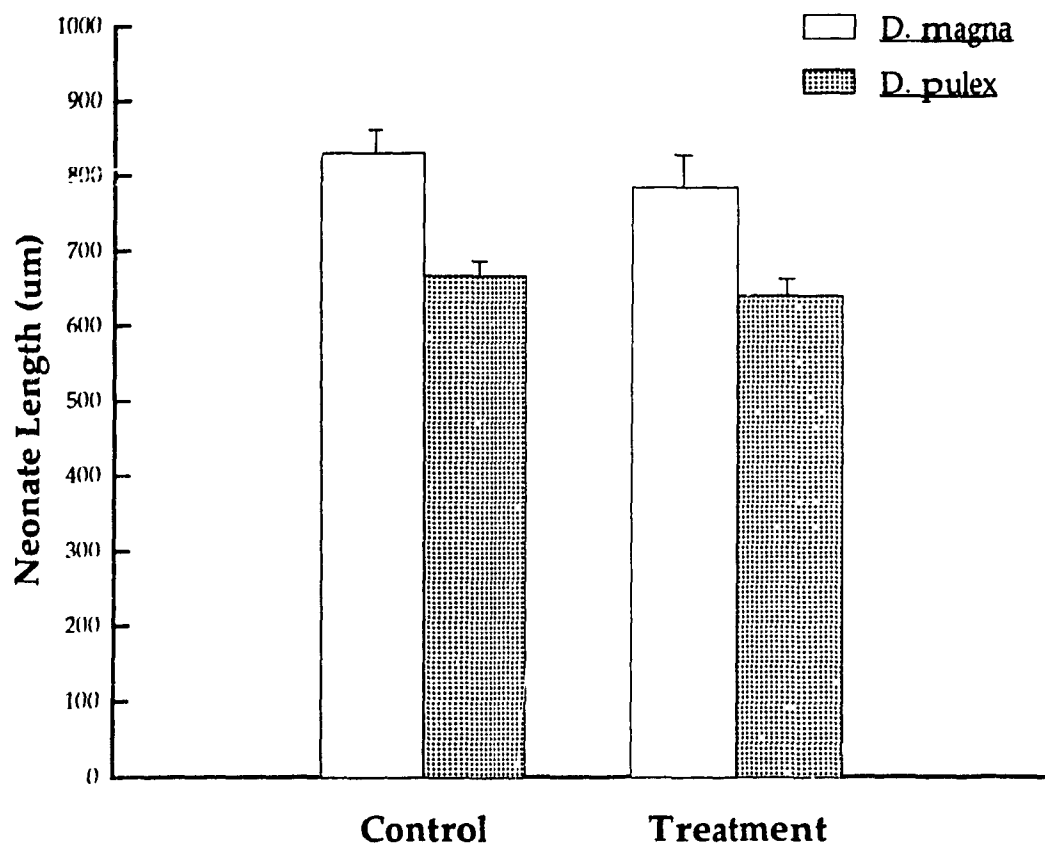
Although ANCOVA indicated that the number of neonates produced by D. magna and D. pulex was not significantly affected by exposure to aluminum sulphate ($F_{1,105}=1.8$; $p=0.187$, Figure 6; Table 3), the interaction between treatment and species was significant ($F_{1,105}=31.9$; $p<0.001$).

There was a significant effect of aluminum sulphate on reproductive output (number of neonates produced by each female multiplied by the neonates' average dry mass) ($F_{1,105}=7.9$; $p<0.01$, Figure 7; Table 3). Furthermore, there was a significant interaction between treatment and species ($F_{1,105}=28.1$; $p<0.001$).

Figure 6. Bar graph of number of neonates of both <u>Daphnia magna</u>

and <u>D</u>. <u>pulex</u> at control and treatment concentration (6.0 mg/L)

of aluminum. The data are presented as mean ± standard
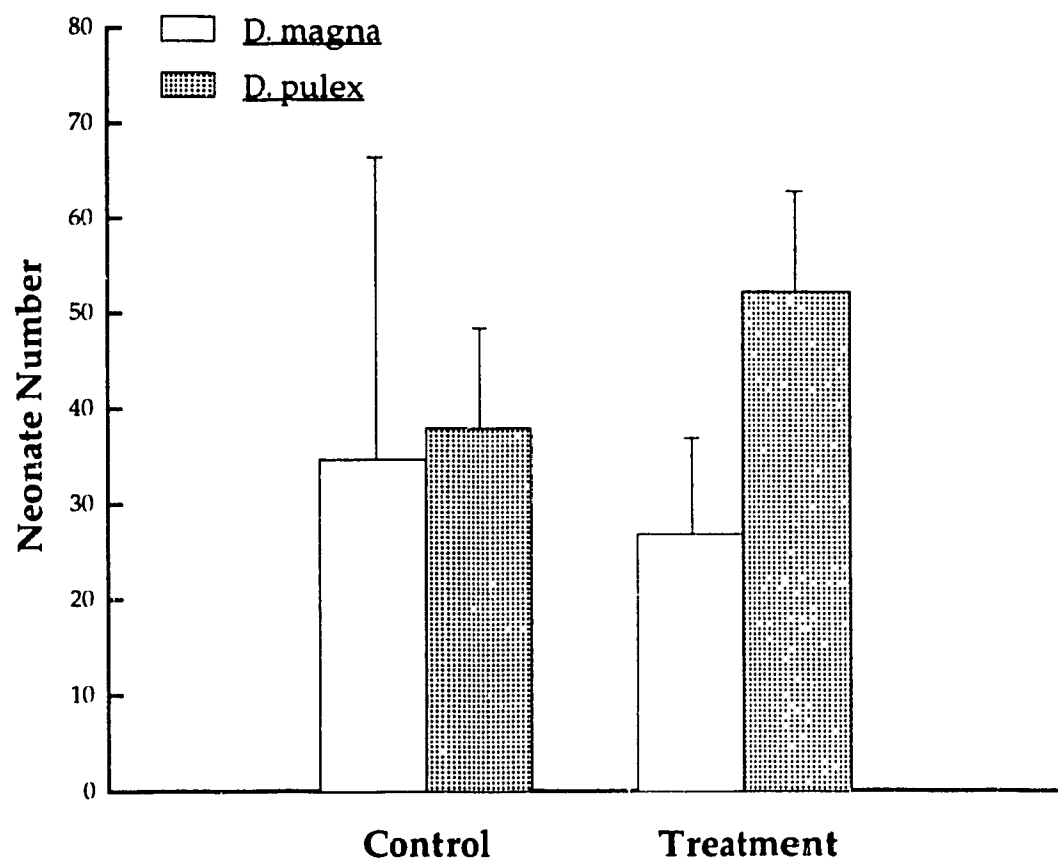
deviation.

Figure 7. Bar graph of reproductive output of both _Daphnia magna_

and _D. pulex_ at control and treatment concentration (6.0 mg/L)

of aluminum. The data are presented as mean ± standard
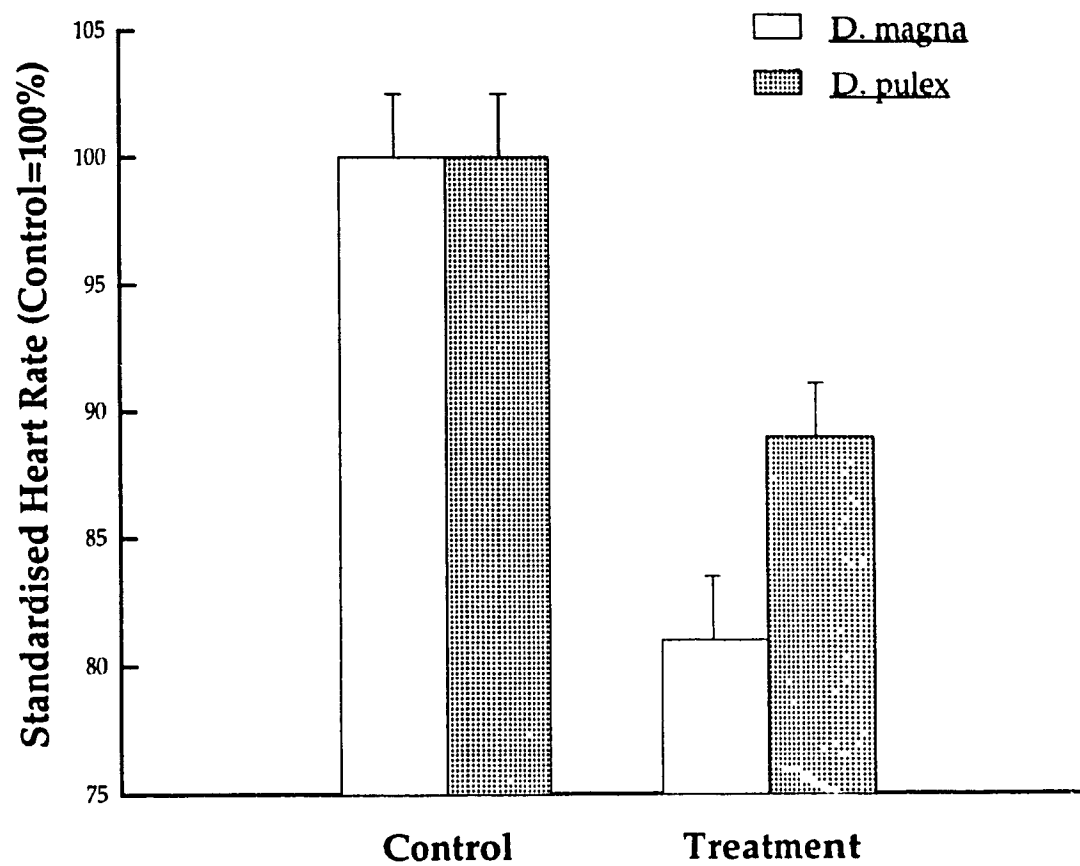
deviation.
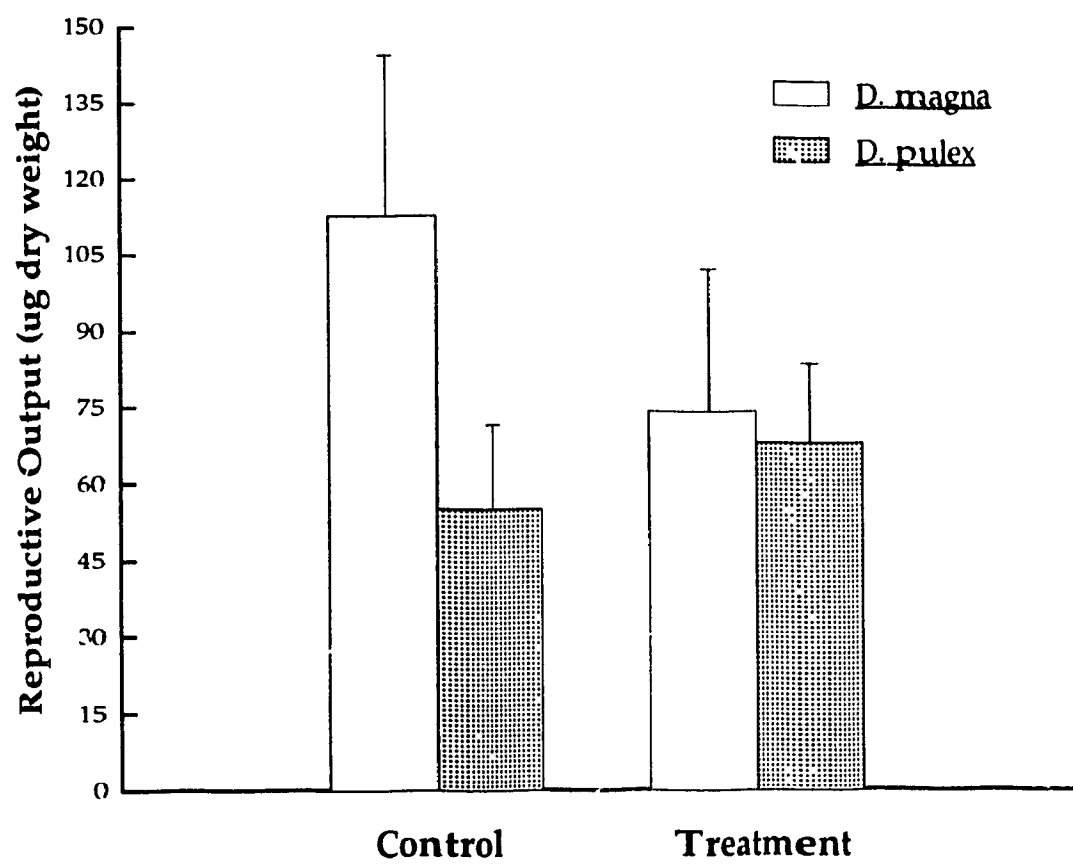
Part 2: Comparison of Heart Rate Observations with Acute Lethality Test Results Using Sodium Chloride as the Toxicant.

Heart Rate

The regressions of standardised heart rate (Percent HR) against the natural log of NaCl concentration (mg/L) were significant in both $\underline{D}.$ magna and $\underline{D}.$ pulex ($F_{1,32}$=48.5; p<0.001 and $F_{1,32}$=38.3; p<0.001, respectively, Figure 8). The regression equations are given in Equation 3 and 4 below:

Species	Regression Equation
$\underline{D}.$ magna	Percent HR=(-13.5)Ln Concentration + 180.0 (3)
$\underline{D}.$ pulex	Percent HR=(-11.3)Ln Concentration + 167.2 (4)

ANCOVA did not reveal any significant interaction between concentration and species ($F_{1,62}$=0.695; p>0.05), indicating that the slopes of the two regressions were not significantly different. The calculated IC10 values were 785 mg/L for $\underline{D}.$ magna and 925 mg/L $\underline{D}.$ pulex. The was not, however, a significant species effect on heart rate between $\underline{D}.$ magna and $\underline{D}.$ pulex ($F_{1,62}$=0.409; p>0.05).

Figure 8. Standardized heart rate (control values represent 100%)

regressed against log concentration of sodium chloride. Each

point of the graph represents 1 individual. The dotted line is

for Daphnia pulex; the solid line is for D. magna.
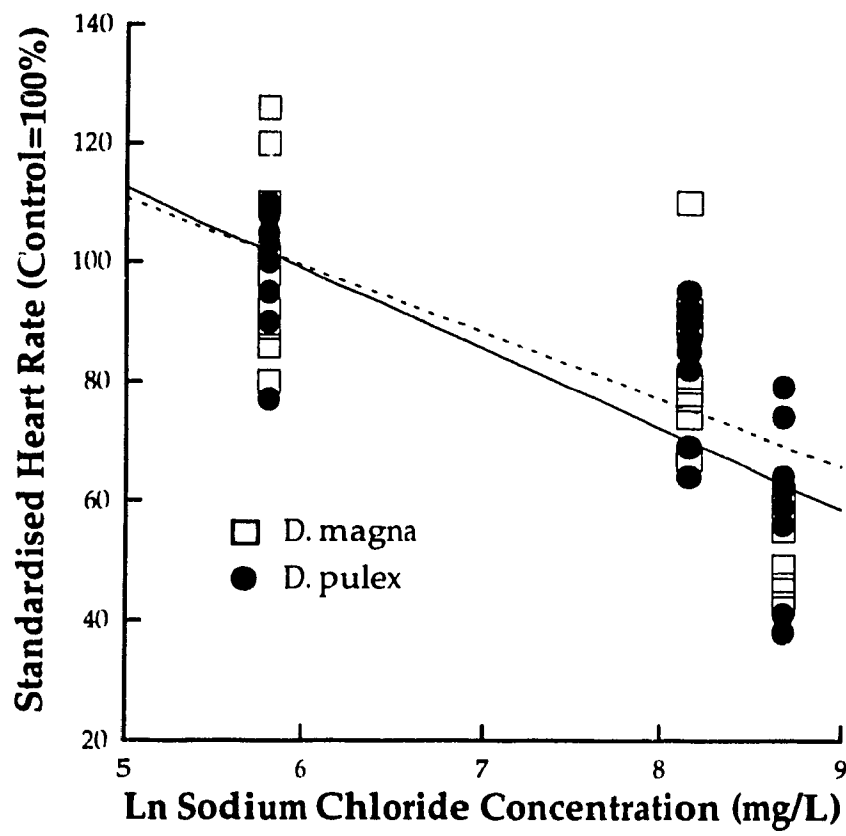
Acute Lethality

The dose-response regression models for D. magna and D. pulex using the Maximum Likelihood Estimation method, are as shown in Figure 9 and Equations 5 and 6:

Species | Maximum Likelihood Regression

D. magna | Probit Mortality=(1.05)Ln Concentration -2.99   (5)

D. pulex | Probit Mortality=(3.08)Ln Concentration - 18.34   (6)

The calculated LC50 values with corresponding 95% confidence intervals, according to the MLE method, are 1998 (1274-2566) mg/L for D. magna and 1943 (1618-2296) mg/L for D. pulex. Only one iteration was run to calculate the LC50.

Figure 9. Probit regression of mortality plotted against log

concentration of sodium chloride for <u>Daphnia</u> <u>magna</u> (r^2=0.95;

p<0.01) and <u>D</u>. <u>pulex</u> (r^2=0.90; p<0.01).

.
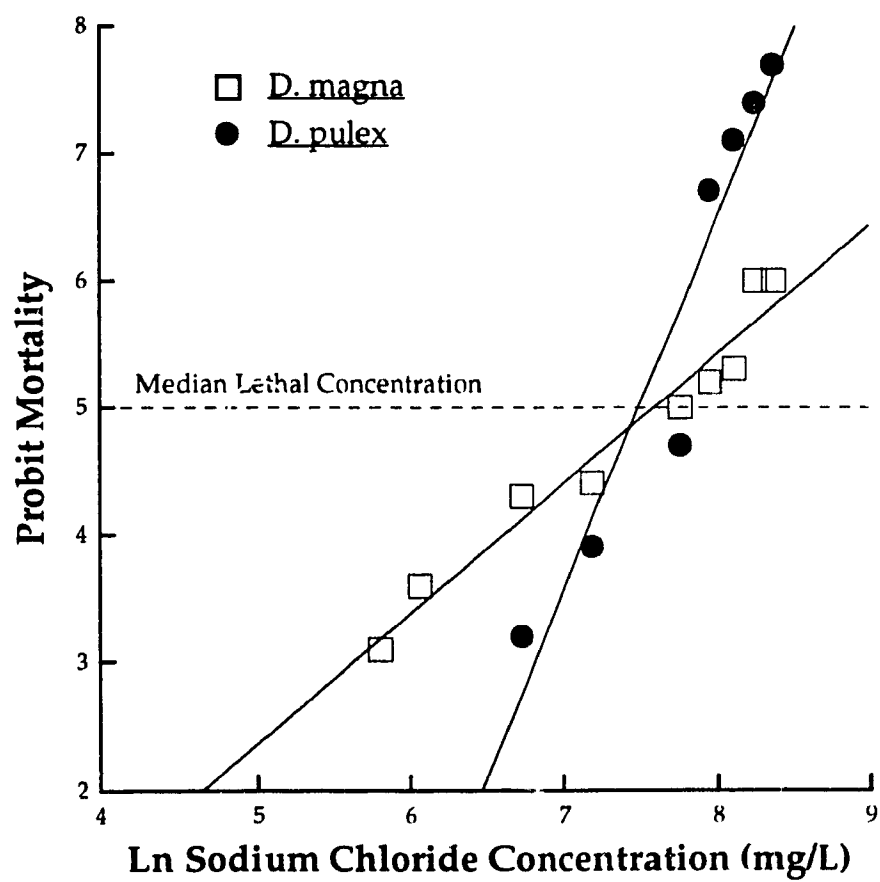
Part 3: Comparison of Heart Rate Observations with Acute Lethality Test

Results Using Cadmium Chloride as the Toxicant.

Heart Rate

Regressions of Percent HR against the natural log of cadmium

concentration were significant in both $\underline{D}$. $\underline{magna}$ and $\underline{D}$. $\underline{pulex}$ ($F_{1,55}=54.7$;

$p<0.001$ and $F_{1,55}=136.5$; $p<0.001$, respectively, Figure 10). The regression

equations are:

Species	Regression Equation	
$\underline{D}$. $\underline{magna}$	Percent HR=(-3.6)Ln Concentration + 82.3	(7)
$\underline{D}$. $\underline{pulex}$	Percent HR=(-3.3)Ln Concentration + 83.7	(8)

ANCOVA did not reveal a significant interaction between concentration

and species ($F_{1,108}=0.195$; $p>0.05$), indicating that the slopes of the two

regressions were not significantly different. The IC10 values were 0.12 and

0.15 for $\underline{D}$. $\underline{magna}$ and $\underline{D}$. $\underline{pulex}$, respectively. Again, there was no significant

species effect on Percent HR ($F_{1,108}=0.035$; $p>0.05$).

Figure 10.   Standardized heart rate (control values represent

100%) regressed against log concentration of cadmium. Each

point of the graph represents 1 individual. The dotted line is

for Daphnia pulex; the solid line is for D. magna.
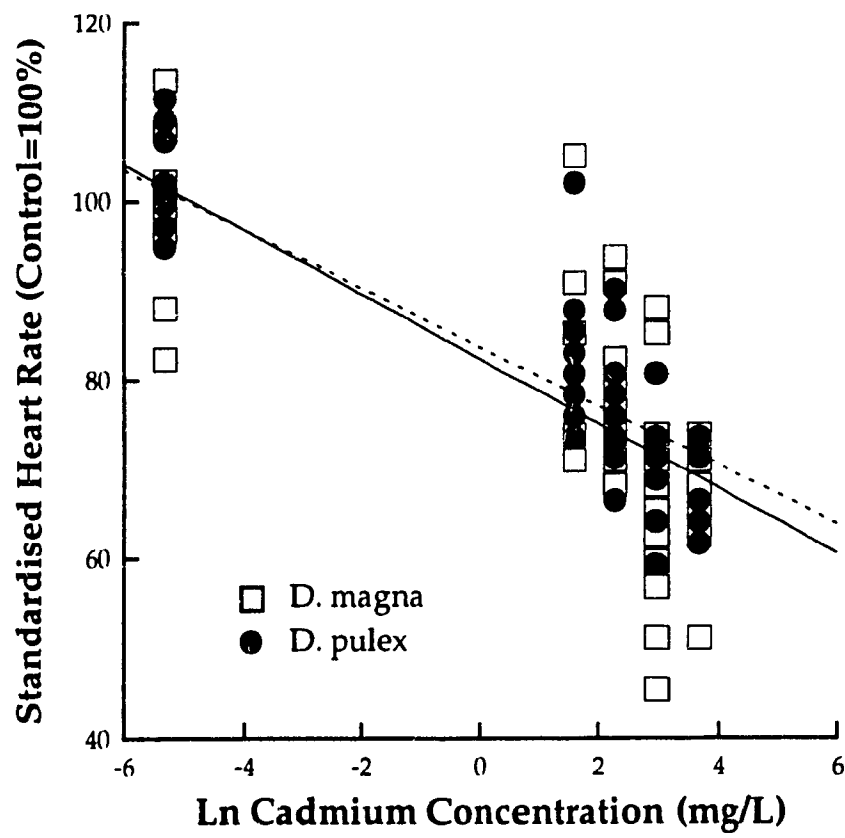
Acute Lethality

The dose-response regressions for the responses of D. magna and D. pulex to cadmium chloride, based on the MLE method, are as shown in Figure 11 and Equations 9 and 10:

Species	Maximum Likelihood Regression	
D. magna	Probit Mortality=(1.09)Ln Concentration +3.99	(9)
D. pulex	Probit Mortality=(0.93)Ln Concentration + 2.76	(10)

The calculated LC50 values with corresponding 95% confidence intervals were 2.5 (0.8-3.2) mg/L for D. magna and 8.67 (4.01-13.8) mg/L for D. pulex. Again, only one iteration of the MLE was run.

Figure 11. Probit regression of mortality plotted against log

concentration of cadmium chloride for <u>Daphnia</u> <u>magna</u>

(r^2=0.97; p<0.01) and <u>D</u>. <u>pulex</u> (r^2=0.91; p<0.05).
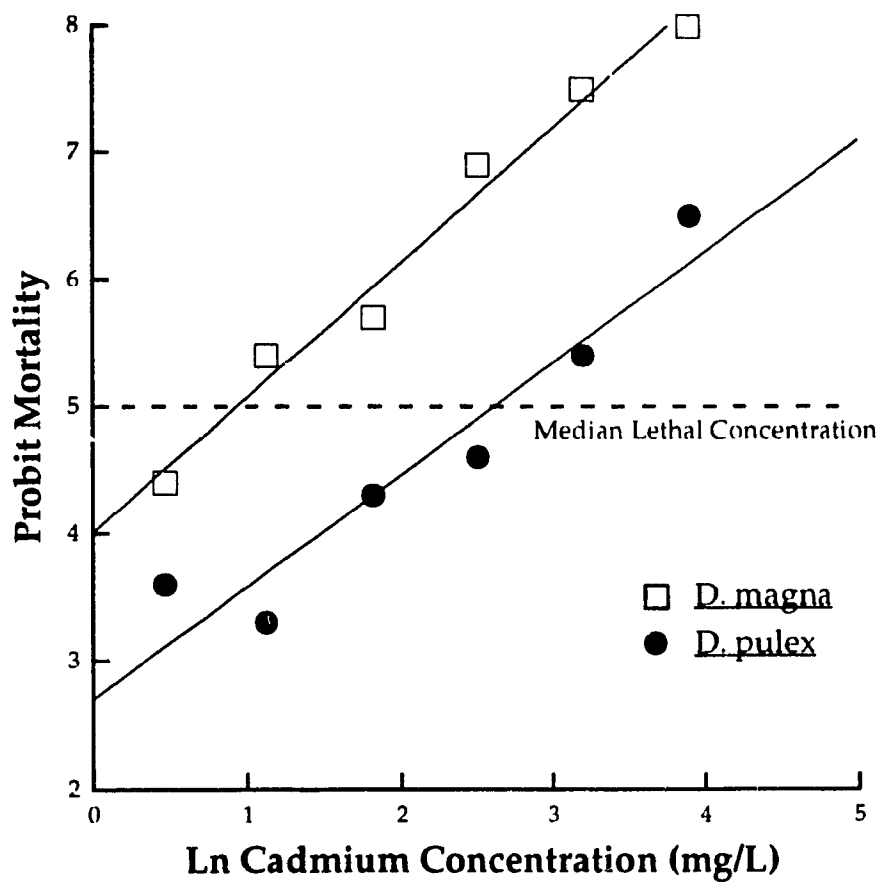
Part 4: A Comparison of Toxicity Ranking Order of Aluminum Sulfate,

Sodium Chloride and Cadmium Chloride According to Heart Rate

Observations

Heart Rate

Although there were no statistically significant differences in effect on

heart rate between D. magna and D. pulex within either sodium chloride or

cadmium chloride, there were significant differences between toxins within a

species (Figure 12). ANCOVA of the effects of treatment concentration and

toxicant on heart rate revealed that the sodium chloride was significantly less

effective than aluminum sulfate ($F_{1,106}$=28.6; p<0.001) and cadmium chloride

($F_{1,85}$=40.4; p<0.001) in reducing heart rate in D. magna. There was no

significant difference between the effects of aluminum sulfate and cadmium

chloride in reducing heart rate in D. magna. The concentration-response

regression slope for sodium chloride was significantly lower than that of

aluminum sulfate ($F_{1,106}$=7.5; p<0.01) and tended to be less than that of

cadmium chloride ($F_{1,85}$=3.6; p=0.06). There was no significant difference in

the regression slopes of aluminum sulfate and cadmium chloride in tests

using D. magna.

In tests using Daphnia pulex, sodium chloride was significantly less

effective at reducing heart rate than cadmium chloride ($F_{1,85}$=44.4; p<0.001).

There was no significant difference in the slopes ($F_{1,85}$=2.2; p>0.05).

Figure 12. Concentration-response regessions for aluminum

sulfate, sodium chloride, and cadmium chloride as derived

from the heart rate observations
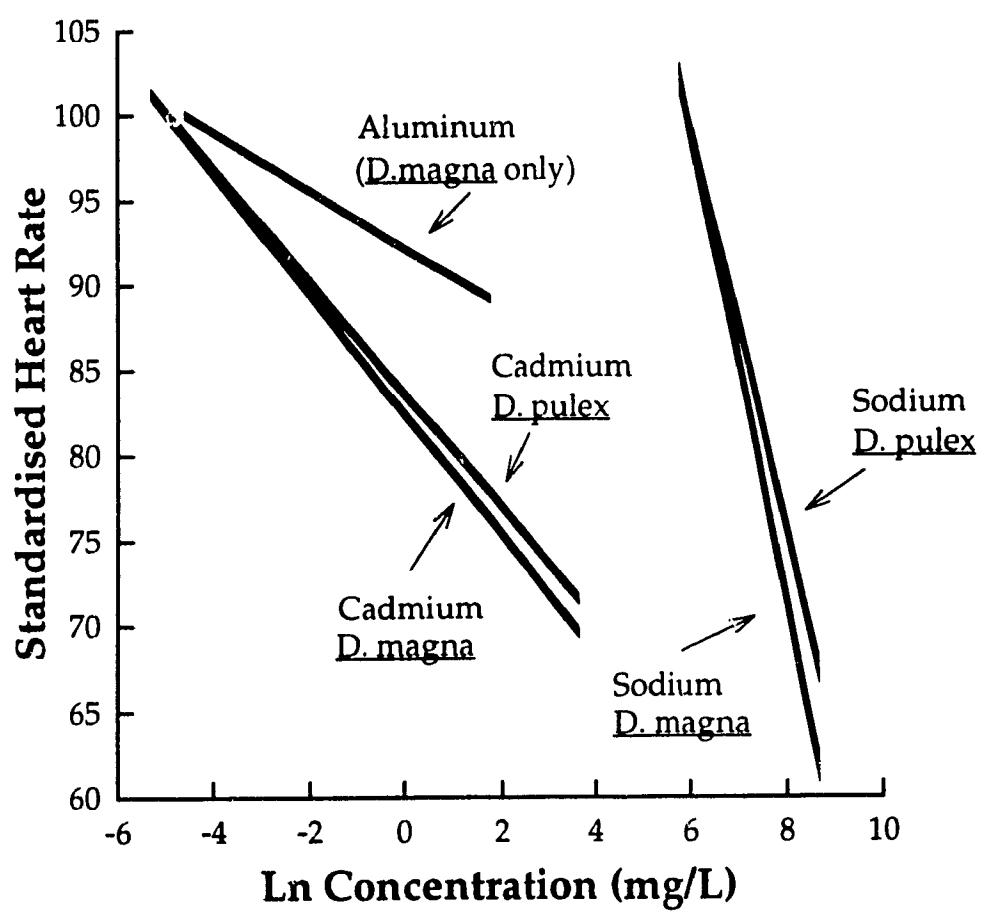
The toxicity ranking of aluminum sulfate, sodium chloride and cadmium chloride for D. magna was: Cd=Al>>Na. For D. pulex, the ranking was Cd>>Na.

DISCUSSION

The results of this investigation clearly show that exposure to aluminum sulfate, sodium chloride and cadmium chloride causes a significant decrease in heart rate for both <u>Daphnia</u> <u>magna</u> and <u>D</u>. <u>pulex</u>. Linear regressions of log concentration against heart rate were significant for <u>D</u>. <u>magna</u> exposed to all three toxicants, and <u>D</u>. <u>pulex</u> exposed to sodium chloride and cadmium chloride, indicating a concentration-dependent relationship. The concentrations corresponding to a 10% reduction of heart rate (IC10) in <u>Daphnia</u> <u>magna</u> and <u>D</u>. <u>pulex</u> were 3.51 mg/L for aluminum, 785-925 mg/L for sodium chloride and 0.12-0.15 mg/L for cadmium. These values are equal to or much less than LC50 values for the three toxicants. With respect to life history traits, although they may be more sensitive than bioassays utilizing heart rate counts, the results obtained in the first part of this study indicate that heart rate observations are potentially more consistent, and require much less time and effort.

The effectiveness of heart rate observations in detecting differences in species sensitivity to toxicants was comparable to the results obtained from measuring neonate length (life history trait), but was superior to the other life history traits examined, specifically, the number of neonates produced, and total reproductive output (average mass of neonates produced by an adult female multiplied by the number of neonates produced). The effects of aluminum on both neonate number and reproductive output were

contradictory when the results from D. magna and D. pulex were compared. As previously mentioned, a major problem with using life history tests, aside from the time involved, is that traits such as number and size of offspring tend to first increase, peak, then decrease. Accordingly, choosing appropriate toxicant concentrations is crucial in order to obtain results that can be compared to those of other studies.

Hormesis, an event whereby low doses of a toxicant cause a result opposite to that expected, may explain why D. pulex had greater number of neonates and greater reproductive output in the treatment than in the control. An example of hormesis can be found in Bodar et al. (1988), where cohorts of D. magna were exposed to concentrations of cadmium ranging from 0 to 10 ppb for 25 days and the number of young produced were recorded. It was found that a concentration between 0 (or control water) and ~7 ppb caused an increase, not a decrease, in the number of young produced. Beyond ~7ppb cadmium, the number of young produced decreased, the effect that was anticipated. Accordingly, the treatment concentration in the 14 day aluminum study may have been lower than the concentration necessary to overcome the effect of hormesis for D. pulex, possibly indicating that D. pulex is more tolerant of aluminum than D. magna.

When standardised heart rate values of D. magna and D. pulex were plotted against Ln concentration of sodium chloride and cadmium chloride, there was no significant difference in the slopes, and no significant species effect. These results indicate that is no detectable difference in the sensitivity

between the two species according to the effect on heart rate, which is consistent with previously published studies examining differential tolerance among Daphnia species (Canton and Adema, 1978; Winner and Farrell, 1976).

Analysis of the median lethal concentration (LC50) also indicated that there was no difference in sodium chloride tolerance between D. magna and D. pulex, with LC50 values of 1998 mg/L (1274-2566) and 1943 mg/L (1618-2296), respectively. The LC50 estimates for cadmium, however, indicated a significant difference in the relative tolerances between the two species. The respective values were 2.5 mg/L (0.8-3.2) for D. magna and 8.7 mg/L (4.0-13.8) for D. pulex. Although the LC50 estimates for cadmium are slightly higher than previously published values (0.04-1.88 mg/L; Appendix 1), considering that there is over a 500 fold difference between the highest and lowest published value, the estimates from this study should not be considered to be aberrant. Furthermore, the huge variation in published LC50 values casts doubt on whether or not there was actually a difference in tolerance to cadmium between the two species.

The IC10 and LC50 results yield a similar toxicity order (Cd>>NaCl) for sodium and cadmium in both species. When aluminum is included in the order by using published 48-hour LC50 values (Appendix 1), it is clearly more toxic than sodium chloride, but may or may not be more toxic than cadmium chloride; the ambiguity is attributed to large variation in published LC50 values (Khangarot et al., 1989; Beisinger et al., 1972). When the aluminum data from the heart rate observations is incorporated, the toxicity order is:

Cd=Al>>NaCl. The slopes of the heart rate concentration-response regressions of the metals were significantly lower than that of sodium chloride in tests using D magna. Toxicants with a steeper concentration-response regression slope indicate much greater sensitivity once a threshold concentration has been exceeded, thus greater caution should be used in setting maximum allowable concentrations for these toxins.

Although a specific hypothesis regarding the effect of toxicants on heart rate was presented in the introduction, there are many additional mechanisms through which exposure to toxicants may affect the daphnid heart rate, including altered ionic concentrations in the hemolympth due to toxicant binding/interference on the ion-exchange sites, impairment of the calmodulin-mediated "calcium pump" within the heart muscle cell, and interference with mitochondria.

The second potential mechanism responsible for decreases in heart rate is altered concentrations of sodium and/or potassium due to toxicant mediated effects on ionic equilibrium. Decreased sodium has been demonstrated to reduce the beating frequency of the crustacean heart (Maynard, 1960). Whether or not the heart of Daphnia spp. conforms to the model crustacean heart described by Maynard is subject to speculation. Havas (1985) reported a net loss of sodium from D. magna exposed to elevated aluminum concentrations. This loss could be due to the binding of aluminum to the daphnids' ion-exchange sites as described by Havens (1990), who noted that acid and aluminum sensitive zooplankton had significantly

greater aluminum bound to their maxillary glands (gas and ion-exchange sites). Cadmium and other metals such as copper and zinc are known to bind to gills of fish (Sorensen, 1992), and presumably could exert a similar affect as aluminum on the sodium balance in crustaceans. Although decreased sodium in the hemolymph may be responsible for the decrease in heart rate in the aluminum and possibly cadmium tests, the daphnids exposed to NaCl most certainly did not suffer from decreased sodium. Therefore, although decreases in sodium may contribute to the observed effect, there must be other mechanisms involved.

A third mechanism by which metal ions may affect the heartbeat frequency of Daphnia spp. is through interference of the calmodulin-mediated removal of Ca^{++} from the heart muscle cell. After a contraction in a normally functioning heart, calcium ions must be pumped out of the muscle cell for relaxation to occur (Guyton, 1976). The "calcium pump" (Ca^{++}/Mg^{++} ATPase) is regulated by calmodulin (Sigel, 1988). Metal ions have been shown to bind to calmodulin (and most other biological enzymes), causing conformational changes (Farnell et al., 1985), which may lead to inactivation or reduced activity. Impairment of the calmodulin-regulated calcium pump would lead to either an increase in intracellular $Ca++$, or an increased time period for removal of $Ca++$ from the cell which would conceivably reduce the frequency of the heart rate.

Although not specifically examined in vivo, a fourth potential mechanism of metal toxicity on the heart (and most other cells in the

organism) is interference with the mitochondria. Mitochondria are organelles specialised to carry out cellular respiration, oxidative phosphorylation, and represent the main source of ATP for most cells (Lehninger, 1982). Metals from group IIb of the periodic table are believed to alter the conformation, stability and function of mitochondria (Brierley, 1976). The reduction in ATP can be as great as 75% or more (Mustafa et al., 1971). A reduction in energy production would obviously effect heart function.

The four potential mechanisms of toxicant effect on heart rate express specific hypotheses regarding the action of aluminum, sodium and cadmium on heart rate. However, since metal ions can bind to practically all physiologically important enzymes to varying degrees, there are thousands of biochemical reactions that could be impeded and result in an overall decrease of metabolism, and heart rate has been demonstrated to be a good descriptor of metabolism in freshwater crustaceans (Ingle et al., 1937), and marine copepods (Pavlova and Minkina, 1983). The ability of a particular metal ion to disrupt normal physiological functions, thus causing toxicity, has been investigated in Daphnia magna (Khangarot and Ray, 1989). Of the 35 physicochemical properties that were investigated, the properties with high correlations with toxicity were electronegativity (r=0.736), melting point (r=-0.933), the negative log of specific heat (r=0.797), thermal conductivity (r=0.794) and equilibrium constants of various amino acids.

Although it is believed that certain metal ions will bind to, and possibly disrupt, catecholamines at nM concentrations (Sigel, 1988), the heart rate

would not likely be significantly affected in <u>Daphnia</u> since their hearts are believed to be myogenic, thus not dependent on neural activity (Maynard, 1960).

Regardless of the mechanism by which heart rate is affected by toxicants, the results from this investigation have shown that heart rate is lowered in a dose-dependent manner, and the results are consistent for both D. <u>magna</u> and <u>D. pulex</u>. The potential effectiveness of heart rate as a toxicity index, based on the 2 hour exposure period, has not been confirmed by the results of this preliminary study. Although statistically significant reductions in heart rate may occur over the range of concentrations found in contaminated waters, comparison with LC50 values indicates that there may be significant mortality before the IC10 index is reached. This does not mean, however, that heart rate measurements cannot be incorporated into a useful bioassay for toxicant stress. Since the concentrations used in the current study were high relative to the LC50 values, future work using lower toxicant concentrations may be able to increase the resolution of the test, allowing IC1 or IC2 concentrations to be determined. Secondly, the 2 hour exposure period may have been insufficient for the effect on heart rate to be maximised. An exposure period of 24 to 48 hours would ensure that the lethal threshold is not exceeded, and would most likely increase the resolution of the test. In order for heart rate to be incorported into a useful toxicity index, further work aimed at increasing the sensitivity of the test, and correlating heart rate to ecologically relavent effects, would be required.

Physiological parameters such as heart rate have the potential to yield information about how organisms are able to cope with their environment, i.e., ability to escape predation, choice of microenvironment (epilimnion vs metalimnion) and/or time to development. Such information is simply not available from acute lethality tests which only show direct action of the toxin being tested. The more physiological parameters that are examined in the study of toxic effects of pollutants, the greater our understanding of how these pollutants will affect the organisms, allowing us to make more informed and intelligent decisions.

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Appendix 1: Published Median Lethal Concentrations of aluminum, sodium chloride, and cadmium.

Study	Toxicant	48hr-LC50 Value
Khangarot and Ray 1989	Aluminum	59.6 mg/L
Biesinger and Christensen 1972	Aluminum	3.9 mg/L
Khangarot and Ray 1989	Sodium	402.6 mg/L
Biesinger and Christensen 1972	Sodium	1640 mg/L
Naylor et al. 1992	Cadmium	0.004-0.020 mg/L
Bodar et al. 1990	Cadmium	0.230-0.495 mg/L
Khangarot and Ray 1989	Cadmium	1.88 mg/L
Fargasova 1994	Cadmium	1.462 mg/L
Enserink et al. 1990	Cadmium	0.1-0.3 mg/L
Biesinger and Christensen 1972	Cadmium	0.065 mg/L

Appendix 2: Preparation of YTC Daphnia Food[*]

Preparing Digested Trout Chow [**]

1. Preparation of trout chow requires one week. Use starter or No. 1 pellets.

2. Add 5.0 g of trout chow pellets to 1 L of deionized (Milli-Q™ or equivalent) water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation should be replaced during digestion. Because of the offensive odour usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.

3. At the end of the digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (e.g. Nitex™, 110 mesh). Combine with equal volumes of supernatant from Cerophyll™ and yeast preparations (see following). The supernatant can be used fresh, or frozen until use. Discard the sediment.

Preparing Yeast

1. Add 5.0 g of dry yeast, such as Fleischmann's™, to 1 L of deionized water.

2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.

3. Combine the yeast suspension immediately (with no settling) with equal volumes of supernatant from the trout chow and Cerophyll preparations (see following). Discard excess material.

Preparing Cerophyll (Dried, Powdered Cereal Leaves)

1. Place 5.0 g of dried, powdered Cerophyll or cereal leaves[***] in a blender. Dried, powdered alfalfa leaves from health food stores have been found to be a satisfactory substitute for cereal leaves.

2. Add 1 L of deionized water.

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[*] From Environment Canada (1992).

[**] Researchers at U.S. EPA's Environmental Monitoring Systems Laboratory in Cincinnati, Ohio recommend using the commercially available tropical fish food "Tetra-min™" as a suitable substitute for trout chow (J.M. Lazorchak and P.A. Lewis, pers. commun., 1991).

[***] Available as "Cereal Leaves" from Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri 63178 (800-325-3010); or as Cerophyll™ from Ward's Natural Science Establishment Inc., P.O. Box 92912, Rochester, New York 14692-9012 (716-359-2502).

3. Mix in a blender at high speed for 5 min,
   or stir overnight at medium speed on a
   magnetic stir plate.

4. If a blender is used to suspend the material,
   place in a refrigerator overnight to settle.
   If a magnetic stirrer is used, allow to
   settle for 1 h. Decant the supernatant
   and combine with equal volumes of
   supernatant from trout chow and yeast
   preparations. Discard excess material.

Preparing Combined YCT Food

1. Mix equal (approximately 300 mL)
   volumes of the three foods previously
   described.

2. Place aliquots of the mixture in small
   (50 to 100 mL) screw-cap plastic bottles
   and freeze until needed.

3. Freshly prepared food can be used
   immediately, or it can be frozen until
   needed. Thawed food is stored in the
   refrigerator between feedings. and is
   used for a maximum of two weeks.

4. It is advisable to measure the dry weight of
   solids in each batch of YCT before use.
   The food should contain 1.7 to 1.9 g
   solids/L. Cultures or test solutions
   should contain 12 to 13 mg solids/L.