Hazard/Risk Assessment

ENERGY-BASED MODELING AS A BASIS FOR THE ANALYSIS OF REPRODUCTIVE DATA WITH THE MIDGE (CHIRONOMUS RIPARIUS)

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Abstract—We propose a biologically based approach to analyze reproductive data for the midge (Chironomus riparius). We showed in a previous study that its larval development can be divided in two distinct phases regarding the use of energy: The somatic growth period and the gametic growth period. We hypothesize here the gametic period to be a crucial period for the energetic investment for reproduction. To test this, we performed several assays with different feeding programs. Our results confirmed that energetic investment for reproduction mainly occurs during the gametic growth period. We could then propose energy-based models to analyze reproductive data. We assumed the effects to result from a perturbation in the use of energy. Two models corresponding to different physiological modes of actions—decreased efficiency of feeding (i.e., feeding decrease model) or increased cost of egg production (i.e., egg-cost increase model)—were built and used for the analysis of data obtained after an exposure to an artificially copper-spiked sediment. During this experiment, different exposure patterns were performed to investigate whether effects on reproduction resulted mainly from an exposure that occurs during the gametic growth period or from an exposure that occurs during the somatic growth period. These exposure patterns led to similar effects on reproduction, which suggests that the toxicant-induced stress persists during the whole life cycle, even in case of exposure occurring only during part of it. Both the feeding decrease model and egg-cost increase model were able to describe the data. We showed that addition of copper in the sediment, even in low quantity (<6.5 mg/kg), might affect reproduction at the individual level. If no-effect concentrations were derived from these results, this may lead to an overprotective value. A relevant no-effect concentration could be derived from further investigations at the population level.

Keywords—Chironomus riparius  Fecondity  Biologically based models  Feeding levels  Copper-spiked sediment

INTRODUCTION

Sediment particles provide shelter and food to many aquatic invertebrates, either during their whole development or during part of it. The amounts of chemicals accumulated in the sediment from surface water constitute an increasing concern for benthic communities. Consequently, sediment toxicity assessment has become a critical issue. Acute and chronic effects of sediment-binding chemicals are commonly studied via bioassays involving Chironomidae [1]. Indeed, these organisms are particularly relevant for sediment toxicity assessment from both an ecological and a toxicological point of view [2]. Chironomidae also have practical advantages (e.g., breeding and handling facilities) [3].

A number of risk assessment studies have focused on survival during an acute exposure at high concentrations of toxicant. Nevertheless, toxicity in field-contaminated sediment often occurs at sublethal levels because of a long-term exposure to lower doses. Furthermore, sublethal endpoints, such as growth and reproduction, are assumed to be integrative variables that respond to most of the physiological disturbances resulting from environmental stressors [4]. Thus, they are very sensitive and ecologically relevant endpoints [3]. Furthermore, the analysis of reproductive output permits assessment of toxic effects at the population level [5], which has been shown to be more relevant than the individual level to the functional complexity of ecosystems [6,7]. Understanding the effects of toxicants on reproduction implies identifying factors affecting this major life-history parameter. We showed in a previous paper [8] that energy is a key factor in analyzing reproductive data. This suggests that the assessment of toxic effects on reproduction should focus on effects regarding energy management by the organism. In Chironomidae, energy allocation to reproduction is a continuous process occurring during larval development, whereas reproduction itself is event driven. This means that the energy allocated to reproduction first accumulates (probably into lipid reserves, as in many insects, including Chironomus tentans [9]) and that these reserves are converted into a clutch of eggs [10]. Characterizing this energy accumulation process for Chironomidae raises two main issues. First, does accumulation of reserves for reproduction also occur during the somatic growth of the larva, or can we isolate a particular investment period once the maximal size (i.e., the end of somatic growth) is achieved? Second, if this investment period for reproduction exists, can the effects of the toxicant on reproduction be linked to an exposure that occurs during this period in particular?

Another issue is to develop mechanistic models to analyze properly the reproductive data obtained from toxicity tests with Chironomidae. Indeed, the modeling approach should be preferred to a classic statistical treatment of the data, because this latter approach is badly adapted to the measurement of chronic effects [11]. Furthermore, statistical analysis usually provides no-effect concentrations (NECs) or lowest-effect concentrations, which have been criticized on both practical and statistical grounds [10,12]. Finally, descriptive analysis (using, e.g., nonlinear regression techniques) consists of a posteriori obtaining the best fit to the bioassay dose–response curve without

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making any biological hypothesis. Consequently, the estimated parameters have hardly any biological meaning, and the fit can never account for previous knowledge or assumptions about the mode of action of the compounds.

Kooijman and Bedaux [13] have shown that mechanistic models are suitable for the analysis of standard reproduction tests with *Daphnia magna*. Their biologically based models, also called DEBtox models, are derived from the dynamic energy budget theory [14], which is a general model that accounts for the use of energy by the organisms. We recently built a similar model for the use of energy by Chironomidae [8]. This model can be used as a relevant basis for the development of effects models for the analysis of reproductive data in Chironomidae.

In the present study, we examine effects on reproduction resulting from a chronic exposure of the midge (*Chironomus riparius*) to artificial sediment spiked with copper. To characterize the use of energy during the larval development for *C. riparius* and to check the existence of an investment period for reproduction, we carried out four bioassays on uncontaminated sediment with different feeding diets (i.e., varying amount of fish food delivered daily during a treatment and between treatments during 21 d of exposure). To test if the effects of copper on reproduction can be linked to an exposition during this investment phase for reproduction in particular, we carried out three bioassays on spiked sediment with different periods of exposure regarding the larval development. Finally, we developed two biologically based models for the mode of action of copper to analyze reproductive data for *C. riparius*.

**MATERIALS AND METHODS**

**Experimental procedures**

Characterization of the larval energetic investment for reproduction in *C. riparius*. In a previous paper [8], we showed that the amount of energy necessary for reproduction is stored during larval development. We also showed that the larval development of *C. riparius* can be divided in two distinct phases: the somatic growth period (divided into four larval instars) and the gametic growth period, i.e., from the end of somatic growth (marked by a stabilization of body length at the fourth instar) to pupation. In the present study, we hypothesized that the gametic growth period may correspond to a period of energetic investment for reproduction, and we tested this assumption. If the energetic investment for reproduction only begins at the end of somatic growth, a change in the amount of food delivered daily to the larvae at this time should affect the fecundity of females.

Four assays on uncontaminated sediment were performed to test this latter hypothesis. We used an artificial sediment (silica sand with the following particle size distribution: 90%, 50–200 µm; 10%, <50 µm). This sediment was incubated for three weeks with water and 1 g of ground Tetramin® (Tetra-werk, Melle, Germany) fish food per 15 L of wet sediment to allow a bacterial development that favors the survival of Chironomidae [15]. The test beakers were filled with 0.1 L of control sediment and 0.4 L of water. The sediment was then allowed to deposit for 3 d before the test under testing conditions of temperature, light, and aeration. The assays were initiated with 4-d-old larvae (beginning of the third larval instar). This starting instar was chosen both because we needed to have comparable experiments with and without copper-spiked sediments and because preexperiments showed that second-instar mortality is high at the copper concentrations we had chosen. Test organisms came from our laboratory culture. At day 0 of the test, 10 organisms were randomly introduced into each glass beaker. The beakers were kept at 21°C in a water bath with a 16:8-h light:dark photoperiod. Water was gently aerated. Conductivity, temperature, pH, dissolved oxygen, and nitrate and ammonium concentrations were measured daily during the tests.

Four diets (chosen according to previous results [8]) were compared. The first diet consisted of Tetramin at 0.2 mg/larva/d from the beginning of the test until the end of the somatic growth and then 0.6 mg/larva/d (ad libitum feeding level determined in Pery et al. [8]) until the end of the test. The other diets consisted of feeding Tetramin ad libitum until the end of growth and then 0.1, 0.2, or 0.3 mg/larva/d. Four replicates per diet were made. Growth models that were developed in the previous work [8] permitted us to predict the end of somatic growth, which corresponded to the day 6 of our experiment (10 d after hatching) for feeding ad libitum and to day 11 for feeding 0.2 mg/larva/d. The beakers were covered with net traps to prevent adults from escaping.

From the very start of emergence, imagos were withdrawn daily from the test beakers by aspiration using a device inspired by Benoit et al. [3] and then counted. Females emerging from each replicate corresponding to a defined diet and concentration were gathered into a bottle (1 L) containing a small quantity of water for egg laying. Bottles were immediately stored at 23°C after addition of two males per female. Because emergence occurs earlier for males than for females, not enough males were available from the test beakers to fertilize the emerging females. Benoit et al. [3] and Sibley et al. [5] showed for *C. tentans* that fecundity mainly depends on the female’s quality, whereas a male’s quality has little influence on this endpoint. We suppose that this might also be true for *C. riparius*. In addition, males that were withdrawn from the test beakers were supposed to be of a quality equivalent to that of males from our laboratory culture. Thus, we only used males from our laboratory culture to fertilize females. The laying obtained in each bottle was removed daily. The number of eggs per egg mass was counted according to the ring count method [3]. For asymmetrical egg masses, we achieved a manual counting of eggs using a binocular microscope. This latter method needed egg masses to be dissociated in an acid solution. Because only one egg mass is laid down per female for *C. riparius*, the mean number of eggs per egg mass corresponds to a measurement of fecundity.

Females of *C. riparius* remain receptive for reproduction during their whole life [16]. Thus, the test ended when all females were dead (day 21 after the beginning of the test).

**Characterization of the effects of copper on reproduction for *C. riparius***. We hypothesized that toxic effects on reproduction might be related primarily to an exposure during the period of energetic investment for reproduction. To test this assumption, we carried out three bioassays on copper-spiked sediment. We used the same artificial sediment as used previously. For each concentration tested, copper was dissolved into 0.8 L of water, and 1.98 kg wet sediment was mixed with this solution (i.e., rolling water) and introduced into a 2-L jar. Jars were then rolled as described in Ditsworth and Schults [17] for 6 h. They were kept at test temperature (21 ± 1°C [± SE]) for 10 days before the test and swirled manually each day to maximize the incorporation of copper into the sediment. According to pre-experiment tests, we chose nominal copper concentrations of 0, 6.5, 9.5, 12.5, 19, 25, and 50 mg/kg dry
sediment. We introduced the corresponding amount of copper into the jars knowing from previous experiments that the ratio between wet and dry sediments is 1.32.

To test the spiking efficiency, the concentration of copper was measured in the rolling water (i.e., the overlying water in the jars) after 10 d of equilibration and also in the spiked sediment as well as in the overlying water at the beginning of the test. The concentrations of copper in water and sediment were measured by atomic absorption photometry with the Analyst 300 flame photometer (Perkin-Elmer, Norfolk, CT, USA). Before analysis, water samples (20 ml) were acidified (with 0.5% v/v nitric acid), and sediment samples (500 mg) were dried (60°C for 48 h), milled, and mineralized (contact with an acid solution with 2.5 ml of nitric acid and 7.5 ml of hydrochloric acid for 12 h at room temperature and then for 2 h at boiling temperature).

Tests were initiated with 15 four-day-old larvae/beaker. Organisms were fed ad libitum (Tetramin, 0.6 mg/larva/d) during the entire test. The test design in these assays was similar to that used for experiments with uncontaminated sediments (see previous paragraph). To take into account the different phases of energy management during larval development, three exposure programs were set up: (1) Exposure covering the third and fourth instars, (2) exposure during somatic growth only, and (3) exposure during the gametic growth period only. For exposure programs 2 and 3, larvae were transferred into new testing media at the end of somatic growth. Four replicates per concentration and program were made. As discussed earlier, the end of the somatic growth in control conditions was determined from growth models developed previously [8]. We did not account for any growth delay resulting from copper, and we chose day 6 of our experiment to perform the transfer of the organisms for programs 2 and 3. Consequently, the exposure duration was 6, 15, or 21 d, respectively, for the three exposure programs. For exposure program 2, organisms were gently aspirated with a Pasteur pipette from the contaminated testing medium and immediately transferred to a control testing medium. For exposure program 3, larvae were transferred from control sediment to a spiked one. Spiked sediment came from the same jars that were used for exposure program 2. Jars had been stored at 4°C until this change of testing medium. New testing media were allowed to equilibrate for 3 d under testing conditions of temperature, light, and aeration before the transfer.

**Modeling the fecundity of *C. riparius*** The theoretical reproduction model was based on three main hypotheses. First, we assumed that the energetic investment for reproduction occurs only during the gametic growth period. Second, we assumed that during the gametic growth period, a fixed part of the energy assimilated by the larva is allocated to reserve storage for reproduction. Thus, in our models, fecundity only depends on the amount of energy that is stored during the gametic growth period (i.e., maintenance costs during the gametic growth period were not taken into account). Therefore, the amount of energy stored during the gametic growth period corresponds to the amount of food that is assimilated by the larva. Third, the energetic cost of egg production is assumed to be constant. Finally, with clean sediment, control mean fecundity ($N_0$) can be predicted from the amount of energy stored during the gametic growth period and the fixed cost of an egg [8]:

$$N_0 = \frac{D_0 \cdot \beta}{\delta_0} \tag{1}$$

where $\beta$ is the easily assimilated fraction of Tetramin for *C. riparius* ($\beta = 0.69$ [8]), $\delta_0$ is the cost for production of an egg in control females, and $D_0$ is the minimum value between the food available and the maximum amount of food possibly ingested per female during the gametic growth period ($D_{\text{max}}$). Consequently, we assume the mean fecundity to reach a limit value ($N_{\text{max}}$) under control conditions and ad libitum feeding:

$$N_{\text{max}} = \frac{D_{\text{max}} \cdot \beta}{\delta_0} \tag{2}$$

**Modeling the effects of copper on fecundity.** In a previous study [18], we showed that DEBtox (i.e., the dynamic energy budget for ecotoxicology model) assumptions [10] for kinetics were correct and permitted proper description of copper effects on survival. Thus, we used this theoretical basis to build our models for reproduction. Our models assume that reproductive effects are the consequence of a perturbation in the use of energy for the organisms that are exposed to the toxicant. They were built on three complementary units deriving chronic effects from the external concentration of the toxicant in the test beakers. Each unit is based on biological assumptions.

In a first step, the internal concentration of copper in the body of the larva is modeled from the copper concentration in the sediment. In the DEBtox model, the uptake of the compound is proportional to its concentration in the sediment, and its elimination is proportional to its concentration in the body (i.e., a linear one-compartment kinetics model). In our models, we assume that internal concentration has already reached equilibrium at the beginning of the gametic growth period. Thus, internal concentration is a constant value that is calculated directly from the concentration in the sediment knowing the bioconcentration factor. To minimize the number of parameters needing to be estimated in the model, the internal concentration was approximated by the external concentration ($c_0$). A significant deviation from this kinetics hypothesis would lead to a significant difference between the results of exposure programs 1 and 3 (i.e., exposure covering the third and fourth instars and exposure during the gametic growth period only).

In a second step, we analyzed how the concentration of the toxicant might affect the use of energy in test organisms. We suggest two toxicity mechanisms that might be responsible for reproductive effects via a perturbation in the use of energy by test organisms. The presence of the toxicant is supposed to lead either to decreased feeding (and, hence, reduced energy stored for reproduction) or to an elevated energy requirement to produce eggs. Note that effects are assumed to occur only above a concentration threshold, the NEC. In essential metals, the NEC does not take into account the physiological internal concentration of metal. Furthermore, as in DEBtox models, the effects are assumed to be proportional to the exposure concentration of the toxicant minus the NEC once the NEC is exceeded.

In the case of decreased feeding, we assume that the decrease in the feeding rate is proportional to ($c_c - \text{NEC}$) once the exposure concentration exceeds the NEC. The maximum amount of food assimilated by the larva in exposed organisms and under ad libitum feeding conditions ($D_{\text{max}}$) can be found as

$$D_{\text{max}} = D_{\text{max}}[1 - b(c_c - \text{NEC})] \tag{3}$$

where $b$ is the level of toxicity of the chemical for *C. riparius* once the exposure concentration exceeds the NEC.
In the case of an increased energy requirement to produce eggs, we assume that the increase of the energetic cost of an egg is also proportional to \((c_e \times NEC)\) once the exposure concentration exceeds the NEC. The cost of an egg in exposed organisms \(\delta\) is related to the cost of an egg in control organisms \(\delta_0\):

\[
\delta = \delta_0 [1 + b(c_e \times NEC)]
\] (4)

In a third step, we analyzed the consequences of each type of energetic perturbation on energetic investment for reproduction. Fecundity in exposed organisms is expressed in reference to the control fecundity \(N_\text{max} \cdot I\). Because we work in ad libitum feeding conditions, we analyze the effects on \(N_\text{max}\). In the case of decreased feeding, Equations 2 and 3 become

\[
N_\text{max} = N_\text{max}_0 [1 - b(c_e \times NEC)]
\] (5)

In the case of an increased cost of egg production, Equations 2 and 4 become

\[
N_\text{max} = \frac{N_\text{max}_0}{1 + b(c_e \times NEC)}
\] (6)

**Statistical analysis**

Statistical analysis of the data we obtained from bioassays was performed for \(\alpha = 5\%\) with the software R version 1.1.1 (AT&T-Bell Laboratories, Murray Hill, NY, USA). The parameters \(b\) and NEC as well as their 95\% confidence intervals were estimated for the two proposed models from bioassay data obtained using a computer program we coded in C++. Maximum likelihood methods were used after checking that the fecundities followed a normal distribution and that the large sample theory of the likelihood ratio statistic would apply. Fit between the model and the data were tested for each point of the dose–response curve by Student’s \(t\) test.

**RESULTS**

**Chemical measurements**

Chemical measurements in the overlying water withdrawn from the test beakers showed that temperature \((21 \pm 0.5°C)\) and pH \((8.1–8.5)\) were constant during all experiments. Conductivity ranged from 300 to 400 \(\mu S/cm\). The percentage of dissolved oxygen was always greater than 80\%. Nitrate and ammonia remained below 2 mg/L.

Copper spiking of sediment was very satisfactory, with a spiking yield of more than 93\%. Approximately 5\% of the total amount of copper introduced into the rolling jars was found in the rolling water. No more than 1\% was found in the overlying water during the tests.

**Analysis of the reproductive data**

**Larval energetic investment for reproduction in C. riparius**

Four feeding diets were tested to characterize energetic investment during the larval development on clean sediment. Whatever the diet, every female withdrawn from the test beakers oviposited. Moreover, we observed no difference in egg sizes between the tested feeding levels. Fecundity in \(C. \ riparius\) was distributed normally. The mean fecundity increased significantly among feeding diets (Kruskall-Wallis test, \(p < 0.05\)), as presented in Figure 1. This increase cannot be related to feeding during somatic growth, because the highest reproductive level is associated with the lowest feeding level during somatic growth. In contrast, the more food that the females received during gametic growth, the more eggs they could produce. We concluded that, as we hypothesized, the larval energetic investment for reproduction occurred mainly during the gametic growth period. We could thus apply our effects models to reproductive data obtained on spiked sediment.

**Effects of copper on reproduction for \(C. \ riparius\)**

To characterize the larval sensibility pattern on copper-spiked sediment, three exposure programs with different periods of exposure during larval development were tested during the third and fourth instars, during somatic growth only, and during gametic growth only. Survival in the control beakers was satisfactory \((75\% \text{ at } 21 \text{ d})\). For treatments 1 and 2, no survivors were observed at the highest concentration tested \((50 \text{ mg/kg dry sediment})\). Only 63\% of females that emerged from contaminated beakers oviposited. This proportion was lower than that in control beakers \((100\%)\), and it did not depend significantly either on the copper concentration in the sediment or on the exposure program. Furthermore, whatever the concentration tested or the exposure program, no visible difference was observed in egg sizes. For females who reproduced, frequency of egg-mass deformities (i.e., destructured masses that do not present the classical C-shape) were also significantly higher for exposed females \((21\%)\) than for control females \((3\%)\), but this frequency did not vary significantly among the copper concentrations tested. Mean fecundity decreased significantly when copper concentration increased, as presented in Figure 2. This decrease of fecundity did not depend significantly on the exposure program. We concluded that an exposure during any period of the larval development would involve equivalent effects on reproduction. The gametic growth period does not represent a particularly sensitive period, although the energetic investment for reproduction occurs during this time.

Furthermore, the duration of exposure to contaminated sediment did not influence significantly the mean fecundity. This latter observation suggests that the effects of copper on reproduction do not depend on the duration of exposure to spiked sediments.

Finally, we showed that in the case of chronic exposure to copper, whatever the duration of this exposure and whatever the period of the larval development at which it occurs, the effects on reproductive output are equivalent to those observed after an exposure during the third and fourth instars.

Independently from the copper concentration in the sediment or from the exposure program, we occasionally obtained...
small egg masses that were approximately twofold smaller (150–250 eggs/mass) than the mean size (414 ± 112 eggs/egg mass). We did not remove those small egg masses from the data set to perform the data analysis.

The exposure scenario had no influence on fecundity (analysis of variance, p = 0.93). Consequently, data resulting from the three experiments were pooled before modeling copper toxicity. Because no survivors were observed in treatments 1 and 3 and a high mortality was found in treatment 2, few data were available to calculate the mean fecundity for the concentration of 50 mg/kg dry sediment. Furthermore, the standard error around mean fecundity is largely greater for this concentration than it is for the other tested concentrations. Thus, the reliability of the observed data for this concentration is questionable. The pooled data were treated with both the feeding decrease model and the egg cost model. Figure 2 presents a comparison of the bioassay data and the estimations of fecundity for the two proposed models.

For both models, estimations of mean fecundity for a copper concentration of 12.5 mg/kg dry sediment differed significantly from the observed data. Other predictions were satisfactory for the two proposed models, and so none of the models (and the corresponding mode of action) could be rejected. However, the model that assumes an increase of reproductive costs provides the best fit to the data: The sum of the squared differences between the data and the model estimations, expressed as the squared number of eggs, was 12,626 for the egg cost model and 22,726 for the feeding decrease model. This observation suggests that effects on reproduction do not result from an exposure during the gametic growth period only. We can thus assume that the increased copper concentration in the tissues of the larva at any period of larval development might induce resistance mechanisms. These mechanisms are assumed to have high energy costs. Indeed, it has been shown that energy precursors, such as carbohydrates, proteins, and lipids, have a key role in overcoming toxic stress [20]. These mechanisms probably involve a regulation of the internal concentration of copper [21]. In particular, we believe that the induced synthesis of metallothionein-like proteins (MLTPs) might be responsible for this regulation. Indeed, MLTPs have a high affinity for several trace metals, including copper [22]. Furthermore, the presence of cadmium-induced MLTPs has smaller than the mean fecundity of control females. These results suggest that the addition of copper in sediment, even at low concentrations and for episodical exposures, may involve sublethal effects on reproduction.

DISCUSSION

Our first goal in the present study was to investigate the pattern of larval energetic investment for reproduction in regard with the larval development of C. riparius. We supposed that the whole amount of energy available during somatic growth was allocated mainly to biomass production and to maintenance costs. Thus, it was assumed that no energy was invested in reproductive reserves until somatic growth was over. Experiments carried out on clean sediment confirmed that energetic investment for reproduction occurred mainly during the gametic growth phase of the larval development. Indeed, we showed that fecundity was related to the amount of food ingested by the larva during this period of energetic investment for reproduction. A similar result has been observed for C. riparius [8] and for C. tentans [9]. These results suggest that a food limitation could act as a confounding factor for bioassays, especially concerning reproductive endpoints. To avoid such experimental bias, we suggest bioassays to be completed under ad libitum feeding conditions so that fecundity under control conditions is optimized. When larvae were fed ad libitum during the gametic growth period, we noticed that the mean number of eggs per egg mass was 422. This result is consistent with the findings of Postma et al. [19] as well as Péry et al. [8], who reported mean fecundity rates of 400 and 410 eggs per egg mass, respectively. This is assumed to be the maximum mean fecundity rate under optimal laboratory conditions (control sediment and ad libitum feeding) for C. riparius.

These assays on clean sediment provided us with a better understanding of energy management during the larval development from both a qualitative and a quantitative point of view. Thus, it permitted us to build more relevant energy-based models for analysis of the effects of copper on fecundity.

Our second goal was to determine if effects on reproduction were mainly caused by an exposure during the gametic growth period and to characterize the sensitivity of larvae to toxicant during this particular period. We showed that larvae exposed to spiked sediment during somatic growth only or during the third and fourth instars showed reproductive disturbances similar to those exposed during the gametic growth period only. This observation suggests that effects on reproduction do not result from an exposure during the gametic growth period in particular, although energetic investment for reproduction mainly occurs during this time. We can thus assume that the larval pattern of sensitivity to copper does not directly depend on the pattern of energy management during larval development. We hypothesize that the increased copper concentration in the tissues of the larva at any period of larval development might induce resistance mechanisms. These mechanisms are assumed to have high energy costs. Indeed, it has been shown that energy precursors, such as carbohydrates, proteins, and lipids, have a key role in overcoming toxic stress [20]. These mechanisms probably involve a regulation of the internal concentration of copper [21]. In particular, we believe that the induced synthesis of metallothionein-like proteins (MLTPs) might be responsible for this regulation. Indeed, MLTPs have a high affinity for several trace metals, including copper [22].

Moreover, we showed that the mean fecundity for exposed females was 14% of the predicted value. The model that assumes an increase for the feeding decrease model and 22,726 for the feeding decrease model. Figure 2 presents a comparison of the bioassay data (points) and the estimations of the mean fecundity for the feeding decrease model (dotted line) and the egg cost increase model (solid line). *Significant differences (p < 0.05, Student’s t test) between the observed data and the predicted value.
been reported for *C. riparius* [23]: MTLP concentrations increased proportionally to the internal cadmium concentration. Such a mechanism could deplete the amount of energy available for reproduction from reserves, thus explaining the observed effects. We showed that exposure to copper during somatic or gametic growth led to similar effects as those observed in case of exposure during 21 d (from the beginning of the third instar). This strongly suggests that in the case of pollutant-induced stress, once the resistance mechanisms have been induced, their activity persists during the entirety of larval development even if exposure to the toxicant ceases. More work is needed to confirm this hypothesis.

The range of variations in fecundity was high (230–588 eggs/egg mass under an ad libitum feeding level). This variability was probably related to individual differences in assimilation and use of energy efficiency. In particular, we occasionally (7 of 181) obtained egg masses that were twice as small as the average size. This observation could not be related to the concentration of copper in sediment, but there could be a physiological explanation for this result. During the experiment, we suffered periodically from a shortage of reproductive males so that some emerging females might not have been fertilized immediately after their transfer into the reproductive chambers but, instead, 1 or 2 d after, when new males were added into the chambers. Downe [16] highlighted an egg resorption process in one of the ovaries for caged *C. riparius* females that were not fertilized 48 h after emergence. However, those females remained receptive to insemination until the ends of their lives. We believe that the small masses may correspond to the laying of these old females. Because of the high variability of fecundity around the mean value, we were not able to define a threshold under which the smallest egg masses could be considered as old-female masses. Thus, these values were not removed from the data set.

Our biologically based models were able to describe properly the reproductive data for *C. riparius*. Indeed, predicted fecundities fit the observed data except for one of the six tested concentrations. These results confirm that modifications of the energy balance constitute a pertinent theoretical basis for modeling toxic effects on reproduction.

Both the feeding decrease model and the egg cost model fit the bioassay data well, and no proposed mode of action could clearly be rejected. Further work is needed to assess the physiological mode of action of copper on reproduction.

It is worth noting that the NEC estimations do not depend on the chosen effect model. This result is consistent with the observations of Kooijman and Bedaux [10] for the analysis of reproductive data with *D. magna*. Both models provided a NEC estimation that did not differ significantly from 0 mg copper/kg dry sediment. In the case of essential metals, such as copper, the NEC does not take into account the physiological internal concentration of metal (which corresponds to 20 mg/kg dry body mass in our cultured Chironomidae). Rather, the NEC corresponds to the quantity of toxicant that can enter the body without involving effects on the reproductive output. Our results suggest that the addition of copper in sediment, even in a low quantity (<6.8 mg/kg on artificial sediment), can substantially affect the reproductive efficiency. Let us stress that the upper limit of the 95% confidence interval for the NEC estimates (4.7 mg/kg in the egg cost model and 6.8 mg/kg in the feeding decrease model) were near the lowest concentration tested (6.5 mg copper/kg dry sediment). A tightening of the range of concentrations tested (i.e., 0–15 mg copper/kg dry sediment) might permit validating and refining the NEC estimations in forthcoming studies. However, this estimation might not represent a realistic threshold for in situ risk management, because it would imply that no copper should be rejected by human activities.

We must note that the decrease in reproduction is not very pronounced (<31% for all concentrations). What are the ecological consequences of such a reduction? To solve this issue, it is necessary to determine if effects that were predicted at the individual level are observed at the population level. Indeed, Forbes et al. [7] highlighted that working at the population level would be more relevant for risk assessment, because analysis of effects at the individual level may lead to overprotection of species. Energetically based models permit us both to link the major life history that might be disturbed by the toxicant (survival, growth, and reproduction) and to characterize those disturbances quantitatively [10]. Population growth rate can easily be calculated for semelparous organisms [24] from this approach. We thus believe that our models could provide a relevant basis for modeling toxicity at the population level.

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