# Effect of Endosulfan and Parathion on Energy Reserves and Physiological Parameters of the Terrestrial Isopod *Porcellio dilatatus*

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The *in vivo* effects of parathion and endosulfan on the isopod *Porcellio dilatatus* were investigated. Feeding parameters (food consumption and assimilation rates), growth, and energy reserves (glycogen, lipid, and protein contents) of pesticide-exposed isopods were compared with those of control animals. Isopods were exposed to a wide range of concentrations of parathion or endosulfan (0.1, 1, 10, 25, 50, 100, 250, and 500 µg/g of food) for 21 days. The route of uptake of the pesticides was through the diet (alder leaves). Results revealed that parathion induces a significant depression of glycogen, lipid, and protein contents. However, no significant effect was observed on either feeding parameters or growth. Animals fed endosulfan-contaminated food had smaller amounts of glycogen and lipid than control animals, while protein levels were similar in all treatments. Endosulfan also induced a significant decrease in food consumption and assimilation rates at the highest concentrations tested. Growth rate was also significantly affected by endosulfan. These results suggest that the isopod *P. dilatatus* is a suitable species for use in toxicity tests and that energy reserves could be used as effect criteria in both laboratory and field studies. © 2001 Academic Press

*Key Words:* glycogen; lipids; protein; feeding; growth; parathion; endosulfan; *Porcellio dilatatus*.

## INTRODUCTION

Continuous or pulse exposure to pesticides may cause serious problems for nontarget organisms, leading to a number of pathological and disturbed biochemical processes, including changes in energy budgets. The organisms can have direct energy costs to resist the toxicant by avoidance, exclusion, or removal; moreover, they may need energy to repair mechanisms and eventually pathological effects. All these energy expenses reduce the amount of energy left to invest in normal life and, therefore, increase the probability of dying from additional stress (Calow, 1989).

Recently a great deal of attention has been devoted to the use of physiological/energetic processes of nontarget organisms (e.g., feeding parameters, growth, respiration, reproduction and energy allocation mechanisms) as sensitive indicators in situations of toxic stress due to exposure to metals (Donker, 1992; Donker *et al.*, 1993; Drobne and Hopkin, 1994; Khalil *et al.*, 1995) and chemicals (Van Straalen and Verweij, 1991; Mohamed *et al.*, 1992; Van Brummelen *et al.*, 1996a). The ecological relevance of these parameters becomes more clear, since short-term exposures can cause long-term effects in the life cycle of nontarget organisms, although some compounds do not persist long in the soil.

Parathion (*O,O*-diethyl-*O*-(4-nitrophenyl) phosphorothioate) is an organophosphate insecticide whose main mode of action is the inhibition of AChE, resulting in an accumulation of acetylcholine in the synaptic terminals, and, therefore, a change in the normal transmission of the nervous impulse (Gallo and Lawryk, 1991; WHO, 1996; Ribeiro *et al.*, 1999). In mammals, severe poisoning can stop breathing due to congestion of the lungs and weakness of the respiratory muscles (Eto, 1974; WHO, 1992; Gälli *et al.*, 1994). Because of the high toxicity of parathion to mammals and humans, its use has been prohibited or severely restricted in many countries. Nevertheless, it is still commonly used in some regions of Portugal to control numerous insect pests.

Endosulfan (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5*a*, 6, 9, 9*a*-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepin-3-oxide) is an organochlorine insecticide. This toxicant is a central nervous system poison (WHO, 1988; Naqvi and Vaishnavi, 1993). Acute intoxication may result in neurological manifestations, such as irritability, restlessness, muscular twitching, and convulsions that may end in death. Due to its low persistence in the environment, it is commonly used in Portugal in several cultures (including rice and corn) to control a wide range of insect pests.

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The terrestrial isopod *Porcellio dilatatus* was chosen to evaluate the ecotoxicity of these pesticides for several reasons: it is quite easy to capture in the field and to maintain in laboratory cultures and it is an important member of the macroarthropod community of the soil. These organisms play an important role in decomposition processes, promoting litter fragmentation and increasing the attack area for fungi and bacteria, and, therefore, promoting microbial degradation. Isopods have also a major role in the mineralization of nutrients necessary for plants, mainly nitrogen and phosphorus (Sutton, 1972; Hopkin, 1991; White *et al.*, 1992; Van Wensem *et al.*, 1993; Van Brummelen *et al.*, 1996a, b).

Soil contamination may affect saprotrophic activities and may thus endanger soil processes (Hopkin and Martin, 1982; Van Straalen and Verweij, 1991; Van Brummelen *et al.*, 1996a, b). For these reasons, terrestrial isopods are suitable candidates to be used as test organisms in both ecological studies and soil ecotoxicological research (Hopkin, 1991; Donker, 1992; Drobne, 1997). In fact, the number of tests with isopods has been increasing in the past few years (Van Straalen and Verweij, 1991; Donker *et al.*, 1993; Léon and Van Gestel, 1994; Drobne and Hopkin, 1994; Crommentuijn *et al.*, 1995; Vink *et al.*, 1995; Van Brummelen *et al.*, 1996a, b; Caseiro, 1997; Ribeiro *et al.*, 1999).

The aims of the present study are to (1) optimize laboratory methodology used to quantify glycogen, lipid, and protein contents in *P. dilatatus*; (2) quantify physiological and biochemical responses with ecological relevance (feeding, growth, and energy reserves) in the organisms exposed to parathion or endosulfan; and (3) relate data obtained with the biochemical parameters to the physiological status of the animals.

## MATERIALS AND METHODS

### Test Organism and Culture Procedures

The specimens of *Porcellio dilatatus* used in these experiments were obtained from a laboratory population, composed of animals collected from a dunar system in central Portugal and fed on alder leaves (*Alnus glutinosa*), previously oven-dried at 60°C for 48 h. This diet was chosen because it provides a higher growth rate and better reproductive performance than others, mainly because of its higher nitrogen content (Caseiro, 1997; Sousa *et al.*, 1998). Isopods were kept in plastic boxes covered with a layer of sand previously autoclaved. Humidity was maintained by regular spraying with distilled water and the ventilation in the boxes was ensured by holes made on the box cover.

### Experimental Setup

In independent experiments, 3- to 4-month-old animals were exposed to parathion or endosulfan. Isopods were confined individually in 5-cm-diameter Petri dishes covered

with a bottom of plaster of Paris and fed *ad libidum* weekly with contaminated alder leaves. Both experiments were conducted in a climate room at a temperature of 20 ± 1°C and a light : dark cycle of 16:8 h. The endosulfan stock solution was prepared in ethanol and the parathion stock solution was prepared in distilled water. Pesticide solutions were previously diluted and added to the leaves. Endosulfan-contaminated leaves were allowed to dry overnight at room temperature to ensure solvent evaporation. Distilled water was applied to a further batch of leaves which acted as controls. Ingestion of the toxicants was the major exposure route, although direct surface contact may also have contributed to exposure. Concentrations used for both chemicals were 0.1, 1, 10, 25, 50, 100, 250, and 500 µg/g of food. In each experiment 10 animals were used per treatment; the period of exposure to the insecticides was 21 days.

### Feeding and Growth Measurements

Isopods were weighed at the beginning of the experiment and weekly. The initial weights ranged from 15 to 55 mg. One day before the start of the experiment and 1 day after the end animals were left without food to empty their gut. The food offered to the animals was weighed at the beginning of every feeding period (1 week). The feces of the animals and the remaining leaf material were removed weekly from the test boxes, dried in the oven (60°C, 48 h) and weighed; then new contaminated food was offered to the animals.

The parameters measured were consumption rate (Cr: mg food consumed/mg animal/day), assimilation rate (Ar: mg food assimilated/mg animal/day), assimilation efficiency (AE: percentage of assimilated food in relation to the consumed food), and growth rate (Gr: biomass gain/average weight over time).

### Energy Reserve Measurements

At the end of the experiment animals were sacrificed. Several biochemical analyses were performed on each sample: glycogen, lipid, and protein contents. Individual samples were homogenized in 1 mL of 0.15 M sodium chloride, using an electrical homogenizer equipped with a speed regulator, and centrifuged for 3 min at 8000 rpm. Samples were kept on ice throughout the homogenization process to avoid protein denaturation and stored at −70°C until the biochemical determinations were performed.

### Glycogen Determination

Glycogen was determined using a modification of the method described by Marshall and Orr (1962) based on that of Dubois *et al.* (1956). Glycogen was separated from soluble sugars by precipitation in the presence of methanol: 10 mL

of methanol was added to 0.2 mL of sample homogenate and shaken on the Vortex mixer. Mixtures were then centrifuged for 15 min at 3000 rpm. Supernatants were discarded, and the precipitates resuspended in 10 mL of methanol and shaken on the Vortex. Suspensions were centrifuged again and the precipitates were used for glycogen quantification. The sulfuric acid method was then used to quantify glycogen in the samples, since glycogen is hydrolyzed into glucose in the presence of hot sulfuric acid (Kemp and Heijningen, 1954); all samples were evaporated to total dryness in a water bath at 100°C. The residue was resuspended with 3 mL of 1.67% phenol solution and shaken on the Vortex. Then 5 mL of concentrated sulfuric acid was mixed and heated at 100°C in a water bath for 10 min. Finally, the absorbance was measured at 490 nm. Standards ranging from 0 to 100 µg of glucose were prepared from a stock solution of 0.1% glucose and received the same treatment as the samples.

### Lipid Determination

The lipid fraction in the samples was extracted using a modification of the Blight and Dyer (1959) method: 2.5 mL of methanol was mixed with 0.5 mL of sample homogenate for 3 min on a Vortex mixer. Then, 2.5 mL of chloroform was added and the mixture shaken for another 3 min. Following this, the mixture was centrifuged for 5 min at 3000 rpm to speed up the sedimentation of solid residues. The sediment was discarded and 1.25 mL of chloroform and 2.5 mL of water were added to the supernatant. The mixture was shaken on the Vortex and centrifuged at 3000 rpm for 5 min to speed up the phase separation process. Then, the lower phase was carefully collected with a pipet equipped with a rubber pump. The sample was dried under a nitrogen stream and resuspended with 2 mL of a 2:1 (v/v) mixture of chloroform:methanol. Total lipids were determined using a modification of the method described by Ahlgren and Uppsala (1991) based on the sulfophosphovanilline method of Zöllner and Kirsch (1962): 0.5 mL of sample was evaporated in a water bath at 30°C under a nitrogen stream. Then 0.5 mL of concentrated sulfuric acid was added and heated at 100°C in a water bath for 10 min. Finally, 2.5 mL of vanilline reagent was added and the absorbance was measured at 528 nm after 2 to 3 h. Standards ranging from 0 to 100 µg of linoleic acid were prepared from a stock solution of 0.1% linoleic acid and received the same treatment as the samples.

### Protein Determination

Before protein determination, samples were diluted 1:10 (v/v) with the sodium chloride solution. The concentration of protein in the samples was determined using the Bradford (1976) method adapted to microplate (Herbert *et al.*, 1995):

250 µL of diluted color reagent (Bio-Rad) was added to 20 µL of all samples and to standards of bovine serum albumin. After 15 min the absorption was measured at 595 nm.

### Pesticides

Parathion-ethyl, 98.9% pure, and endosulfan sulfate, 97.6% pure, were purchased from Dr. Ehrenstorfer GmbH, Augsburg, Germany.

### Statistical Analysis

Feeding and growth data were analyzed by comparing consumption and assimilation rates of different treatments using analysis of variance (ANOVA); whenever applicable initial biomass of isopods was used as covariable. Growth and energy reserve data were also analyzed using ANOVA. The arcsine transformation was used to achieve normality and homoscedasty when necessary. These were verified by Kolmogorov–Smirnov and Bartlett tests, respectively (Zar, 1984). Lipid content was compared with a Kruskal–Wallis one-way analysis of variance, since no homoscedasticity was met even after data transformation. Post hoc testing was carried out using the Tukey test. A significance level of 0.05 was used for all statistical tests.

## RESULTS

### Mortality

For the control groups no mortality was observed. In the endosulfan experiment no significant mortality was observed even at the highest concentrations. By contrast, in the parathion experiment the mortality increased with the pesticide concentration and reached 100% at the highest concentrations (Table 1). For this reason, for the sixth, seventh, and eighth treatments with parathion results were not considered in the feeding and growth measurements, since too many individuals had already died. Nevertheless, biochemical analysis were also performed in the dead isopods.

### Effect of the Insecticides on Physiological Parameters

Feeding parameters and growth in *P. dilatatus* were not significantly affected by parathion at the concentrations considered (0.1–50 µg/g of food) (Figs. 1–3). Contrastly, endosulfan induced a significant change in both consumption and assimilation rates at the highest concentrations tested (100, 250, and 500 µg/g of food) (Fig. 1). Moreover, food assimilation efficiency was also significantly affected by this toxicant ($F(8, 76) = 4.6$, $P < 0.05$) (Fig. 2). These changes in feeding behavior significantly affected growth rate at the highest concentrations ($F(8, 79) = 7.07$, $P < 0.05$) (Fig. 3).

| | % Mortality | |
| --- | --- | --- |
| | Parathion | Endosulfan |
| Ctr | 0 | 0 |
| C1 | 10 | 10 |
| C2 | 10 | 10 |
| C3 | 20 | 20 |
| C4 | 30 | 0 |
| C5 | 30 | 0 |
| C6 | 70 | 0 |
| C7 | 100 | 10 |
| C8 | 100 | 0 |

[a] Ctr., control group; C1 to C8, treatment groups (for concentrations refer to the text).

The average assimilation efficiency values obtained in both toxicants (around 60%) are much higher than the values reported by Van Straalen and Verweij (1991) and Khalil *et al.* (1995) for *P. scaber* (25.6 and 30%, respectively). Present values are also higher than the value reported for *P. laevis* by Khalil *et al.* (1995), who found an assimilation efficiency of 42.3%. Nevertheless, these results agree with those obtained for *P. dilatatus* by Sousa *et al.* (1998), which averaged 80% when fed alder leaves.

*Effects of Parathion and Endosulfan on Energy Reserves*

Protein, glycogen, and lipid contents of animals cultured in the presence of parathion were significantly lower than those of animals cultured in the absence of the insecticide ($F_{(8, 95)} = 10.1$, $P < 0.05$ for protein; $F_{(8, 46)} = 13.9$, $P < 0.05$ for lipids) (Figs. 4A, 4C, 4E). The effect of parathion on glycogen content is concentration dependent, being the differences between the control group and all the other groups statistically significant ($F_{(8, 59)} = 22.37$, $P < 0.05$). A significant decrease in both glycogen and lipid contents was also noted in endosulfan-treated woodlice ($F_{(8, 56)} = 10.87$, $P < 0.05$ for glycogen; $F_{(8, 51)} = 11.23$, $P < 0.05$ for lipids) (Figs. 4B, 4D). Protein content, however, was similar to that of the control group ($F_{(8, 89)} = 1.16$, $P > 0.05$) (Fig. 4F).

## DISCUSSION

Feeding parameters and growth were not affected by parathion at the concentrations considered (0.1–50 µg/g food). One reason for this absence of effects could be the short exposure period (21 days). Similar results on growth due to short-term exposures were also obtained by Vink *et al.* (1995) and Crommentuijn *et al.* (1995). Vink *et al.* (1995), when exposing *P. pruinosus* to benomyl and diazinon, observed a significant decrease in weight gain only after 3 and 2 weeks of exposure, respectively. Crommentuijn *et al.* (1995), analyzing the influence of cadmium on *P. scaber*, observed a significant effect on growth only after 308 days of exposure.

Another explanation for these results may be related to the narrow border that exists between sublethal and lethal effects in isopods. Crommentuijn *et al.* (1995) calculated a small sublethal sensitivity index (SSI) for cadmium in *P. scaber*, which indicates the closeness of sublethal and lethal concentrations. If this feature is maintained with other chemicals, it may imply that sublethal effects are not always observable if a large spacing factor is used; in the present case no effects were observed at 50 µg/g food, and significant mortality was verified at the next concentration tested (100 µg/g food).
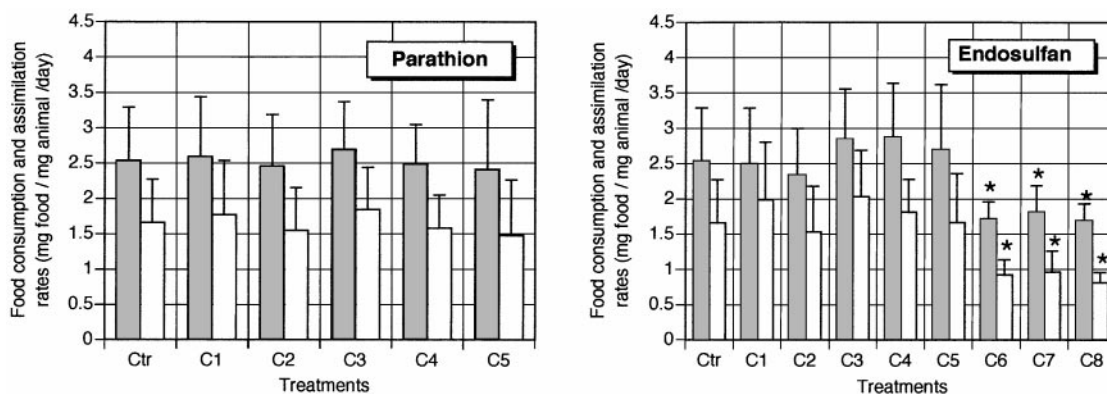


**FIG. 1.** Food consumption (gray bars) and assimilation (white bars) rates of *P. dilatatus* cultured for 21 days in several concentrations of endosulfan or parathion. Ctr., control group; C1 to C8, treatment groups (for concentrations refer to the text). Results are expressed as the mean activity with associated standard deviation bars. Asterisks indicate significant differences in relation to control.
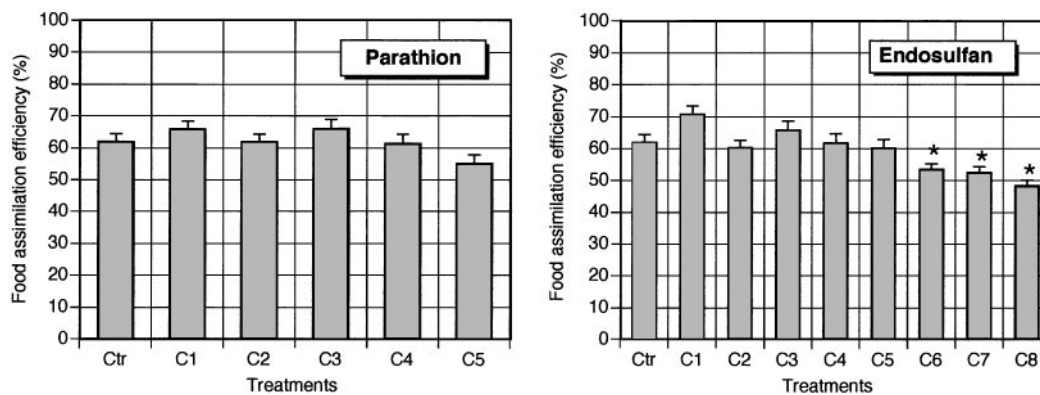
**FIG. 2.** Assimilation efficiency of *P. dilatatus* cultured for 21 days in several concentrations of endosulfan or parathion. Ctr., control group; C1 to C8, treatment groups (for concentrations refer to the text). Results are expressed as the mean activity with associated standard deviation bars. Asterisks indicate significant differences in relation to control.

Endosulfan, on the other hand, significantly affected the same feeding parameters at the highest concentrations. This may indicate a feeding inhibition situation, with the isopods regulating the intake of the pesticide by reducing consumption rates and this affecting thus other related parameters, including growth. This strategy, however, is commonly used to avoid poisoning with heavy metals (Donker, 1992; Drobne and Hopkin, 1994; Bibic et al., 1997); with organic chemicals the strategy is to have high elimination rates to remove the toxicants from the organism. This was verified in *P. scaber* exposed to PAHs (Van Brummelen and Van Straalen, 1996) and in *P. pruinosus* exposed to lindane (Sousa et al., 2000).

The present study indicates that as a result of exposure to the pesticides the biochemical constituents were reduced. A significant decrease in both glycogen and lipid contents was observed at sublethal concentrations of parathion and endosulfan as low as 0.1 $\mu$g/g of food.

The present results agree with those of Vink et al. (1995), who observed a decrease in these energy reserves in isopods exposed to diazinon. Depletions in glycogen and lipid contents were also verified after exposure to pesticides on mice (Dere and Yanikoglu, 1988, in Naqvi and Vaishnavi, 1993), eels (Sancho et al., 1998), and snails (Rambabu and Rao, 1994) and exposure to metals on isopods (Donker, 1992; Sørensen et al., 1997).

Depletion of glycogen may be due to direct utilization of this compound for energy generation, as a result of pesticide-induced hypoxia. Glycogen is rapidly catabolized, resulting in a rapid decrease in this energy reserve. Lipid content also decreased during exposure to both pesticides due to its use as an energy reserve, parallel to glycogen (Sancho et al., 1998; Rambabu and Rao, 1994).

The decrease in protein content of parathion-intoxicated isopods also indicates a physiological adaptability to compensate for pesticide stress. To overcome the stress
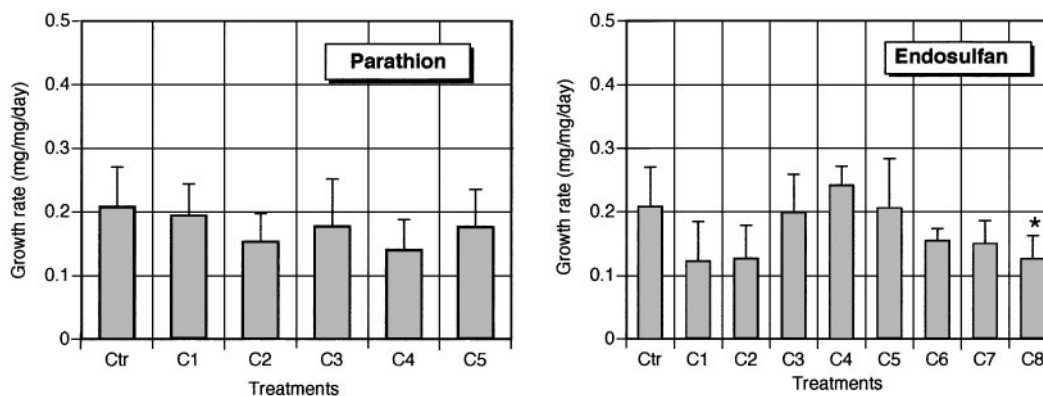


**FIG. 3.** Growth rate of *P. dilatatus* cultured for 21 days in several concentrations of endosulfan or parathion. Ctr., control group; C1 to C8, treatment groups (for concentrations refer to the text). Results are expressed as the mean activity with associated standard deviation bars. Asterisk indicates significant difference in relation to control.
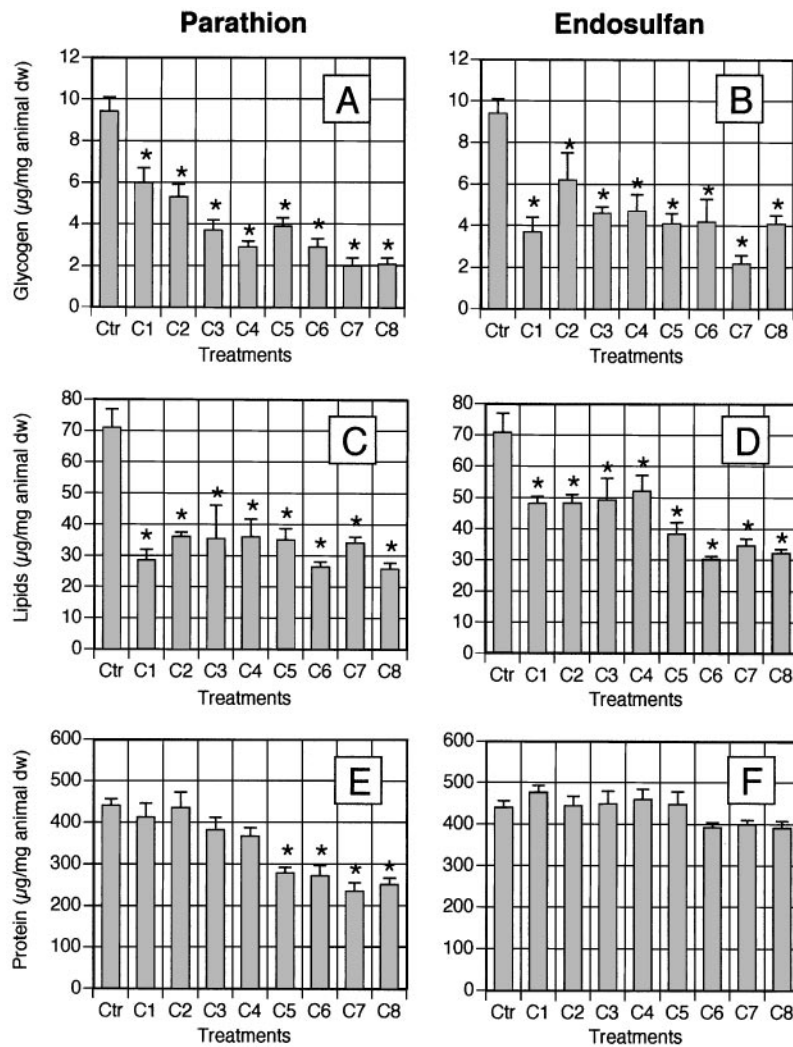
**FIG. 4.** Glycogen (A, B), lipids (C, D), and protein (E, F) in homogenates of *P. dilatatus* cultured for 21 days in several concentrations of parathion (A, C, E) or endosulfan (B, D, F). Ctr., control group; C1 to C8, treatment groups (for concentrations refer to the text). Results are expressed as the mean activity with associated standard deviation bars. Asterisks indicate significant differences in relation to control.

situation, animals require high energy and this energy demand may have led to the simulation of protein catabolism. Furthermore, this decrease in protein content might also be due to a mechanism of lipoprotein formation, which will be used to repair damaged cell and tissue organelles (Sancho *et al.*, 1998; Rambabu and Rao, 1994). A similar decrease in protein content was also observed by Van Brummelen and Stuijfzand (1993) in *P. scaber* and *O. asellus* exposed to benzo(*a*)pyrene and by Van Brummelen *et al.* (1996a) in *O. asellus* exposed to fluorene and benz(*a*)anthracene; this decrease was followed by a reduction in growth.

On the other hand, in the endosulfan experiment no significant reduction was found in protein levels of insecticide-exposed animals. Perhaps a greater concentration of endosulfan and a higher exposure period would have produced observable effects.

## CONCLUSIONS

The results of the present investigation suggest that the most commonly measured physiological parameters (feeding inhibition-related parameters) may not always provide *per se* the most sensitive criteria for establishing ecotoxicological effects. This is particularly evident when considering pesticide exposure scenarios, and perhaps the relevance of these parameters could be enhanced by integrating them into physiological mechanistic models that could predict long-term effects on individuals.

On the other hand, biochemical parameters seem quite promising to assess and, to a certain extent, predict the effects of toxicants on nontarget organisms. However, the use of such parameters as biomarkers requires better knowledge of the physiological significance of the changes

induced by the exposure to pesticides. Furthermore, these results indicate that the isopod *P. dilatatus* is a suitable organism for use in toxicity tests and suggest that it is a sensitive nontarget species that could be used in monitoring studies.

The authors are currently evaluating the effect of other environmental contaminants from different chemical groups on the same type of parameters of *P. dilatatus* to investigate their potential for use both in chemical testing and in specific assessment of soil quality.

## ACKNOWLEDGMENTS

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