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# Environmental risk assessment of carbosulfan on target and non-target beetles when used as white grub larvicide in the cork oak forest of Mamora (Morocco)

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The protection of new cork oak plantations against the larvae of the beetle *Sphodroxia maroccana* (Melolonthidae), which destroy the roots of newly planted trees, was provided for many years in the forest of Mamora in Morocco by the use of carbosulfan, usually formulated as dry granules for direct incorporation into the soil at planting. Ecotoxicological tests were conducted in the laboratory on *S. maroccana* and on two non-target beetle species, namely *Pachychila obtusecostata* and *Pimelia platynota* (Tenebrionidae), to measure the acute effects of carbosulfan and evaluate the potential risk associated with inappropriate practices. The mortality was measured at both 4 d and 7 d after ingestion of a known quantity of carbosulfan. The recommended dose by the manufacturer was exceeded by at least four-fold in the Mamora Forest, with obvious environmental risks. Risks were identified for wildlife when the pesticide is misused and spread on the soil around the plants.

**Keywords:** carbosulfan; cork oak; environmental risk; Melolonthidae; Morocco; mortality; *Pachychila obtusecostata*; *Pimelia platynota*; *Sphodroxia maroccana*; Tenebrionidae

## 1. Introduction

The Mamora Forest (6°00'–6°45'W; 34°00'–34°26'N) in Morocco covers a large area in the region of Salé, Kenitra and Tifelt. This vegetation type is in marked decline (Bouslihlim 1996), with many old *Quercus suber* L. trees in poor condition (most trees are more than one century old) and serious problems in natural regeneration. In the previous century, the forest lost nearly two-thirds of its original area, while in the remaining parts many oak trees declined in number due to great human pressure (e.g. clearing, pruning, stripping, collection of acorns, and domestic animal grazing in the undergrowth), combined with insect attack, including that by gypsy moth (*Lymantria dispar* [L.]) and cork ant (*Crematogaster scutellaris* [Olivier]) (Formicidae), as well as several borers, including the beetles *Cerambyx cerdo* L. (Cerambycidae) and *Platypus cylindrus* (Fabricius) (Platypodidae) (Fraval and Villemant 1997). Overall, the forest resembles a wooded park planted with even-aged stands of old oak trees, the foliage of which is periodically destroyed below 2 m by cattle and the lateral branches often pruned for cattle feeding in drought periods. At the end of the twentieth century, the area occupied by cork oaks covered only 60,000 to 70,000 ha (Bendaanoun 1998; Benzyane 1998). In this context, natural regeneration was very difficult and the artificial reconstitution of the forest became necessary (Dahmani 2005). The plantation was soon faced with high mortality of seedlings (from 41% to 68% mortality during the first year, depending on the blocks), mostly due to larvae (white grubs) of *Sphodroxia*

*maroccana* Ley (Melolonthidae) (Montreuil et al. 2004; Ghaïoule et al. 2007, 2010). This species is widespread throughout the Mamora Forest as it is endemic to this region, with an outbreaking population mainly due to imbalances of anthropogenic origin (especially domestic animal grazing in the undergrowth and the subsequent disappearance of the grass cover, whose roots normally repulse and divert attacks by white grubs against the roots of young trees) (Ghaïoule et al. 2007, 2010). To combat white grubs, the technicians responsible for the forest plantings for many years used carbosulfan before it was replaced by its metabolite carbofuran (OMS-864). Carbosulfan, usually formulated as dry granules (10%) or microgranules for direct incorporation into the soil at planting, was often misused and spread by technicians on the soil surface around the plants, posing real risks for wildlife that might encounter it. Inadequate understanding of the potential risks that this pesticide posed to the environment, associated with inappropriate practices, resulted not only in high mortality of granivorous birds, but also of many non-target beetles such as detritivorous darkling beetles (Tenebrionidae) (pers. obs.). Birds died from ingesting the granules, which resemble grain seeds in size and shape, as already reported many times (US EPA 1991, 2006; Almeida et al. 2010). As a result, granular formulations of carbofuran were banned in 1994 by the Environmental Protection Agency to protect the avifauna. Separately, the European Union banned the use of carbosulfan in 2007 (EU 2007).

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Carbosulfan (2,3-dihydro-2, 2-dimethyl-7-benzofuryl [(dibutylamino) thio] methylcarbamate) (CASRN—55285-14-8 PubMed) is a carbamate with insecticidal and nematocidal properties that is absorbed by the roots of treated plants and has contact and oral activity. This insecticide has similar toxic mechanisms compared to organophosphates. It blocks transmission of nerve impulses by irreversible inhibition of the enzyme acetylcholinesterase at cholinergic synapses, causing insect death (Fukuto 1990).

The aims of our study were (i) to assess the acute effects of carbosulfan on the larvae of the pest species *Sphodroxia maroccana* and on two non-target darkling beetles *Pimelia platynota* Fairmaire and *Pachychila obtusecostata* subspecies *trisulcata* Antoine (Tenebrionidae); and (ii) to examine how carbosulfan was used at the time of planting cork oak in Morocco before the recent ban on its use.

## 2. Materials and methods

### 2.1. Test species

*Sphodroxia maroccana* larvae were collected during winter when they were active near the soil surface (Ghaïoule et al. 2007, 2010; Fegrouche et al. 2012a, 2012b). Larvae were reared individually in 2-L capacity plastic boxes filled with sand (130–150 µm; Texier et al. 1992) which was kept slightly moist (40–60% humidity, based on previous experiments; Fegrouche et al. 2012b). Larvae were fed with fragments of potato (which the polyphagous larvae are known to feed on). Ecotoxicological tests were conducted in the laboratory between February and late March 2010, when the feeding activity was highest.

*Pimelia platynota* and *Pachychila obtusecostata* are widespread and abundant detritivorous beetles in the Mamora Forest. The ease of maintenance under laboratory makes these non-target species good candidates for toxicity testing. Adults were collected in the spring (March) using pitfall traps (Barber model) buried to their rim in the sandy soil at random locations in different parts of the forest. Individuals were placed in 3-L capacity plastic boxes with 5 cm of dry sand from the site. The boxes, containing about 30 individuals, were maintained at laboratory temperatures ( $25 \pm 2^\circ\text{C}$ ) and light–dark regime 14 h L : 10 h D. The beetles were fed weekly with a spoonful of flour mixed with bread.

### 2.2. Exposure to carbosulfan

Carbosulfan is not soluble in water. Hence, it was dissolved in acetone to obtain the desired concentrations by serial dilution. Preliminary tests covered a wide concentration range to test lethal and sublethal effects. Test dilutions were prepared from a stock solution containing 250 mg carbosulfan in 50 mL acetone. Toxicity tests were performed using technical grade carbosulfan (Pestanal<sup>®</sup>, Sigma-Aldrich Company, 94.4% purity, batch 5137X). The values mentioned below in the text have been adjusted to 100% purity.

Tests with *S. maroccana* larvae were performed in 400 mL plastic boxes covered with lids, with one larva per box. Before starting the test, only 0.10 g of crushed potato, whatever the dose and the size of larvae, was placed at the bottom of each box and the appropriate dose of carbosulfan solution was added to food. Boxes were first left open (without a lid) for 12 h to allow any residual solvent (acetone) to evaporate, and then 20 µl of water were added to obtain optimal moisture for food. The tests with white grubs (large and small larvae were tested separately, 2.7 and 5.7 g, respectively) comprised 10 treatments: 2 controls (water and solvent) and 8 doses of carbosulfan which ranged from 0.047 to 47.2 µg (0.047; 0.095; 1.42; 2.36; 3.77; 4.72; 14.16; 47.2 µg carbosulfan per 0.10 g food). These values were selected on the basis of a range-finding test performed at nominal carbosulfan concentrations of 0.047, 1.42, 14.16 and 47.2 µg per 0.10 g food together with an untreated control and solvent control, using five individuals per concentration. The results of the range-finding test showed that the mortality (as end-point) had to be recorded twice, after 96 h (4 d) and 168 h (7 d). The palatability to larvae of food containing the highest doses of carbosulfan was low, so that more larvae than needed (depending on the dose) were tested in order to have the right number of individuals which would ingest the pesticide in totality (dose–effect relationship regarding the feeding on potato: 2 and 5 more larvae for doses 14.16 and 47.2 µg carbosulfan, respectively). Only larvae that had consumed all the food provided containing a known dose of carbosulfan were retained. Therefore, carbosulfan ingested with food was exactly the required dose for each individual. The larvae that refused to feed (or consumed only a part of food) were excluded from the trial, as it was considered that the required dose had not been totally ingested. Ten and 22 larvae of small and large size, respectively, fulfilled these criteria for each dose.

The tests performed with adults of *Pimelia* and *Pachychila* comprised 11 treatments: 2 controls (water and solvent) and 9 doses of carbosulfan, ranging from 0.047 to 47.2 µg (0.047; 0.47; 1.42; 2.36; 3.77; 4.25; 4.72; 14.16; 47.2 µg carbosulfan). A small piece of bread (0.10 g) was placed at the bottom of the vessel (Petri dish), and carbosulfan was added to the food to obtain the right dose. As was the case for larvae of *S. maroccana*, the palatability, for darkling beetles, of food containing the highest doses of carbosulfan was low, and more insects were necessary for the experiments. Only individuals that had consumed all the provided food containing a known dose of carbosulfan were retained; beetles that refused to feed (or consumed only a part of food) were excluded from the trial, as it was considered that the required dose had not been totally ingested by the individuals. Thirty and 27 adults of *Pimelia* and *Pachychila*, respectively, were retained for each dose.

All tests were performed at room temperature ( $25 \pm 2^\circ\text{C}$ ) and lasted for 7 d. The numbers of surviving darkling beetles (*Pimelia* and *Pachychila*) and *S. maroccana* (large and small larvae) were recorded daily – the results are given for 4 d and 7 d exposures.

Table 1. Live weight of tested individuals (*Sphodroxia maroccana*, *Pachychila obtusecostata* and *Pimelia platynota*) and comparison of batches, including control and solvent. The normality of groups was tested with the Anderson–Darling test (using the Pearson correlation coefficient).

Species	No. batch	No. individuals tested/batch	Max. weight (g)	Min. weight (g)	Average weight (g)	Standard deviation	Normality of batches
<i>Sphodroxia</i> larvae (large size)	10	22	6.26	5.50	5.71	1.09	n.s.
<i>Sphodroxia</i> larvae (small size)	10	10	2.85	2.55	2.68	0.43	n.s.
<i>Pimelia</i> (adults)	11	30	1.03	0.70	0.84	0.14	n.s.
<i>Pachychila</i> (adults)	11	27	0.15	0.08	0.10	0.07	n.s.

n.s. = not significant.

### 2.3. Statistical analysis

The critical effect and threshold concentration for tests were calculated using Toxrat<sup>®</sup> software (version 2.10). The LOED (Lowest Observed Effect Dose) and NOED (No Observed Effect Dose) values were determined using Fisher's exact binomial test with Bonferroni correction. The values for doses causing 50% mortality (LD<sub>50</sub>) with 95% confidence limits were determined with a Probit analysis, using linear maximum likelihood regression. The tests were considered as valid when the mortality in the controls did not exceed 5.0%. The pairwise comparisons between the two controls (water and solvent) were performed using a Fisher's exact binomial test (alpha = 0.05; two-sided). In all cases there was no significant difference between control water and solvent, and no mortality at all was observed. The control mortality at 4 d and 7 d was compensated using Abbott's formula. To ensure the samples were comparable (the susceptibility of individuals depending in large part on their size), four groups were segregated according to the weight of individuals: darkling beetles (*Pimelia* and *Pachychila*) and large and small larvae of *S. maroccana*. The normality of group data was tested with the Anderson–Darling test. (This test measures the least squares distance between the points of the diagram and the regression line. The least squares estimate is calculated using the Pearson correlation coefficient.) Statistical analyses were conducted using Minitab<sup>®</sup> 13 Statistical Software.

## 3. Results

### 3.1. Homogeneity of biological material

All samples were uniform in weight distribution, without significant difference between batches of the same size class (Table 1).

### 3.2. Mortality

The mortality was recorded initially after 4-d exposure to check any changes of activity exhibited by the treated *Sphodroxia* larvae (small and large size) and adults of the darkling beetles *Pimelia* and *Pachychila*. Final mortality was recorded at 7 d (Table 2). All the tests were valid.

#### 3.2.1. *Sphodroxia* larvae

The first symptoms of poisoning appeared rapidly: between one hour and half a day after ingestion of contaminated food. Larvae vomited large volumes of a dark liquid when the first muscular contractions (curvature of abdomen) appeared. The movements became increasingly erratic and uncoordinated (sublethal). The evolution of symptoms continued with hyperexcitement and convulsions leading finally to paralysis until death of the larvae. After ingestion of carbosulfan, the mortality of larvae varied depending on their size and the doses. After 4 d exposure, the mortality of small larvae reached 80% with the ingestion of 3.77 µg carbosulfan per 0.1 g food (Figure 1a). In the case of large larvae, 3.77 µg carbosulfan resulted in a mortality of 22.7%; the mortality reached 77.2% with 14.16 µg (Figure 2a). The mortality was 100% when 47.2 µg carbosulfan per 0.1 g food was ingested, irrespective of the size of larvae. No mortality was observed in the controls (solvent and water). The results showed a clear dose–response relationship in all the tests and significant effects with increased doses of carbosulfan. The LD<sub>50</sub> values (median lethal dose with 95% confidence limits) after 4 d were 1.63 (95% CL: 0.73–3.18 µg / 0.1 g food) and 5.84 µg carbosulfan for small and large *Sphodroxia* larvae, respectively, with an NOED of 0.09 and 2.36 µg carbosulfan per 0.1 g food per

Table 2. Test results (4 d and 7 d) with *Sphodroxia maroccana* larvae (small and large larvae) and with *Pachychila obtusecostata* and *Pimelia platynota* adults (data in µg carbosulfan / 0.1 g food). Lethal dose LD<sub>50</sub> (50% effect) including the 95% confidence limits (CL) in parentheses, and NOED and LOED values.

Species	0–4 days			0–7 days		
	LD <sub>50</sub> (95% CL)	NOED (µg)	LOED (µg)	LD <sub>50</sub> (95% CL)	NOED (µg)	LOED (µg)
<i>Sphodroxia</i> (small larvae)	1.63 (0.73–3.18)	0.09	1.42	1.50 (0.67–2.88)	0.09	1.42
<i>Sphodroxia</i> (large larvae)	5.84 (n.d.)	2.36	3.77	4.44 (n.d.)	2.36	3.77
<i>Pachychila</i> (adults)	3.30 (2.22–4.85)	0.47	1.42	2.86 (1.93–3.97)	0.47	1.42
<i>Pimelia</i> (adults)	4.53 (2.7–9.61)	2.36	3.77	3.97 (2.48–6.82)	0.47	1.42

n.d. = not determined.

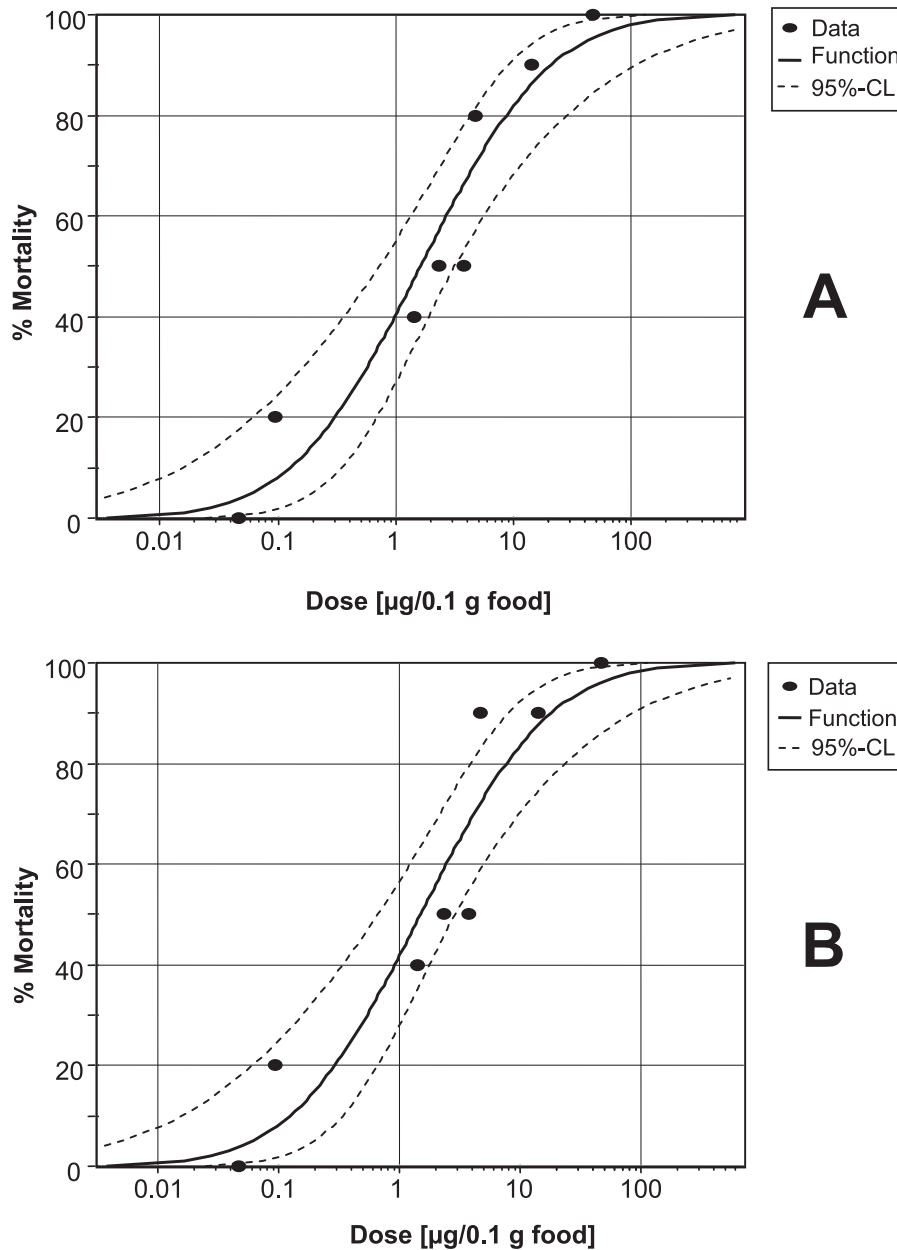


Figure 1. Concentration–effect curves showing the effect of carbosulfan ( $\mu\text{g}/0.1\text{ g food}$ ) on mortality (%) of small *Sphodroxia marocana* larvae. A: 4 d after ingestion; B: 7 d after ingestion.

larva for small and large individuals, respectively (Table 2).

After 7 d exposure, the probit analysis revealed  $\text{LD}_{50}$  values of 1.50 (0.67–2.88) and 4.44  $\mu\text{g}$  carbosulfan per 0.1 g food per larva for small and large individuals, respectively. The NOED was 0.09 and 2.36  $\mu\text{g}$  carbosulfan per 0.1 g food per larva for small and large individuals, respectively (Table 2). Between the endpoints at 4 d and 7 d, the NOED and LOED values remained unchanged, both for small and large larvae. The dose-effect varied according to the size (Figures 1b and 2b). When  $\text{LD}_{50}$  values were reported to the same body weight (1 kg body weight), the susceptibility of small larvae was higher than for large larvae. At 4 d post-ingestion, the  $\text{LD}_{50}$  was 583  $\mu\text{g}$  carbosulfan per kg small larvae compared to 1023  $\mu\text{g}$  carbosulfan per kg large larvae.

Similarly, at 7 d post-ingestion,  $\text{LD}_{50}$  values were 560  $\mu\text{g}$  carbosulfan per kg small larvae against 777  $\mu\text{g}$  carbosulfan per kg large larvae. For comparison, the oral  $\text{LD}_{50}$  in rats is 250 mg carbosulfan per kg body weight; (Dawson et al. 2010).

### 3.2.2. Darkling beetles

The behavioural sequence observed in adults of *Pimelia* and *Pachychila* post-ingestion was similar to that reported above for *Sphodroxia* larvae. In both species, the first symptoms of poisoning appeared immediately after consuming toxic food. The insects vomited a more or less brownish liquid and displayed dysfunctional locomotion and phases of hyper-excitement, leading to complete paralysis and death of individuals. After 4 d exposure,

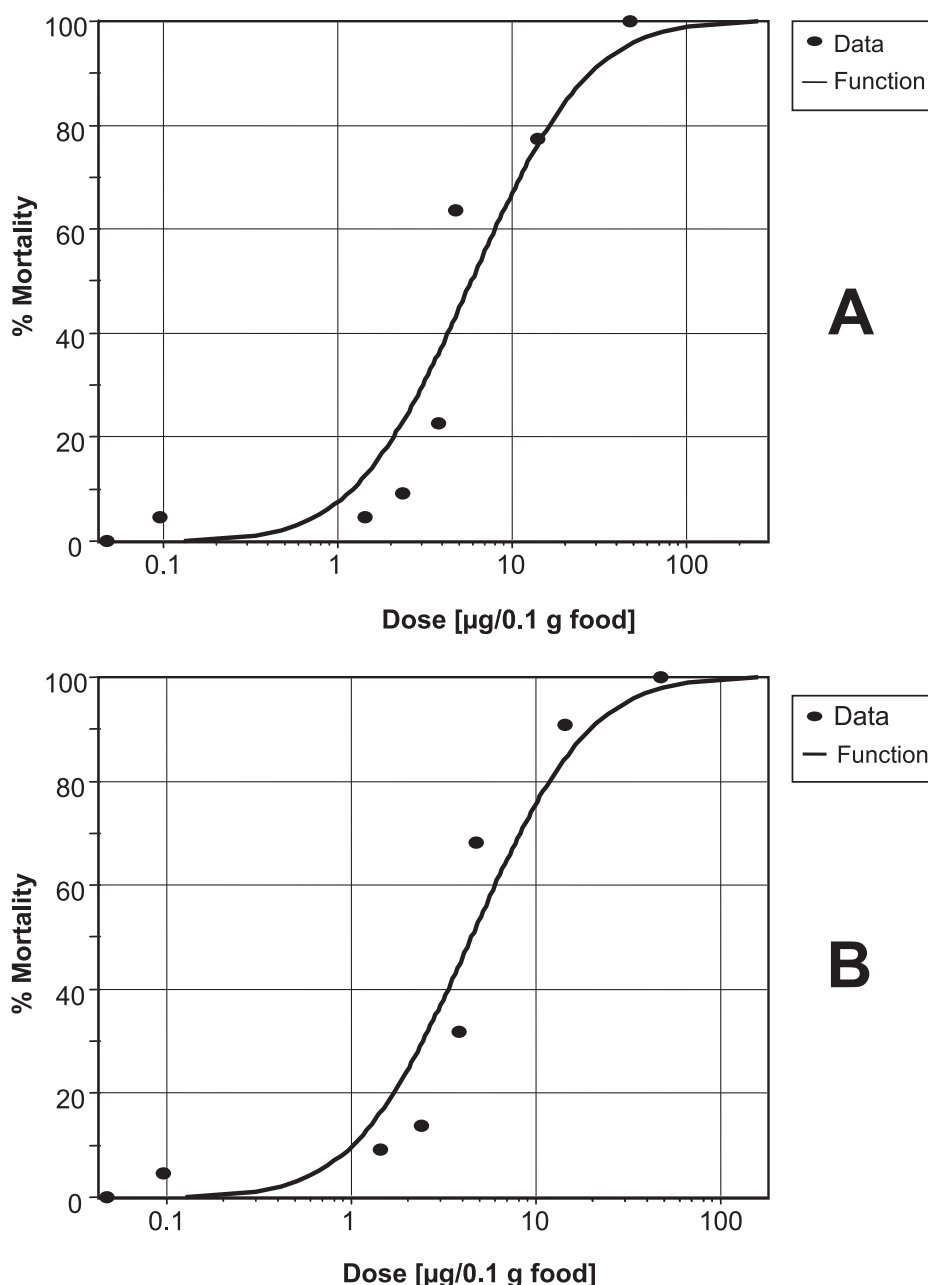


Figure 2. Concentration–effect curves showing the effect of carbosulfan ( $\mu\text{g}/0.1\text{ g food}$ ) on mortality (%) of large *Sphodroxia marocana* larvae. A: 4 d after ingestion; B: 7 d after ingestion.

*Pimelia* beetles remained as active as in controls after ingestion of  $0.047\ \mu\text{g}$  carbosulfan per  $0.1\text{ g food}$ . The first symptoms appeared with a dose 10-fold higher ( $0.47\ \mu\text{g}/0.1\text{ g food}$ ), which corresponds to the LOED value. 53.3% mortality was obtained with the absorption of  $4.72\ \mu\text{g}$  carbosulfan present in  $0.1\text{ g food}$ , and mortality of all individuals with a dose of  $14.16\ \mu\text{g}$  carbosulfan in  $0.1\text{ g food}$ , which corresponds to  $14.16\ \mu\text{g}$  carbosulfan ingested by each individual. As for *Pimelia*,  $0.047\ \mu\text{g}$  carbosulfan in  $0.1\text{ g food}$  had no significant effect on *Pachychila* beetles. After 4 d, the mortality reached 55.6% with ingestion of  $4.25\ \mu\text{g}$  carbosulfan per  $0.1\text{ g food}$ , and 100% with  $14.16\ \mu\text{g}$  carbosulfan per  $0.1\text{ g food}$ .

At low doses ( $3.77\ \mu\text{g}$  carbosulfan/ $0.1\text{ g food}$ ), the resistance of *Pachychila* beetles and *Sphodroxia* small

larvae was not significantly different, with 44.5% and 50.0% mortality, respectively. At higher doses the species susceptibility was different: 100% mortality was attained with  $14.16\ \mu\text{g}$  carbosulfan per  $0.1\text{ g food}$  for *Pachychila*, and  $47.2\ \mu\text{g}$  carbosulfan per  $0.1\text{ g food}$  for *Sphodroxia* small larvae. The type of food (potato for *Sphodroxia* larvae; bread for darkling beetles) was not of particular importance in so far as food was completely consumed by individuals ( $0.1\text{ g food}$  in all cases) and was used only to allow ingestion of carbosulfan.  $\text{LD}_{50}$  values (95% CL) calculated for *Pachychila* and *Pimelia* after 4 d were 3.30 and  $4.53\ \mu\text{g}$  carbosulfan, respectively, with NOED values of 0.47 and  $2.36\ \mu\text{g}$  carbosulfan for *Pachychila* and *Pimelia*, respectively (Table 2).  $\text{LD}_{50}$  values (95% CL) after 7 d post-ingestion were lower when compared to 4 d

values, with 2.86 and 3.97  $\mu\text{g}$  carbosulfan per individual for *Pachychila* and *Pimelia*, respectively, with the same values for NOED (0.47  $\mu\text{g}$  carbosulfan/0.1 g food) (Table 2). If LD<sub>50</sub> values were reported to the same body weight (bw) in kilograms (for comparison with other species), the difference in susceptibility was significant: 33.00 and 5.39 mg carbosulfan per kg bw for *Pachychila* and *Pimelia*, respectively at 4 d, and 28.60 and 4.73 mg carbosulfan per kg bw, respectively, after 7 d.

#### 4. Discussion

Carbosulfan is an insecticide that has been widely used in agriculture and forestry. In its dry granule formulation, it has been recommended by the manufacturers for use against many pests. It was used, for example, against the pine bark beetle *Hylaster ater* (Paykul) (Scolytinae) which compromised forest regeneration of *Pinus radiata* D. Don in New Zealand (Reay and Walsh 2002a; Reay et al. 2012), but is no longer in operational use (Reay and Walsh 2002b). Other studies have demonstrated the efficacy of carbosulfan against *Hylaster ater* (Lempérière and Julien 1989; Mrlina et al. 1994; Heritage et al. 1997) and against the large pine weevil *Hylobius abietis* (L.) (Lempérière and Julien 2003). This carbamate was also used against wireworms (elaterid beetle larvae) in corn and against the larvae of common cockchafer beetle *Melolontha melolontha* (Scarabaeidae) (Bednarek et al. 2004). Other studies have confirmed the efficacy of carbosulfan against pyrethroid-resistant mosquitoes (Guillet et al. 2001).

Correct utilization of carbosulfan in regeneration plots at the Mamora Forest reduced significantly the damage by *S. marocana* larvae. During a field test campaign, the mortality of seedlings under carbosulfan protection (the granules were buried, in contact with the roots) was 8.3% against 73.5% for the controls (untreated seedlings) (Ghaioule et al. 2010). According to our results, LD<sub>50</sub> values ranged between 1.63 and 5.84  $\mu\text{g}$  carbosulfan per larva after 4 d and between 1.50 and 4.44  $\mu\text{g}$  carbosulfan per larva after 7 d for small and large *S. marocana* larvae, respectively. The recommended dose by the manufacturer, intended to prevent damage by white grubs when planting, was 10 g carbosulfan granules per seedling (Reay and Walsh 2002b; Ghaioule et al. 2010). However, most often in the Mamora Forest this recommended dose was exceeded by the forest technicians by at least four-fold (42 g granules per seedling carbosulfan 10%, i.e. 4.2 g active substance), with obvious environmental risks (pers. obs.).

The protection of seedlings is necessary for at least four years so that trees attain a sufficient size to resist damage by white grubs (Ghaioule et al. 2007). The results of our experiment showed that the dose recommended by the manufacturer is still high and should be reduced to half. It is crucial that the use of insecticides in granular form has to be accurate, whatever compound may be recommended for plantations in the Mamora Forest. Under normal conditions, the incorporation of granules at the root level (about 50 cm deep, allowing the transfer of the systemic pesticide into the plant) is essential to prevent birds and other non-

target organisms having access to granules (Almeida et al. 2011). However, several cases of granules misuse have been observed in the Mamora Forest (pers. obs.). Some technicians were casting granules over the soil surface, or arranging them in a circle around the seedlings. In both cases this procedure is completely ineffective because white grubs move back and forth into the soil at the interface between dry and moist sand (Fegrouche et al. 2012b). As a consequence, mortality of many non-target insects was observed at the soil surface (mostly darkling beetles) and granules were consumed by granivorous birds. Even though the incorporation of granules into the soil reduces the risk to the non-target fauna living on the soil surface, the organisms living into the soil, such as darkling beetles larvae, can be adversely affected. In this forest, the density of *S. marocana* was particularly low when the degree of aridity of soils or climate was high. The highest white grub densities were observed in sites where the soils showed the highest capacity to retain water (Ghaioule et al. 2007; Fegrouche et al. 2012a). Only these sites would merit chemical protection of seedlings.

In arid ecosystems, darkling beetles constitute a key group in the use and recycling of plant litter but their beneficial role may be threatened by the widespread use of pesticides. Bad practices are not compatible with the conservation of the environment and the organisms that provide vital ecological services. Our results confirmed the high toxicity of carbosulfan against white grubs and side effects on non-target species. It is not surprising that carbosulfan was banned in Europe in 2008 and in Morocco in 2011. *Sphodroxia marocana* constitutes an excellent example of a discrete species, previously present at low density (“low noise” species) in the forest ecosystem, which has become a pest in recent decades because of imbalances related to human activities. In the future, the challenge for forest technicians will be to control this endemic species without complete eradication. For now, this control is achieved using mainly pesticides. Alternative methods are being tested, for example: plantations in alternate rows by keeping the natural vegetation intact; chemical treatments restricted to areas at risk where larval densities are the highest.

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