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Short Communication

Preliminary Soil and Aquatic Ecotoxicity Evaluation of [6]Helicene

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Abstract

Helicenes are polycyclic π-conjugated systems consisting ortho-fused aromatic rings. Since the technology of helicenes is still in its infancy, the expected environmental inputs and levels are currently unknown. However, the very promising properties of helicenes predict many potential applications in various fields. The present study investigates ecotoxic properties of [6]helicene to aquatic (*Daphnia magna, Desmodesmus subspicatus, Vibrio fischeri*) and soil (*Folsomia candida, Enchytraeus crypticus*) organisms. In the case of the aquatic species, no significant effect was observed even at the highest nominal concentration tested of 0.63 mg/L. In the case of the soil species, the higher of the two tested concentrations of 1.7 mg/g showed an inhibition of reproduction of 51% and 39% of *Enchytraeus crypticus* and *Folsomia candida*, respectively. The soil toxicity of [6]helicene is higher than expected from its lipophilicity, which might suggest a specific toxicity mechanism. Further research is necessary to fully understand the toxicity of helicenes.

Keywords: polyaromatic hydrocarbons, helicenes, ecotoxicity, aquatic, terrestrial

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are important environmental contaminants, and many of these substances are within the list of priority pollutants of the United State Environmental Protection Agency [1]. A number of toxicological studies have proven PAHs to be toxic to both aquatic [2, 3] and terrestrial [4-6] organisms, and many PAHs have also shown carcinogenic or mutagenic properties [7, 8]. Apart from the conventional PAHs that have been subjected to various research activities for many years, new polyaromatic substances are being developed, such as the helicenes.

Helicenes are polycyclic π-conjugated systems that consist of ortho-fused aromatic rings with helical chirality [9-11]. These substances have been known for about 100 years but have not attracted broader attention until approximately the mid-1990s [12]. They have attracted great interest owing to their unique structural features, molecular chiroptical properties, and wide potential applications in chiral materials [13-16], asymmetric molecular recognition [17-21], asymmetric catalysis [22-25], liquid crystal molecules [26], and nanomaterial sciences [27-30]. Up to now, the most promising field of use is electronic and opto-electronic devices [31]. Helicenes

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might also have biological applications. There are several studies on the interaction of helicenes with molecules of DNA or cholesterol [31]. However, the preparation of helicenes is still cost- and time-demanding, mainly because of expensive reagents and complicated synthesis [32]. It was not until early 2013 that the helicenes became commercially available, but they are still available only in small quantities [33]. It is therefore difficult to predict the potential contamination sources or environmental concentrations. However, it is important to investigate toxic and ecotoxic properties of new substances from the very early phase so that necessary precaution measures can be adapted to minimize the potential negative effects. To the best of our knowledge, no studies have been performed investigating the ecotoxic effects of helicenes.

The aim of the presented study is to assess the ecotoxicity of [6]helicene to both aquatic (*Daphnia magna*, *Desmodesmus subspicatus*, *Vibrio fischeri*) and soil (*Folsomia candida*, *Enchytraeus crypticus*) organisms.

Materials and Methods

Test Substance

The [6]helicene (Fig. 1) was prepared by the oxidative photocyclization procedure described elsewhere [34] and purified by crystallization. The purity of the final product was 98.8% (analyses by GC-MS, Focus DSQ, Single Quadrupole GC-MS, Thermo Electron Corp., USA; and LC-NMR, Varian ProStar 230, Varian INOVA 500 MHz spectrometer, Agilent Technologies, Inc., US). Physical and chemical properties estimated using the EPIWEB software [35] are shown in Table 1. For the aquatic tests, an aqueous stock solution was prepared by dissolving an appropriate quantity of the sample in acetone. The acetone solution was added to distilled water in a volumetric flask; the acetone was left to evaporate by stripping the solution with nitrogen for 10 min and the volume was adjusted to the graduation mark. Before helicene precipitation, appropriate volumes of the stock solution were dosed in the test vessels.

In most PAH soil toxicity studies, acetone is used as the carrier. The solubility of the toxicant in acetone was therefore estimated by adding small quantities of [6]helicene in 10-min intervals to 25 ml acetone in a test tube placed in a thermostat-controlled 25ºC water bath until the first crystals appeared. The experiment was repeated five times and the average solubility was 8.5±0.1 mg/mL. The volume of acetone necessary to make the wanted soil concentration would therefore be too high. The helicenes were therefore added directly into the soil, which was then hand mixed with a spoon. The mixture was then shaken on an overhead shaker for 24 hours in a glass flask to achieve maximum homogenization.

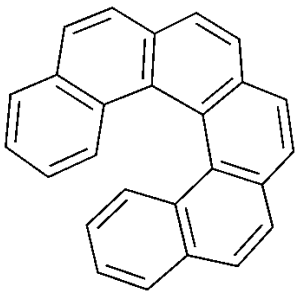

Fig. 1. The [6]helicene molecule.

Table 1. Physical and chemical properties of [6]helicene tested for its ecotoxicity to different soil and water organisms predicted by the EPIWEB 4.1 software by the U.S Environmental Protection.

	[6]helicene
$\log K_{ow}$	7.87
$\log K_{oc}$	7.32
water solubility	0.12 μg/L
Henry constant	$8.59 \cdot 10^{-4}$ Pa·m^{-3}/mol

Aquatic Tests

The crustacean test evaluates the immobilization of *Daphnia magna* Straus and was performed according to the ISO 6341:1996 method [36]. The daphnids were kept under a 16:8-h light:dark photoperiod at 20ºC and fed with algae. The test was performed in triplicate with female individuals of *D. magn*a – up to 24 hours old exposed to four concentrations of the tested sample for 48 h.

The *Vibrio fischeri* test, which measures a decrease in bacterial respiration as a function of reductions in luminescence, was performed according to the ISO 11348-2:2007 method [37]. A liquid-dried culture of *V. fischeri* (Bruno Lange/Hach Lange, Düsseldorf, Germany) was used for the test, which was performed two times in duplicate at four concentrations of the sample as dilutions in 20 g/L of NaCl solution (p.a., Penta, Czech Republic). The luminescence (tube luminometer, model LM02Z, Immunotech, Prague, Czech Republic) was measured after 5, 15, and 30 min using an NaCl solution (20 g/L) as control.

The algae (*Desmodesmus subspicatus*) test measures the inhibition of growth of the algae and was performed according to the ISO 8692:2012 method [38]. The algae were cultivated in the standard medium under continuous illumination (6,000 lux, 27ºC) in 250 ml flasks with constant aeration (the air was purified with an antibacterial filter). The test was performed in triplicate in 25-ml Erlenmeyer flasks capped with cellulose caps, which were continuously shaken on an orbital shaker (240 rpm). The concentration of the algae was evaluated every 24 h for 3 d using a light microscope and a counting chamber.

For all of the aquatic tests (*Daphnia magna*, *Desmodesmus subspicatus*, and *Vibrio fischeri*), we used the same nominal concentration range (mg/L): 0.63, 0.3, 0.14, and 0.07.

Soil Tests

The soil used in the tests was a standard soil prepared according to OECD guideline No. 317 [39], which consists of 69.2% fine quartz sand, 20% clay (CLUZ, Czech Republic), 10% peat (Agro CS, Czech Republic; air-dried and sieved through a 2 mm sieve), and 0.8% calcium carbonate (p.a., Penta, Czech Republic). The content of organic and inorganic carbon was 6.14% and 0.03%, respectively (LiquiTOC II, ELEMENTAR Analysensysteme, Germany). The soil pH (0.01M $CaCl_2$) was 6.4±0.1.

The springtail (*Folsomia candida*) test measures the effects of substances on reproduction and was performed according to the ISO 11267:1999 method [40]. Springtails were raised in plastic boxes on an 8:1 mixture of plaster (Kittfort, Czech Republic) and activated charcoal (p.a., Fluka Analytical, France) under a 16:8-h light:dark photoperiod (500 lux) at 20ºC and fed with dry yeast. The enchytraeids (*Enchytraeus crypticus*) test measures the effects of substances on reproduction and was performed according to the ISO 16387:2004 method [41]. Earthworms were raised in plastic boxes in standard soil under a 16:8-h light:dark photoperiod (500 lux) at 20ºC and fed with mashed oatmeal. The quantity of the sample that was available for testing was limited. The highest soil concentration that was possible to test was 1.7 mg/g soil (dry weight). Apart from that, a concentration 10 times lower, 0.17 mg/g, was also tested. Both of the tests were performed in triplicate in 100-ml glass beakers at the same conditions as the cultivation. Due to a small quantity of the sample available, in order to obtain the desired concentrations it was necessary to lower the quantity of the test soil. Therefore, we tried to perform the tests with 20 and 10 g of standard soil in every beaker for *F. candida* and *E. crypticus*, respectively, and compare it with the standardized quantities (30 and 20 g, respectively). We observed no statistically relevant difference in the results (data not shown; independent samples *t*-test; $p>0.05$). The soil was moistened to 50% of the water-holding capacity and the beakers were covered with a plastic film. Every 7 d, the beakers were aerated, the soil humidity checked, and food (yeast or oatmeal) added. After 28 d, the number of juveniles was counted. The enchytraeids were immobilized by ethanol, coloured with Bengal red, and hand counted. The springtails in every beaker were photographed after the addition of a mixture of water and black ink and counted on a computer using the UTHSCSA ImageTool [42].

Results and Discussion

The helicenes are very promising substances with many potential applications, but the production price is still high and the substances are therefore available only in small quantities. The sample available for our study was limited so it was not possible to perform a full ecotoxicity study, but only a preliminary one to indicate the possible ecotoxic properties of helicenes. Since to our knowledge no ecotoxic data on helicenes were published up to now, five basic standardized ecotoxicological biotests have been chosen for which studies on other PAHs have been published to allow comparison. The study was performed using three aquatic (crustaceans *Daphnia magna*, microalgae *Desmodesmus subspicatus*, bacteria *Vibrio fischeri*) and soil (springtails *Folsomia candida* and enchytraeids *Enchytraeus crypticus*) organisms.

Based on the estimation from US EPA software EPIWEB (Table 1), the [6]helicene is a highly lipophilic substance. This implies a low toxicity to both aquatic and terrestrial organisms because of low water solubility and a high affinity toward carbonaceous soil constituents, resulting in decreased bioavailability. In general, our data confirm the trends that have been established in studies with other PAHs. However, a few differences might suggest a possible different mode of action or behaviour of the helicenes, possibly resulting from their unique structure.

Aquatic Tests

The aquatic solubility of [6]helicene is supposedly very low, i.e. 0.12 μg/L based on the EPIWEB software. Other studies with aquatic organisms suggest that low or no toxicity could therefore be expected [3, 43, 44]. The aquatic tests were therefore performed using higher concentrations with the toxicant in excess to make sure the maximum aquatic concentration was achieved. The actual aquatic concentration of the [6]helicene was not measured during this study, but if the solubility is in the range of the predicted value it would most probably be below the detection limit. This, however, will need to be verified in future studies.

All of the aquatic tests did not show any measurable acute effect of the [6]helicene (data not shown). In the case of the Daphnia test, after 24 h and for the two highest concentrations, a thin layer of the helicene was observed at the bottom of the test vessel. The daphnids tend to forage at the bottom looking for food, which means they were in intense contact with the helicene layer. With their filter-feeding nature, it is also expected that they would ingest some part of it. Even though no measurable acute effect was observed. Precipitation of the toxicant was expected also during the algae test, even though it could not be visually verified. But because of the constant shaking of the test vessels the precipitate most probably did not sediment. Again, the algal cells were in close contact with the toxicant, which could be adsorbed on the cell surface. However, it did not affect the growth of the algae in any way.

Soil Tests

Both of the soil tests were valid since the mean mortality was lower than 20% and the number of juveniles was higher than 100 per control for both organisms. For both of the soil tests (*Enchytraeus crypticus*, *Folsomia*

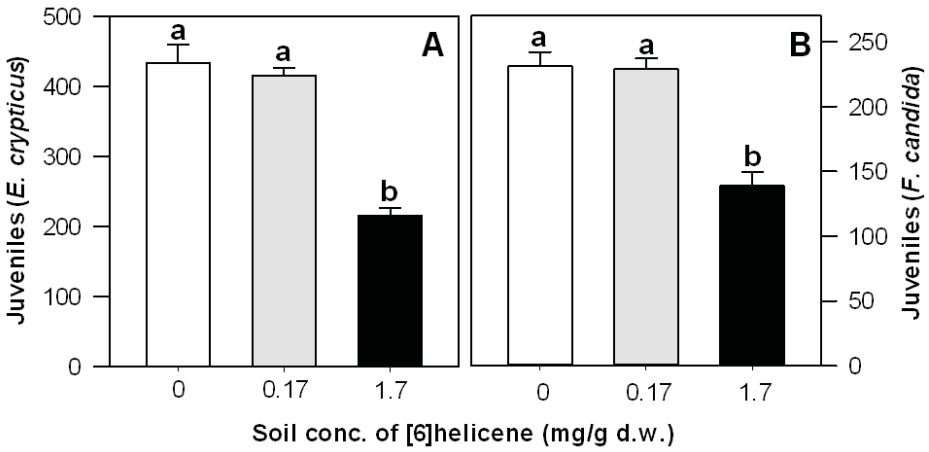

Fig. 2. Inhibition of reproduction (number of juveniles) of *Enchytraeus crypticus* (A) and *Folsomia candida* (B) exposed to control (white bars), 0.17 (grey bars), and 1.7 (black bars) mg/g d.w. of [6]helicene for 28 d. Data are mean±one standard deviation, n=3. Different lowercase letters indicate significant differences (ANOVA, $p \leq 0.05$).

candida), we used two concentrations of 0.17 and 1.7 mg/g (dry weight). The results of the tests are presented in Fig. 2. The lower concentration did not cause any significant effect on the reproduction of both species (ANOVA Dunnet, $p>0.05$). On the other hand, the higher concentration caused an important effect (ANOVA Dunnet, p=0.005 and 0.013 for *E. crypticus* and *F. candida*, respectively) with an inhibition of reproduction of 38.4±11.1 % and 50.7±2.8 % (mean±one standard deviation, n=3) for *E. crypticus* and *F. candida*, respectively.

The PAHs do not possess any functional groups and thus it is believed that the mechanism of toxicity consists of nonpolar narcosis when fluidity and function of cell membranes is affected [5, 43]. Even though soil ingestion has been suggested as a possible route of exposure, it has been shown that soil organisms are exposed to PAHs mainly through pore water [5, 6, 43]. The concentration of PAHs in the pore water depends on several factors, especially lipophilicity of the substance (represented by the K_{ow}, the octanol-water partition coefficient), its soil sorption properties (represented by the K_{oc}, the soil organic carbon-water partition coefficient), and the content of organic carbon in the soil in question [5, 43]. The predicted log K_{ow} and log K_{oc} of [6]helicene (Table 1) as well as the TOC of the OECD soil used in this study (6.1%) are very high. It can therefore be expected that the adsorption of [6]helicene to the soil would be very important and that the pore water concentration would be very low. Other studies on toxicity of PAHs on soil invertebrates suggest that toxicity of such substances is not important. For example, Sverdrup et al. tested toxicity to *Folsomia fimetaria* of 16 different PAHs with log K_{ow} ranging from 3.32 to 6.70 [43]. The PAHs with log K_{ow}>5.5 did not show a significant effect on reproduction even at the highest concentration tested, which was around 1 mg/g for all the substances. Similarly, Droge at al. tested toxicity of 6 PAHs to *F. candida* and *E. crypticus*. For example benzo[a]pyrene (log K_{ow}=6.02, log K_{oc}=6.27) did not have a significant effect on reproduction even at 3.7 mg/g [4]. Accepting this presumption, in this study the [6] helicene should not be toxic – even at the higher of the two tested concentrations. However, we have observed an important inhibition of reproduction, which in the case of *E. crypticus* exceeded 50%. Because of a limited quantity of the sample available for testing, we were able to test only two concentrations. It was therefore not possible to calculate any ecotoxicological indexes. But the results suggest that the EC50 for both organisms would be around the tested 1.7 mg/g. This might suggest that [6]helicene might possess a different mode of action than the non-polar narcosis. This was also suggested, for example, for anthracene, which has proven to be more toxic that would be expected from its properties [4, 5]. Another explanation might be that the actual log K_{ow} is lower that the value predicted by the EPIWEB software. However, more research is necessary to fully understand the toxicity of [6]helicene and its mechanisms.

Conclusion

The presented study is to our knowledge the first investigation of the ecotoxicity of helicenes, a very promising group of substances with many potential applications. The results show no toxicity of [6]helicene towards the water species (*D. magna*, *D. subspicatus*, *V. fischeri*) within the tested nominal concentration range (0.07-0.63 mg/L). In the case of the soil species (*E. crypticus*, *F. candida*), the tests show toxic effects at 1.7 mg/g. These toxic effects are higher than would be expected based on the lipophilicity of [6]helicene, which might point to different toxicity mechanisms of the helicenes compared to planar PAHs. Further investigation of especially the chronic or special (i.e. mutagenity, etc.) toxic effects as well as of the properties and behaviour in environmental media is, however, necessary to fully understand the toxicology of helicenes.

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