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Citation for the published paper:

Gustafsson, Kerstin; Blidberg, Eva; Karlsson Elfgren, Irene; Hellström,
Anna; Kylin, Henrik; Gorokhova, Elena. (2010) Direct and indirect effects
of the fungicide azoxystrobin in outdoor brackish water microcosms.

Ecotoxicology. Volume: 19, Number: 2, pp 431-444.

<http://dx.doi.org/10.1007/s10646-009-0428-9>.

Access to the published version may require journal subscription.

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Published in *Ecotoxicology* (2010) 19:431–444

DOI 10.1007/s10646-009-0428-9

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Direct and indirect effects of the fungicide azoxystrobin in outdoor brackish water microcosms

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Abstract

The effects of the strobilurin fungicide azoxystrobin were studied in brackish water microcosms, with natural plankton communities and sediment. Two experiments were conducted: Experiment 1 (nominal conc. 0, 15 and 60 µg/L, 24-L outdoor microcosms for 21 days) and a second, follow-up, Experiment 2 (nominal conc. 0, 3, 7.5, 15 µg/L, 4-L indoor microcosms for 12 days). The microcosms represent a simplified brackish water community found in shallow semi-enclosed coastal areas in agricultural districts in the Baltic Sea region. Measured water concentrations of the fungicide (Experiment 1) were, on average, 62 and 83% of nominal concentrations directly after application, and 22 and 26% after 21 days, for the low and high dose treatments, respectively, corresponding to mean DT50-values of 15.1 d and 25.8 d, for low and high dose treatments, respectively. In Experiment 1, direct toxic effects on calanoid copepods at both test concentrations were observed. Similarly, in Experiment 2, the copepod abundance was significantly reduced at all tested concentrations. There were also significant secondary effects on zooplankton and phytoplankton community structure, standing stocks and primary production. Very few ecotoxicological studies have investigated effects of plant protection products on Baltic organisms in general and effects on community structure and function specifically. Our results show that azoxystrobin is toxic to brackish water copepods at considerably lower concentrations than previously reported from single species tests on freshwater crustaceans, and that direct toxic effects on this ecologically important group may lead to cascade effects altering lower food webs and ecosystem functioning.

Keywords: Baltic Sea; strobilurin fungicides; Copepoda; indirect effects; model ecosystems; toxicity

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Introduction

Pesticides used in agriculture are regularly detected in aquatic environments throughout the world. One of the major groups of pesticides, the fungicides, constitutes a chemically very diverse group of compounds used against fungal infection in crops, and is in some regions of the world the quantitatively most important pesticide group (Castillo et al., 1997). In Sweden, fungicides are the second largest group, after herbicides, of pesticides used for crop protection, accounting for about 11% of the amount sold (Swedish Chemicals Agency, 2007). Fungicide usage in temperate regions such as Sweden is highly dependent on weather conditions during the summer months, with increased usage during rainy periods (Swedish Chemicals Agency, 2003), i.e. the same conditions as may also increase the pesticide runoff from fields to adjacent aquatic ecosystems. The fungicide azoxystrobin was introduced on the world market in 1996 and has since then been registered for use on a wide range of crops (Tomlin, 2000; Bartlett et al., 2002). Based on sales figures, azoxystrobin dominated the world market for fungicides in 1999 (Bartlett et al., 2002). In Sweden, azoxystrobin accounted for approximately one fifth, 40 tons active ingredient (a.i.), on average of the annual total amount of fungicides used in agriculture over the years 1999 - 2002 (Swedish Chemicals Agency, 2003), but the annual amounts sold have in latter years dropped to around 7.3 tons a.i. on average (years 2003 - 2007) (Swedish Chemicals Agency, 2008). Azoxystrobin is a broad-spectrum systemic fungicide belonging to the group β -methoxyacrylate strobilurins. The biochemical mode of action for strobilurin fungicides is the same as for the naturally occurring strobilurins; they inhibit the electron transport in the respiration pathway in mitochondria, thereby disrupting all energy demanding processes in the target organisms (Tomlin, 2000; Bartlett et al., 2002).

The toxic effect of insecticides on non-target aquatic invertebrates such as crustaceans is well recognised, as these compounds are designed to target arthropods. However, many substances used as fungicides can also be very toxic to non-target organisms, such as crustaceans, as seen in reported toxicity data (e.g. Tomlin, 2000), and may also affect the structure and function of biological communities. For example, the fungicide carbendazim has been shown to alter the composition of freshwater model ecosystems, with cladocerans being the most susceptible zooplankton group (van den Brink et al., 2000; Slijkerman et al., 2004), although cyclopoid copepods were also affected (van den Brink et al., 2000). To our knowledge, no information is available on the effects of strobilurin fungicides on aquatic systems of higher biological complexity than single species tests.

Azoxystrobin is highly toxic to several aquatic organisms. Acute LC₅₀-value (48h) for *Daphnia magna* has been determined to 190 $\mu\text{g/L}$ and the chronic (21d) no-observed effect concentration (NOEC) to 44 $\mu\text{g/L}$ (European Commission Peer Review Programme, 1997). Reported NOEC (48h) for the copepod *Macrocyclops fuscus* is 62 $\mu\text{g/L}$ (Elf et al., 1997). Other crustaceans have been shown to be even more sensitive, e.g. mysid shrimp *Mysidopsis bahia*, with reported LC₅₀ (96h) of 56 $\mu\text{g/L}$ and NOEC (28d) of 9.5 $\mu\text{g/L}$ (Pesticide Ecotoxicity Database, 2001). Azoxystrobin was also highly toxic to the green microalga, *Raphidocelis subcapitata* (former name: *Selenastrum*

capricornutum), with a growth rate NOEC (96h) of 38 µg/l (European Commission Peer Review Programme. 1997).

The Baltic Sea has a number of properties that make it particularly vulnerable to anthropogenic disturbances (e.g. review by Jansson, 2003). It is the largest brackish water body in the world. Its drainage basin covers a densely populated area, and considerable amounts of anthropogenic contaminants will inevitably end up in the Baltic Sea. As the water retention time is high (25-30 years), the effects of chemical substances may be long-lasting.

Furthermore, owing to its young evolutionary history and moderate salinity levels (approx. 2 – 10 psu, practical salinity units), the species diversity in the Baltic Sea is generally lower than in most true marine (high salinity) or freshwater ecosystems. Thus, the functional redundancy, i.e. the extent to which multiple organisms in a system contribute to the same ecosystem function, can be assumed to be low. If the impact of a toxic substance suppresses susceptible species, there may not be any less sensitive species available to maintain the specific ecosystem function. These and other properties make it particularly important to study the effects of anthropogenic toxicants on Baltic Sea model ecosystems. Agricultural runoff may be an important pesticide source to estuarine systems (Hapeman et al., 2002). In the Baltic Sea, residues of old-type persistent organochlorine insecticides, e.g. Σ DDT and HCHs, as well as newer pesticides, such as of triazine herbicides, have been detected (Bester and Hühnerfuss, 1993; Pempkowiak et al., 2000). No available studies have addressed the occurrence of azoxystrobin in the coastal Baltic Sea region, but in fresh surface water samples from agricultural regions in southern Sweden, the substance is regularly detected, at concentrations ≥ 1 µg/L (Adielsson and Kreuger, 2008).

The aim of this study was to investigate the effects of the fungicide azoxystrobin on Baltic Sea coastal model ecosystems using simple, small-scale microcosms consisting of Baltic Sea water, plankton and sediment. As azoxystrobin acts upon the basic biological function (respiration pathway in mitochondria), its toxic effects were expected to be manifested at several different trophic and functional levels in the model communities. The concentrations chosen for Experiment 1 were based on the existing toxicity data. The high treatment (60 µg/L) was similar to the reported NOEC for copepods, while the low treatment (15 µg/L) was 2-3 times lower than the reported NOEC for the green alga and cladoceran, respectively. However, the impact on key components of the system was found to be severe already in the low dose treatment and therefore we conducted a second follow-up experiment, Experiment 2, to test the community responses at even lower concentrations. Experiment 2 was designed to focus entirely on the effects on the zooplankton community structure and was performed in smaller microcosms for a shorter time period than Experiment 1 (12 days). In Experiment 2 we chose a concentration range starting with the low dose for Experiment 1 and going down to 3 µg/L

1. Materials and methods

1.1. Experiment 1

1.1.1. Experimental design of microcosm study

The study was conducted outdoors at the Askö Marine Laboratory (58° 49' N, 17° 38' E, Stockholm Marine Research Centre, Stockholm University), in the southern Stockholm Archipelago, northern Baltic proper, Sweden. Sediment (organic content 2%) was collected from a shallow bay at Askö on 1 April, 2003, sifted through a 5-mm sieve and stored covered with an aerated water phase in darkness at 4 °C for approx. three months prior to the experiment. This procedure aimed at maintaining the pre-spring bloom characteristics of the sediment, including high abundances of resting stages for different plankton. In mid-June, the sediment and water was transferred to a large open hatching tank placed outdoors in ambient light. After two weeks a graft of living plankton, natural unfiltered surface water (100 L), together with a concentrated graft of living plankton produced by sieving Baltic Sea water (40 L) through a 100 µm mesh net, was added to the hatching tank. Approximately one week later, microcosms (white plastic 30-L containers, 18 microcosms in total) were constructed by adding sediment (380 ml; 375 g dry wt) and water (14 L, sieved through a 4 mm sieve) from the hatching tank, together with unfiltered surface water (10 L), to each microcosm. The microcosms were placed outdoors in three parallel troughs, 6 microcosms in each, with constantly flowing seawater serving as a cooling system, and were left to stabilise for two days. The zooplankton densities and dominance structure in the experiment corresponded well to the ambient zooplankton communities in July-August in coastal areas of the northern Baltic proper, where zooplankton abundance reach densities 100-500 ind./L and dominate heavily by juvenile copepods (see Fig. 4 in Gorokhova et al. 2000, and Fig 2B in Gorokhova et al. 2004).

During the three week experimental period, evaporated water in the microcosms was replaced with deionised water on two occasions. At mid-day at the start of the experiment (day 0), the average microcosm temperature was 19.8 °C and the salinity was 6.1 psu (see Table 1 for mean values over the whole experimental period). The weather during the experimental period was mostly dry; during few episodes of rain, the whole experimental system was covered with a tarpaulin.

We used three treatments of the test substance azoxystrobin (Dr. Ehrenstorfer GmbH), with the nominal concentrations 0 µg/l (control treatment; CT), 15 µg/L (low treatment; LT) and 60 µg/L (high treatment; HT), each with six replicates placed in random order. The test substance was added from a stock solution with acetone as the solvent, and equal amounts of acetone (not exceeding 0.01% of volume, as recommended by OECD guidelines, e.g. *Daphnia magna* Reproduction Test, OECD 2008,) was added to all microcosms, including controls. The application was done in the evening of July 19 (Day 0), and the microcosms were monitored for 21 days.

1.1.2. Physical and chemical variables

Temperature, salinity, dissolved oxygen and pH in the microcosms were, with a few exceptions, monitored daily, during mid-day, using a Multi 340i (WTW). Filtered (0.7

μm) water samples for dissolved nutrient analyses ($\text{NH}_4\text{-N}$, $(\text{NO}_2+\text{NO}_3)\text{-N}$, $\text{PO}_4\text{-P}$) were taken at the end of the experiment (day 21), and stored frozen ($-20\text{ }^\circ\text{C}$) until analysis at the Chemistry Laboratory, Dept. of Systems Ecology, Stockholm University, according to Ranger (1993).

1.1.3. Zooplankton community structure

Zooplankton samples were taken from the microcosms on day 3, 7, 16 and 20. Water was gently stirred and then sampled (2-4 L of water on different occasions) and filtered through a $48\text{ }\mu\text{m}$ mesh net. The retrieved organisms were rinsed into a 100-ml glass bottle and preserved by adding formaldehyde (final concentration 4%). Sampled water was returned to the microcosms after filtration. The zooplankton samples were analysed using an inverted microscope (Leitz fluovert FS, Leica) and 10-ml counting chambers. Copepods were separated into three groups, viz. nauplii, younger copepodites (C I-III) and older copepodites (C IV-VI, including adults identified to species). Cladocerans and rotifers were identified to genus or species level, when possible.

1.1.4. Phytoplankton community structure

Samples were taken on day 7 and 20 by submersing opened sample bottles (100 ml) into the microcosms while gently stirring the water column. The samples were preserved with Lugol solution and then counted and identified using an inverted microscope, 40 x magnification, and Utermöhl chambers. The biovolume of algal cells was calculated using taxon specific formulae and converted to biomass assuming unit specific gravity (HELCOM, 1988).

1.1.5. Phytoplankton primary production

The phytoplankton primary production, estimated from ^{14}C -incorporation, was analysed on nine occasions (day 0, 1, 2, 4, 8, 11, 14, 17, 20). From each microcosm, two replicate water samples (10 ml) were filtered through a $160\text{ }\mu\text{m}$ sieve, transferred to glass scintillation vials, and $100\text{ }\mu\text{l}$ $\text{NaH}^{14}\text{CO}_3$ (Amersham Biosciences; specific activity 60 mCi/mol , activity in solution $5\text{ }\mu\text{Ci/ml}$) was added to each vial. The two replicate samples were placed in a shallow tub with flowing water that allowed cooling, one sample in ambient light conditions while the other was incubated in darkness as a control of non-photosynthetic incorporation. Incubation was performed during mid-day for 240-312 minutes and was stopped by addition of a few drops of HCl. After air-bubbling for 30-60 min, Lumagel Safe scintillation liquid (10 ml) was added and the samples were kept in darkness until analysis on a liquid scintillation counter (1214 Rackbeta, LKB Wallac). Primary production was calculated according to Parsons et al. (1984).

1.1.6. Chlorophyll a concentration in the water

Water samples were taken on day 3, 7, 11, 15 and 20 from the microcosms after gentle stirring of the water column, and filtered through a $160\text{ }\mu\text{m}$ sieve. Then, 500-1000 ml of sampled water was filtered onto a cellulose-nitrate filter (Sartorius, $0.8\text{ }\mu\text{m}$). The filters were stored frozen ($-20\text{ }^\circ\text{C}$) until analysis. 3 ml 90% acetone was used for extraction, and the extracts analysed spectrophotometrically according to Swedish standard methods (SIS, 1980).

1.1.7. Chlorophyll a in periphyton

Unglazed ceramic tiles (ca. 2.3 x 2.3 cm, total area 69-95 cm² per microcosm) were placed in plastic (HD polyethylene) frames and attached close to the walls of the microcosms. At the end of the experiment, the tiles were removed and gently brushed clean in tap water. The suspensions were filtered onto cellulose-nitrate filters and Chl *a* determined as described for phytoplankton, but using 7 ml of acetone.

1.1.8. Bacterioplankton activity

For the bacterial activity in the water phase, the incorporation of ¹⁴C-leucine (Amersham Biosciences; specific activity 295 mCi/mmol, activity in solution (approx. 1.2 µCi/ml) was analysed. Water samples of 1 ml (triplicate samples of which one served as an abiotic control) sieved through 160 µm sieve, were taken on day 1, 2, 4, 8 and 14 from all microcosms, and on day 0 from three randomly selected microcosms and placed in Eppendorff tubes. To the abiotic control samples, 50 µl cold 100% TCA (trichloroacetic acid) was added, and 4 µM ¹⁴C-leucine (50 µl) was added to all samples. Incubation lasted for 50-90 minutes in darkness, ambient temperature, and was terminated by addition of 100 µl cold formalin. In the next step, cold 100% TCA (50 µl) and skimmed milk (15 µl) were added to the tubes. They were centrifuged for 10 min at 13000 x *g* at 4 °C, the supernatant was removed and 1 ml 5% TCA was added. After another centrifuge session (13000 x *g*, 5 min at 4°C), the supernatant was removed and 1 ml Lumasolve scintillation liquid was added. After 24 h at room temperature, the samples were analysed by liquid scintillation counting. The results are presented as disintegrations per minute (dpm) per incubation minute and can be seen as a relative measure of the bacterioplankton activity in the samples at each sampling occasion.

1.1.9. Degradation of organic material

Stems and leaves of *Ranunculus baudotii* were used as a substrate to study the ecological function of biological degradation in the microcosms. Plants were harvested in the beginning of July in the same shallow bay where the sediment was taken, and dried at 70 °C to constant weight. On day 0, dried plant material was weighed, put in stainless steel holders, weighed again, and placed in the microcosms, one with stem and one with leaf material in each microcosm. At the end of the experiment, the holders were dried and weighed and the percent degraded calculated.

1.1.10. Fungicide residue analyses

Unfiltered water (100 ml) was taken from the low and high treatment microcosms at the start of the experiment (day 0), from five of the six replicates, transferred to 500-ml glass bottles and stored frozen (-20 °C) until analysis. The corresponding water volume was removed from the remaining microcosms. At the end of the study (day 21), the procedure was repeated but now 250 ml of water was sampled and stored in 1000-ml bottles. In addition, sediment samples (approx. 250 ml) from all fungicide treated microcosms were taken at the end of the study, and stored frozen in plastic bottles until analysis.

Water samples (50 ml) were extracted with dichloromethane (3x20 ml) after addition of sodium chloride (5 g) and ethion as surrogate standard. The high treatment water samples were diluted five times before extraction. The combined dichloromethane extracts were dried with sodium sulphate and filtered.

Sediment samples were centrifuged at 1500 rpm for 5 minutes, after which subsamples (10-15 g) were thoroughly mixed with Hydromatrix desiccant (Varian) at the ratio 3:1. The sediment-hydromatrix mix (10 g) was extracted with acetone:dichloromethane (1:1) in a Soxtec Avanti 2050 autosystem (Foss Tectator, Höganäs, Sweden) after addition of ethion as surrogate standard. After changing the solvent to cyclohexane: dichloromethane (1:1) and filtration the extracts were cleaned-up by size exclusion chromatography (Biobeads SX-3, Bio-Rad, Hercules, California) using cyclohexane:dichloromethane (1:1) as eluent. The dry matter contents were determined in parallel sediment subsamples after the centrifugation.

Quantifications were performed on a Thermo Finnigan Trace GC 2000 gas chromatograph equipped with a HP-5 MS column (30 m x 0.25 mm) and a mass spectrometric detector (Thermo Finnigan Trace DSQ) using three masses for identification in the selected ion monitoring mode. A five-point calibration curve was used for quantification.

Dissipation rates in water over the 21 days were used to calculate DT_{50} -values for low dose and high dose treatment. The recovery of azoxystrobin from the whole microcosm system (low and high dose respectively) was calculated as the ratio between the sum of the amounts found in the water and sediment compartments at day 21, and the amount added to the microcosms at day 0.

1.2. Experiment 2

This experiment focused on the effects of the fungicide on zooplankton and was conducted in a temperature controlled chamber (15 °C) with an artificial light:dark regime of 16:8 h. Hatching of organisms from sediment collected and stored as described for Experiment 1 started three weeks prior to the microcosm construction. In addition, water and associated organisms were collected from shore in the middle Stockholm archipelago (59° 13' N; 18° 23' E), northern Baltic proper, Sweden, in September 2003. A water mixture was prepared by adding a concentrated sample of zooplankton, obtained by filtering Baltic Sea water (36 L) through a 100 µm mesh net, to a mixture of Baltic Sea water (150 L) and hatching tank water (15 L). The microcosms (5-L white plastic containers) were constructed by placing sediment (approx. 80 ml; 80 g dry wt) in the bottom, and gently filling up with the water mixture (4 L). The microcosms were left to stabilise for three days before the test substance was added at the nominal concentrations: 0, 3, 7.5 and 15 µg azoxystrobin/L, with five replicates each. The azoxystrobin solution was prepared and applied in the same way as in Experiment 1. One extra microcosm was used for sampling of dissolved nutrients (same procedure as described above) and measurements of salinity and pH (Multi 340i (WTW) at the start of the study (day 0).

The zooplankton community was sampled after 12 days of exposure. Water (approx. 3.5 L) was carefully removed from each microcosm using a siphon and filtered through a 48 µm sieve. The collected organisms were preserved with Lugol solution and identified

and counted as described for the main experiment. For logistic reasons, no fungicide residue levels were analysed in Experiment 2.

1.3. Statistical analyses

1.3.1. Univariate analyses

Data on dissolved nutrients, primary production, chlorophyll a content in plankton and periphyton, bacterial activity and degradation process were analysed using analysis of variance (ANOVA) on log-transformed data, while the zooplankton analysis of abundance of copepods and rotifers were performed on 4th root transformed data, both for Experiment 1 and Experiment 2, as recommended for abundance data when there are lots of zeros and few large values (Quinn & Keough, 2002). Tests showing significant differences were followed by Dunnett's post hoc test to compare treatments with controls. Variance homogeneity was tested according to Cochran's C test. All comparisons were made between treatments on separate sample occasions and all univariate statistics were performed using Statistica 6.0 for Windows (StatSoft Inc., USA). Unless specified otherwise, data are reported as mean values \pm SE.

1.3.2. Multivariate analyses

The structure of the zooplankton community on day 3 and 20 and the phytoplankton community on day 7 and 20 in Experiment 1, and the structure of the zooplankton and phytoplankton community in Experiment 2, was analysed using multivariate statistical methods using Primer v5 software package. Analysis of similarity (ANOSIM), followed by pairwise comparisons between treatments and controls, were used to investigate the differences between the communities. Bray-Curtis similarity measure and square root transformed data were used. Analyses of similarity percentages (SIMPER) were used to determine taxa contributing the most to the observed dissimilarities in the data sets.

2. Results

2.1. Experiment 1

2.1.1. Physical and chemical variables

Mean values of temperature, salinity, dissolved oxygen, pH and dissolved nutrients in the microcosms are presented in Table 1. At day 21, NH₄-N and PO₄-P were both significantly higher in the high dose fungicide treatments compared with controls (Dunnett's post hoc test, $p \leq 0.05$). No differences between treatments were seen for (NO₂+NO₃)-N ($p > 0.05$).

2.1.2. Zooplankton community structure

The zooplankton community of control microcosms was dominated by calanoid copepods *Eurythemora affinis* and *Acartia bifilosa*, with nauplii being the most numerous. The total copepod abundance was significantly lower compared with controls in both fungicide concentrations on day 3 and day 7 (ANOVA followed by Dunnett's post hoc test, $p \leq 0.001$; Figure 1a). The total rotifer abundance was significantly higher on all sampling occasions except for day 3, in both low and high dose treatments

compared with controls (Dunnett's post hoc test, $p \leq 0.01$ in all cases; Figure 1b). On day 7, the number of rotifers increased nearly 4-fold in the low dose treatment, and more than 5-fold in the high dose treatments, compared with controls. The composition of the zooplankton community on day 3 and 20 is presented in Table 2.

Analysis of similarity (ANOSIM) performed on the zooplankton community structure on day 3 revealed a significant difference between the treatments (global $R=0.748$, $p \leq 0.001$). The pairwise tests showed that both low- and high-dose treatments differed significantly from the controls ($R=0.448$, $p \leq 0.01$ and $R=1$, $p \leq 0.01$, for low and high dose treatments, respectively). The SIMPER analysis showed that a drastic decrease of the copepod nauplii contributed the most to the observed effects, and accounted for over 85% of the dissimilarities between both low and high dose fungicide treatments, compared to controls.

On day 20, the zooplankton communities remained different between the treatments (ANOSIM global $R=0.702$, $p \leq 0.001$) and pairwise comparisons showed that both low dose treatment ($R=0.552$, $p \leq 0.01$) and high dose treatment ($R=1$, $p \leq 0.01$) differed significantly from controls. However, more groups were responsible for the observed differences than on day 3. Decreased abundances of nauplii (33% explained), older copepodites (12%) and the cladoceran *Ceriodaphnia* spp. (12%), were the three groups that contributed with more than 10% each to the observed differences between the low dose treatments and the controls. For the high dose treatments, the decrease of nauplii (33%), increase of the rotifer *Euchlanis dilatata* (13%) and decrease of older copepodites (10%), explained most of the differences.

2.1.3. Phytoplankton community structure

The structure of the phytoplankton community was significantly altered on day 7 (ANOSIM global $R=0.259$, $p \leq 0.05$) and on day 20 (ANOSIM Global $R=0.442$, $p=0.001$). The pairwise tests between controls and low dose treatments resulted in $R=0.572$, $p \leq 0.01$ and $R=0.461$, $p \leq 0.05$, for the two sampling days, respectively, while the results of the comparison between controls and high dose treatments was $R=0.409$, $p \leq 0.05$ and $R=0.694$, $p \leq 0.01$, respectively.

On day 7, *Chrysochromulina* spp. and dinophyceans together with an unidentified small algae, contributed with more than 65% to the differences in abundance between both treatments and controls (SIMPER). Similarly, on day 20, *Chrysochromulina* spp. and dinophyceans, now together with pennate diatoms, were the most important contributors to the differences in community structure (over 80%). However, the increase of *Chrysochromulina* spp. observed in fungicide exposed microcosms on day 7, had on day 20 been changed to a significantly lower abundance compared with the controls (Figure 2a). Dinophyte abundance was elevated in fungicide treated microcosms at both sampling occasions, compared with controls (Figure 2b), while pennate diatoms were most abundant in the control microcosms on day 20 (Figure 2c).

2.1.4. Phytoplankton primary production

The primary production was significantly higher on day 8 (ANOVA $F=8.3$, $p \leq 0.01$), with both low dose and high dose treatments differing from controls ($p \leq 0.01$). On day 11, there was marginal significance for the observed difference (ANOVA $F=3.6$, $p =$

0.056), with $p = 0.061$ and $p = 0.075$ for the low and high dose treatments, respectively, as compared with controls (Figure 3). There were no significant differences between treatments on any other sampling day.

2.1.5. Chlorophyll a concentration in the water

On day 7, the Chlorophyll *a* content in the water was higher in experimental treatments than in controls (ANOVA $F = 8.68$, $p \leq 0.01$; Dunnett's post hoc $p \leq 0.01$ in low treatment; $p \leq 0.05$ in high treatment). On day 11, there was a marginally significant (ANOVA $F = 3.58$, $p = 0.054$) effect of treatment and a significant difference between high treatment microcosms and controls (post hoc test, $p \leq 0.05$) (Figure 4). For all other sampling days, there were no significant differences between treatments (ANOVA $p > 0.05$).

2.1.6. Chlorophyll a in periphyton

There were no significant differences in periphytic Chlorophyll *a* between the treatments (ANOVA, $p > 0.05$), with 38.8 ± 6.8 , 29.9 ± 2.3 and 36.6 ± 3.0 ng Chl *a*/cm, in controls, low and high dose treatments, respectively.

2.1.7. Bacterioplankton activity

There was a slight trend of increased bacterial activity on day 1 and 2 in azoxystrobin exposed microcosms, although not statistically significant (ANOVA, $p > 0.05$; Figure 5). However, on day 4 the effect was the opposite and the bacterial ^{14}C -leucine incorporation was significantly lower in exposed microcosms (ANOVA $F = 6.38$, $p \leq 0.05$ in both cases; Figure 5). For all other sampling days, there were no significant differences between treatments (ANOVA $p > 0.05$).

2.1.8. Degradation of organic material

The degradation of organic material in the microcosms was not affected by treatment (ANOVA, $p > 0.05$). On average (all treatments), $72 \pm 1.2\%$ of the leaf material, and $67 \pm 1.1\%$ of the stem material, was degraded during the course of the experiment.

2.1.9. Fungicide residue analyses

The actually measured water concentrations were markedly lower than the nominal concentrations in both low and high dose treatments directly after application (approx. 80% and 60% of nominal concentration in LT and HT, respectively; Table 3). During the course of the experiment, the water concentrations decreased further. At the end of the study, less than one third of the initial measured concentrations remained in the low treatment, and less than half in the high dose treatment microcosms. This corresponds to a dissipation rate of $3.3\% / \text{d}$ ($2.8 - 3.6\%$, min; max) for the low dose, and $1.9\% / \text{d}$ ($1.5 - 2.3\%$, min; max) for the high dose treatments, resulting in DT50-values of 15.1 d ($13.8 - 17.6$ d, min; max) and 25.8 d ($21.9 - 32.5$ d, min; max), for low and high dose treatments, respectively. The fraction that ended up in the sediment varied markedly between the two treatments, with a considerably higher percentage in the high treatment (mean values 4.1% in low dose, 17.9% in high dose; Table 3). The recovery of azoxystrobin from the

whole microcosm system at day 21 was found to be approx. 26% in the low dose and approx. 44% in the high dose microcosms.

2.2. Experiment 2

The zooplankton community (Figure 6) displayed a similar response as in Experiment 1 (Figure 1a and 1b). The total copepod abundance was significantly reduced for all tested concentrations compared with controls (ANOVA followed by Dunnett's post hoc tests, $p \leq 0.05$ in all cases. The apparent increase of rotifers at higher concentrations was not significant ANOVA, $p > 0.05$). The ANOSIM test showed that the zooplankton community structure was significantly altered after 12 days of exposure (Global $R = 0.465$, $p \leq 0.001$), when zooplankton from all tested concentrations became different from controls ($R = 0.312$, $p \leq 0.05$; $R = 0.512$, $p \leq 0.05$; $R = 0.912$, $p \leq 0.01$ for the tested nominal concentrations 3, 7.5 and 15 $\mu\text{g/L}$, respectively). The SIMPER analyses showed that the decrease of nauplii and younger copepodites and the increase of the rotifers *Synchaeta* spp. and *Keratella* spp. accounted for more than 60% the differences in all three concentrations compared with controls. The composition of the zooplankton community is presented in Table 4. The salinity and pH in the reference microcosm day 0 were 5.5 psu and 8.09, respectively, and nutrient concentrations were as follows: $\text{NH}_4\text{-N}$: 170.8 $\mu\text{g/L}$, $(\text{NO}_2+\text{NO}_3)\text{-N}$: 30.2 $\mu\text{g/L}$, $\text{PO}_4\text{-P}$: 22.1 $\mu\text{g/L}$, $\text{SiO}_2\text{-Si}$: 1682 $\mu\text{g/L}$.

3. Discussion

The exposure to azoxystrobin altered the structure and function of model ecosystems at the concentrations tested. Calanoid copepods were particularly sensitive towards the substance. In Experiment 1, the zooplankton community exhibited significant alterations already 3 days after the fungicide application. This suggests that the fungicide exerted an acute and direct toxicity on the copepods. The youngest life stage of copepods, the nauplii, was the group that responded most to the fungicide exposure. The test-concentrations in Experiment 1 were selected based on existing toxicity data for other crustaceans (e.g. the low dose treatment almost three times lower than reported chronic NOEC on *Daphnia magna*; 44 $\mu\text{g/L}$ (European Commission Peer Review Programme, 1997). Thus, the observed effects on the copepods occurred at much lower concentrations than those expected from published toxicity data. In the follow-up experiment (Experiment 2), we investigated if even lower concentrations of azoxystrobin could affect natural copepod populations. The results showed that all tested concentrations, including the lowest (nominal concentration 3 $\mu\text{g/L}$), clearly altered the structure of the zooplankton community, primarily by reducing the abundance of copepod nauplii and increasing the abundance of the rotifers *Synchaeta* spp. Clearly, the standardised toxicity tests on microcrustaceans fail to properly assess the toxicity of azoxystrobin on calanoid copepods. This finding is supported by Lauridsen and coworkers (2003), who performed a series of acute and sub-chronic toxicity tests with azoxystrobin on several different freshwater zooplankton and macroinvertebrate species and found that the calanoid copepod *Eudiaptomus graciloides* was the most sensitive among the tested taxa. Although the EC_{50} (48h) was 38 $\mu\text{g/L}$ in the acute toxicity test, in the subsequent reproduction test, all individuals were killed after 48 h in 10 $\mu\text{g/L}$, and 5 $\mu\text{g/L}$ significantly reduced the number of nauplii (Lauridsen et al., 2003). In the tests with the

cyclopoid copepod *Cyclops vicinus*, all individuals died within 48 h when exposed to 20 µg/L or higher, and the NOEC for reproduction was 10 µg/L. Notably, the least sensitive of all crustacean zooplankters tested was the water flea *D. magna*, commonly used for toxicity testing (Lauridsen et al., 2003). Even so, all zooplankton species, including *D. magna*, were much more sensitive than macroinvertebrates, e.g. *Chaoborus flavicans*, which was not affected by azoxystrobin concentrations up to 6 mg/L. It is apparent that the sensitivity/tolerance spectrum for this chemical is very wide for different arthropod species (Lauridsen et al., 2003).

The variation within species can also be large. In a recently published study, azoxystrobin toxicity on *Daphnia magna* clones from different Danish lakes was compared (Warming et al., 2009). The most sensitive clone had a 48h LC₅₀ of 71 µg/L and the least sensitive 277 µg/L. However, physiological responses such as increased respiration rates were seen for all three *D. magna* clones at a remarkably low concentration, 0.026 µg/L (Warming et al., 2009).

In the present study, the copepod populations showed signs of recovery on day 20 in the low-dose treated microcosms, with a slight increase in nauplii abundance compared with controls (Figure 1a and Table 2). It is likely that the fungicide concentration at this point was low enough for eggs and nauplii to have a higher degree of survival than previously. Indeed, at the end of Experiment 1, the azoxystrobin concentration in water had decreased to 3.8 µg/L, i.e., approximately one third of the initial low-dose concentration. This concentration is in the same range as the lowest nominal concentration used in Experiment 2 (3 µg/L). Unfortunately, no fungicide residue analyses could be performed on samples from Exp. 2, but if we use the data from the measured water concentration at the end of Exp. 1 (low treatment, Table 3), and assume the dissipation rate and distribution between water and sediment compartments to be linear and comparable between the two studies, the water concentration in the lowest treatment of Exp. 2 can be estimated to be in the range of 1.7 – 2.0 µg/L at day 12. This low concentration reduced the average copepod abundance with more than 50%. It is worth noting that the Swedish water quality objective for azoxystrobin, 0.9 µg/L (Swedish Chemicals Agency, 2009), was based on a reported NOEC (28 d) of 9.5 µg/L for *Mysidopsis bahia* (Pesticide Ecotoxicity Database, 2001). Our data imply that this water quality objective is too high.

The phytoplankton community was also affected by the azoxystrobin exposure. In both high and low dose treatments the composition of the phytoplankton community was altered compared to controls at the different sampling occasions. On day 7, both *Chrysochromulina* spp. and dinoflagellates had increased in the fungicide treated microcosms (Figures 2a and b). In the high-dose treatments, *Chrysochromulina* spp. reached abundances of 8800 cells/ml, which is approx. 30 times above the maximum value recorded in depth-integrated water samples in the Askö area (Hajdu et al., 1996). The peak of *Chrysochromulina* spp. in fungicide exposed microcosms was also reflected in the increase of Chlorophyll *a* on day 7 (Figure 4) and the primary production values on the following day (Figure 3). On day 20, the abundance of dinoflagellates was still elevated in the fungicide treated microcosms (Figure 2b), while *Chrysochromulina* spp. abundance had decreased substantially and was now significantly lower in the exposed microcosms than in the controls (Figure 2a).

Dinoflagellate species are usually a good food source for calanoid copepods if they are of a suitable size (Koski et al., 1998). The dominating dinoflagellates in this study were large, approx. 12 μm in diameter, and they were undoubtedly grazed upon by copepods in the control microcosms. Although some species of *Chrysochromulina* and other haptophytes such as *Prymnesium* are potentially toxic (Moestrup, 1994), they are also suitable food source for copepods when given in mixtures with other phytoplankton species (Koski et al., 1999) or during natural blooms (Hansen et al., 1995). Thus, it is likely that the reduced grazing pressure after fungicide application favoured these flagellates, as well as the dinoflagellates and the observed increase in abundance of flagellates represents an indirect effect of the fungicide exposure. Moreover, as many *Chrysochromulina* spp. are mixotrophic, i.e. able to utilize particulate and dissolved organic matter as a carbon source in addition to photosynthesis, they might have had a competitive advantage at the beginning of the incubation. If the initial mortality of crustaceans temporally increased the concentration of particulate and dissolved organic matter in the fungicide treated microcosms, mixotrophic flagellates might have benefited from this, either directly by osmotrophic uptake of dissolved organic matter, or indirectly, via an increased availability of particulate food, e.g. detritus and bacteria. There was indeed a clear trend of elevated ^{14}C -leucine incorporation by bacteria in water samples from the fungicide exposed microcosms one day after application (Figure 5). However, after the initial peak, the trend was reversed and on day 4 the bacterial activity was significantly lower in the two fungicide treatments compared to controls. Furthermore, the reduction of *Chrysochromulina* spp. in fungicide treated microcosms on day 20 is likely to be an indirect response to the increased grazing pressure.

The rotifer abundance markedly increased in the fungicide treated microcosms towards the end of the study (Figure 1b), which likely resulted in a corresponding increase in grazing on *Chrysochromulina* spp. Top-down control and altered interspecific interactions, both within and between functional groups, may also explain the changes seen for the large pennate diatoms. Less suitable for copepod grazing owing to their large size (approx. 50 μm in length), these diatoms become abundant in the control microcosms on day 20 (Figure 2c). In the fungicide treated microcosms, the less abundant pennates were probably outcompeted by smaller phytoflagellates, which high surface to volume ratios provides an advantage in nutrient uptake (Sournia, 1982).

As the concentrations of dissolved nutrients were measured only at the end of the study, the nutrient availability during the course of the experiment is not known. At the end of the study, the nutrient concentrations were low in all microcosms, although with significantly higher ammonium and phosphate concentrations in the high-dose treated microcosms than in the controls. Moreover, it cannot be ruled out that the diatoms, or other phytoplankton species, were directly sensitive to the toxic substance, thus resulting in lower abundance and biomass in exposed microcosms. However, the main driving force behind the observed effects on the phytoplankton community was most likely an altered top-down control by reduced copepod grazing. Clearly, indirect effects changing the ecological interactions seem to be an important consequence of the fungicide exposure. Accordingly, as periphyton grazers, e.g. gastropods, were uncommon in the microcosms, the grazing pressure on periphytic algae had not been altered, and the

periphytic algal biomass was not affected by the fungicide treatment, neither directly nor indirectly.

The recommended application rates for azoxystrobin varies between 100-450 g active ingredient (a.i.)/ha for different agricultural practices (Elf et al., 1997, Tomlin 2000). Worst case environmental concentrations calculated as a spray boom passing over a 30 cm deep water surface (European Commission, 2001) would be in the interval of 33.3 – 150 µg azoxystrobin/L. If a spray drift of 2.77% is expected (Rautmann et al. 2001), the theoretical water concentration 1 m from the field would range between 0.9 and 4.2 µg/L. For early spraying on fruit crops, where a higher spray drift (29.2%) can be expected (Rautmann et al. 2001), concentrations of 10-44 µg/L might be expected within 3 m from the trees. Indeed, in a stream water sample from northern Germany, a concentration of 29.7 µg azoxystrobin/L has been reported (Berenzen et al, 2005). Even if such high concentrations might not be expected to occur in the coastal zones of the Baltic Sea, the exposure concentrations used in the present study are clearly within the range of what should be considered environmentally relevant.

How aquatic micro- and mesocosm experimental studies in ecotoxicology are best designed, constructed and interpreted have been a subject of discussion for many years (e.g. Cairns, 1986, Taub, 1997, Giddings et al., 2002, Daam and van den Brink, 2007). The model communities presented here are reasonably simple, yet with a relatively high ecological realism compared to single species tests, or aquatic microcosms without the sediment compartment. The microcosms represent a simplified brackish water community found in shallow semi-enclosed coastal areas in agricultural districts in the Baltic Sea region. Very few ecotoxicological studies have investigated effects of plant protection products on Baltic organisms in general and effects on community structure and function specifically, yet there is a risk that these communities may be exposed to agrochemical run-off during the growth season. The specific properties of the Baltic Sea, as previously described, make extrapolation from studies on freshwater or truly marine systems insufficient. The difficulty of drawing conclusions for Baltic conditions based on data from freshwater or marine organism single species tests is clearly shown by the present studies. No $NOEC_{community}$ could be calculated, as all tested concentrations were shown to have severe effects on the zooplankton community, although the choices of experimental conditions and test concentrations were made with the intention to include a $NOEC_{community}$. This study demonstrated toxic effects of the fungicide azoxystrobin on copepods at markedly (>10 x) lower nominal concentrations than previously reported from standard toxicity tests on other crustaceans, both for acute and chronic exposure. Also, the phytoplankton community composition, primary production and Chlorophyll *a* content were affected. Most of these effects most likely occurred as secondary indirect effects cascading from the direct toxicity exerted on the copepods and would not have been detected in single species experiments.

Acknowledgement

We would like to express sincere gratitude to the staff at Askö Marine Laboratory. Stephen Mdachi is acknowledged for his work with the pesticide analyses. The study was kindly supported by financial contributions from Stiftelsen Lars Hiertas Minne and Alice och Lars Siléns fond.

References

- Adielsson, S. and Kreuger, J. 2008. Bekämpningsmedel (växtskyddsmedel) i vatten och sediment från typområden och åar samt i nederbörd under 2007. [Pesticides (plant protection products) in water and sediment from agricultural type-areas and rivers and in precipitation during 2007.] *Ekohydrologi* 104. Report, Swedish University of Agricultural Sciences, ISSN 0347-9307. 52 pp. *In Swedish*.
- Adielsson, S., Törnquist, M. and Kreuger, J. 2007. Bekämpningsmedel (växtskyddsmedel) i vatten och sediment från typområden och åar samt nederbörd under 2006. [Pesticides (plant protection products) in water and sediment from agricultural type-areas and rivers and in precipitation during 2006.] *Ekohydrologi* 99. Report, Swedish University of Agricultural Sciences, ISSN 0347-9307. 30 pp. *In Swedish*.
- Bartlett, D.W., Clough, J.M., Goodwin, J.R., Hall, A.A., Hamer, M., Parr-Dobrzanski, B., 2002. The strobilurin fungicides. *Pest Manag. Sci.* 58, 647-662
- Berenzen, N., Lentzen-Godding, A., Probst, M., Schulz, H., Schulz, R. and Leiss, M. A comparison of predicted and measured levels of runoff-related pesticide concentrations in small lowland streams on a landscape level. *Chemosphere* 58, 683-691.
- Bester, K., Hühnerfuss, H., 1993. Triazines in the Baltic and North-Sea. *Mar. Pollut. Bull.* 26, 423-427.
- Cairns, J. J. 1986. The myth of the most sensitive species: Multispecies testing can provide valuable evidence for protecting the environment. *BioScience* 36(10): 670-672.
- Caquet, T., Lagadic, L., Monod, G., Lacaze, J-C., Couté, A., 2001. Variability of physiochemical and biological parameters between replicated outdoor freshwater lentic mesocosms. *Ecotoxicology* 10, 51-66.
- Castillo, L.E., de la Cruz, E., Ruedert, C., 1997. Ecotoxicology and pesticides in tropical aquatic ecosystems of Central America. *Environ. Toxicol. Chem.* 16, 41-51.
- Daam, M. A. and P. J. Van den Brink 2007. Effects of chlorpyrifos, carbendazim, and linuron on the ecology of a small indoor aquatic microcosm. *Archives of Environmental Contamination and Toxicology* 53(1): 22-35.
- Elf, A., Sandberg, T., Bager, Y., 1997. Azoxystrobin. Fact sheet, Swedish Chemicals Inspectorate, Stockholm, Sweden.
- European Commission Peer Review Programme. 1997. Azoxystrobin Monograph. Rapporteur Member State: Germany.
- European Commission, 2001. Guidance document on aquatic ecotoxicology in the frame of the directive 91/414/EEC. Sanco/3268/2001, Health and consumer protection directorate-general.
- Giddings, J., Brock, T., Heger, W., Heimbach, F., Maund, S., Norman, S., Ratte, H.T., Schäfers, C., and Streloke, M. 2002. Community-Level Aquatic System Studies- Interpretation Criteria (CLASSIC). Pensacola, FL, USA: Society of Environmental Toxicology and Chemistry.

- Gorokhova E, Aladin N, Dumont H. 2000. Further expansion of the genus *Cercopagis* (Crustacea, Branchiopoda, Onychopoda) in the Baltic Sea, with notes on the taxa present and their ecology. *Hydrobiologia* 429: 207-218.
- Gorokhova E, Fagerberg T, Hansson S (2004) Predation by herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) on *Cercopagis pengoi* in a western Baltic Sea bay. *ICES Journal of Marine Science* 61: 959-965.
- Hajdu, S., Larsson, U., Moestrup, O., 1996. Seasonal dynamics of *Chrysochromulina* species (Prymnesiophyceae) in a coastal area and a nutrient-enriched inlet of the northern Baltic proper. *Botanica Marina* 39, 281-295.
- Hansen, P.J., Nielsen, T.G., Kaas, H., 1995. Distribution and growth of protists and mesozooplankton during a bloom of *Chrysochromulina* spp (Prymnesiophyceae, Prymnesiales). *Phycologia* 34, 409-416.
- Hapeman, C.J., Dionigi, C.P., Zimba, P.V., McConnell L.L., 2002. Agrochemical and nutrient impacts on estuaries and other aquatic systems. *J. Agric. Food Chem.* 50, 4382-4384.
- HELCOM, 1988. Guidelines for the Baltic Monitoring Program for the third stage; Part D. Biological determinants, Baltic Marine Environment Protection Commission, Helsinki Commission.
- Jansson, B-O., 2003. The Baltic Sea. In: Hempel, G., Sherman, K. (Eds.) *Large Marine Ecosystems of the World*, Elsevier, Amsterdam, pp. 145-170.
- Kannan, N., Tanabe, S., Tatsukawa, R., Phillips, D., 1989. Persistency of highly toxic coplanar PCBs in aquatic ecosystems: Uptake and release kinetics of coplanar PCBs in Green-lipped mussels (*Perna viridis*, Linnaeus). *Environ. Pollut.* 56, 65-76.
- Koski, M., Rosenberg, M., Viitasalo, M., Tanskanen, S., Sjölund, M., 1999. Is *Prymnesium patelliferum* toxic for copepods? - Grazing, egg production, and egestion of the calanoid copepod *Eurytemora affinis* in mixtures of 'good' and 'bad' food. *ICES J. Mar. Sci.* 56 (Suppl.), 131-139.
- Koski, M., Breteler, W.K., Schogt, N., 1998. Effect of food quality on rate of growth and development of the pelagic copepod *Pseudocalanus elongatus* (Copepoda, Calanoida). *Mar. Ecol. Prog. Ser.* 170, 169-187.
- Lauridsen, T.L., Friberg-Jensen, U., Cristoffersen, K., 2003. Effekter af cypermethrin, azoxystrobin og bentazon på limniske invertebrater. Rep. 76, Miljøstyrelsen, Miljøministeriet. In Danish.
- Moestrup, Ø., 1994. Economic aspects: 'blooms', nuisance species, and toxins. In: Green, J.C. (Ed.) *The Haptophyte Algae*. BSC Leadbeater, Oxford, pp. 265-285.
- OECD, 2008. OECD (Organization for Economic Cooperation and Development), 2008. OECD guidelines for testing of chemicals, Section 2, Guideline 211, *Daphnia magna* Reproduction Test. OECD Environment Directorate, Paris
- Parsons, T.R., Maita, Y., Lalli, C.M., 1984. A manual of chemical and biological methods for seawater. Pergamon, Oxford.
- Pempkowiak, J., Tronczynski, J., Pazdro, K., 2000. Spatial and temporal gradients of triazines in the Baltic Sea of Poland. *Mar. Pollut. Bull.* 40, 1082-1089.
- Pesticide Ecotoxicity Database, 2001. Environmental Fate and Effects Division of Office of Pesticide programs, U.S.EPA. Washington, D.C.

- Quinn, G.P., Keough, M.J., 2002. *Experimental Design and Data Analysis for Biologists*, Cambridge University press, Cambridge, UK.
- Ranger, C., 1993. Determination of low level nutrients in environmental water samples using flow injection analysis. *Internat. Environ. Technol.* 3(4), 21-23.
- Rautmann, D., Streloke, M., Winkler, R., 2001. New basic drift values in the authorisation procedures for plant protection products. In: Forster R & M Streloke eds, *Workshop on risk assessment and risk mitigation measures in the context of the authorization of plant protection products (WORMM)*, 27-29 September 1999. *Mitt. Biol. Bundesanst. Land-Forstwirtschaft*, 133-141. Berlin-Dahlem, Heft 381.
- Sanderson, H., Boudreau, T.M., Scott, A., Mabury, S.A., Woo-Jay, C., Solomon, K.R., 2002. Ecological impact and environmental fate of perfluorooctane sulfonate on the zooplankton community in indoor microcosms. *Environ. Toxicol. Chem.* 21, 1490-1496.
- Slijkerman, D.M.E., Baird, D.J., Conrad, A., Jak, R.G., van Straalen, N.M., 2004. Assessing structural and functional plankton responses to carbendazim toxicity. *Environ. Toxicol. Chem.* 23, 455-462.
- Sournia, A., 1982. Morphological bases of competition and succession. *Can. J. Fish. Aquat. Sci.* 210, 339-346.
- Swedish Chemicals Agency, 2003. Sold quantities of pesticides 2002. Sveriges Officiella Statistik. Kemikalieinspektionen, Solna. In Swedish.
- Swedish Chemicals Agency, 2008. Sold quantities of pesticides 2007. Sveriges Officiella Statistik. Kemikalieinspektionen, Solna. In Swedish.
- Swedish Chemicals Agency, 2009 Proposed water quality objective. Azoxystrobin. Kemikalieinspektionen, Solna. (2009-02-27)
<http://www.kemi.se/templates/Page.aspx?id=3294>.
- Swedish Standard, 1980. Determination of chlorophyll in water - Extraction with acetone - Spectrophotometric method. SS028146, SIS.
- Taub, F. B. 1997. Unique information contributed by multispecies systems: Examples from the standardized aquatic microcosm. *Ecological Applications* 7(4): 1103-1110.
- Tomlin, C.D.S., 2000. *The pesticide manual*. British Crop Protection Council, UK.
- Ulén, B., Kreuger, J., Sundin, P., 2002. Undersökning av bekämpningsmedel i vatten från jordbruk och samhälle år 2001. Rapport 2002:4. *Ekohydrologi* 63. In Swedish.
- van den Brink, P.J., Hattink, J., Bransen, F., Van Donk, E., Brock, T.C.M., 2000. Impact of the fungicide carbendazim in freshwater microcosms. II. Zooplankton, primary producers and final conclusions. *Aquat. Toxicol.* 48, 251-264.

Table 1. Abiotic conditions in the microcosms in Experiment 1 (mean over the experimental period \pm SE, except for dissolved nutrients which are mean \pm SE at day 21). Control treatment (CT); 0 μ g/L Low treatment (LT); 15 μ g/L, High treatment (HT); 60 μ g/L added (all in nominal initial concentrations).

	<i>CT</i>	<i>LT</i>	<i>HT</i>
Temperature ($^{\circ}$ C)	20.6 \pm 0.2 (n=114)	20.7 \pm 0.2 (n=114)	20.7 \pm 0.2 (n=114)
Salinity (psu)	5.8 \pm 0.03 (n=84)	5.7 \pm 0.03 (n=84)	5.8 \pm 0.03 (n=84)
pH	8.81 \pm 0.03 (n=114)	8.81 \pm 0.03 (n=114)	8.80 \pm 0.03 (n=114)
Diss. oxygen (mg/L)	11.0 \pm 0.2 (n=114)	10.7 \pm 0.2 (n=114)	10.5 \pm 0.2 (n=114)
NH ₄ -N (μ g/L)	3.5 \pm 0.3 (n=6)	4.4 \pm 0.7 (n=6)	5.2 \pm 0.8 (n=6)
(NO ₂ +NO ₃)-N (μ g/L)	0.6 \pm 0.04 (n=6)	0.7 \pm 0.04 (n=6)	0.7 \pm 0.08 (n=6)
PO ₄ -P (μ g/L)	0.7 \pm 0.08 (n=6)	1.0 \pm 0.2 (n=6)	1.6 \pm 0.4 (n=6)

Table 2. Abundance of zooplankton (individuals per litre \pm SE, n=6) in microcosms after 3 and 20 days of azoxystrobin exposure to three nominal initial concentrations (0, 15 and 60 μ g/L) (Experiment 1).

Treatment	0		15		60	
Sample occasion	T3	T20	T3	T20	T3	T20
Taxa						
Copepoda						
Nauplii	216 \pm 24	397 \pm 25	92 \pm 12	260 \pm 169	16 \pm 2.9	4.7 \pm 3.4
Copepodites I-III	9.8 \pm 1.8	31 \pm 13	2.9 \pm 0.8	13 \pm 4.6	1.8 \pm 0.3	0.1 \pm 0.1
Copepodites IV-VI ^a	9.5 \pm 1.6	37 \pm 7	6.7 \pm 1.7	5.5 \pm 3.1	1.9 \pm 0.2	0.1 \pm 0.1
Cyclopid copepod	0.3 \pm 0.1	0	0.4 \pm 0.2	0	0.3 \pm 0.2	0
Tot Copepoda	236\pm27	465\pm28	101\pm14	279\pm175	19\pm3.1	4.8\pm3.5
Cladocera						
<i>Chydorus sphaericus</i>	0.5 \pm 0.2	1.1 \pm 0.8	0.4 \pm 0.2	3.2 \pm 1.0	0.6 \pm 0.2	1.6 \pm 0.5
<i>Ceriodaphnia</i> spp.	0.8 \pm 0.4	36 \pm 12	1.1 \pm 0.4	54 \pm 18	0.7 \pm 0.3	28 \pm 12
Tot Cladocera	1.3\pm0.4	37\pm13	1.5\pm0.5	57\pm18	1.3\pm0.3	30\pm13
Cirripedia						
<i>Balanus</i> larvae	0.2 \pm 0.2	0	0.8 \pm 0.5	0	0.3 \pm 0.1	0
Rotifera						
<i>Euchlanis dilatata</i>	0.1 \pm 0.1	1.7 \pm 1.4	0.1 \pm 0.1	12 \pm 5	0	87 \pm 41
<i>Cephalodella</i> sp1.	0.3 \pm 0.2	1.1 \pm 0.8	0.3 \pm 0.3	6.7 \pm 2.1	0.2 \pm 0.1	53 \pm 36
<i>Cephalodella</i> sp2.	0.1 \pm 0.1	1.1 \pm 0.4	0	4.9 \pm 1.4	0.3 \pm 0.2	17 \pm 7.3
<i>Keratella quadrata</i>	3.4 \pm 1.0	0	3.4 \pm 0.9	0.3 \pm 0.3	1.6 \pm 0.3	0.2 \pm 0.1
<i>Keratella</i> spp.	0	0	0	0	0	0.6 \pm 0.1
<i>Lecane</i> spp.	0	0	0	0.4 \pm 0.2	0.1 \pm 0.1	0.6 \pm 0.4
<i>Colurella oblonga</i>	0	0.3 \pm 0.3	0	1.7 \pm 0.7	0	10 \pm 3.2
<i>Notommata</i> spp. ₂	0.1 \pm 0.1	0.6 \pm 0.4	0	16 \pm 4	0	7.3 \pm 4
<i>Trichotria</i> spp.	0	0.3 \pm 0.3	0.1 \pm 0.1	0.2 \pm 0.2	0	0.5 \pm 0.5
<i>Notholca</i> spp.	0	0	0	0.1 \pm 0.1	0	0.7 \pm 0.5
Unidentified rotifers	0.3 \pm 0.2	0	0.1 \pm 0.1	30 \pm 8.5	0.1 \pm 0.1	27 \pm 7.8
Tot Rotifera	4.1\pm1.0	5\pm2.4	3.9\pm1.0	72\pm8.3	2.3\pm0.4	203\pm84
Total zooplankton	241\pm26	507\pm21	107\pm15	408\pm189	23\pm3	238\pm95

^a Including adults of *Acartia bifilosa* and *Eurythemora affinis*

Table 3. Concentration of the fungicide azoxystrobin in experimental microcosms containing natural sediment and water from the Baltic Sea. Water samples (mean μg azoxystrobin/l \pm SE) were taken directly after application (T0, Start) and after 21 days (End). Sediment samples (mean ng azoxystrobin/g sediment \pm SE) were taken at the end of the experiment. Low treatment (LT): nominal initial concentration 15 $\mu\text{g/L}$; High treatment (HT): nominal initial concentration 60 $\mu\text{g/L}$. n=5 for all water samples, n=6 for sediment samples.

Treatment	Sampling occasion	LT		HT	
		Concentration	% of nom. Addition	Concentration	% of nom. addition
Water	Start (T0)	12.5 \pm 2.6	83.2 \pm 17.1	37.3 \pm 2.9	62.2 \pm 4.8
	End (T21)	3.8 \pm 0.2	22.1 \pm 1.2	18.1 \pm 0.8	26.4 \pm 1.2
Sediment	End (T21)	39.7 \pm 2.3	4.1 \pm 0.2	171.8 \pm 21.3	17.9 \pm 2.2

Table 4. Abundance of zooplankton (individuals per litre \pm SE, n = 5) in microcosms after 12 days of azoxystrobin exposure in four nominal initial concentrations: 0, 3, 7.5 and 15 $\mu\text{g/L}$ (Experiment 2).

Treatment	0	3	7.5	15
Taxa				
Copepoda				
Nauplii	450 \pm 67.5	197 \pm 47.2	201 \pm 42.7	3.3 \pm 1.0
Copepodites I-III	25.3 \pm 6.9	14.4 \pm 5.2	2.3 \pm 1.7	1.1 \pm 0.2
Copepodites IV-VI ^a	21.7 \pm 4.2	10.9 \pm 2.1	2.4 \pm 0.4	2.1 \pm 0.3
Tot Copepoda	497\pm71	222\pm53	206\pm44	6.5\pm1.1
Cladocera				
<i>Chydorus sphaericus</i>	0.5 \pm 0.1	0.8 \pm 0.5	0.7 \pm 0.3	1.7 \pm 0.4
<i>Ceriodaphnia</i> spp.	0	0	0	3.2 \pm 2.7
Tot Cladocera	0.5\pm0.1	0.8\pm0.5	0.7\pm0.3	4.9\pm3
Rotifera				
	0.1 \pm 0.1	0	0.1 \pm 0.1	0.3 \pm 0.2
<i>Euchlanis dilatata</i>	0.2 \pm 0.2	0.5 \pm 0.2	0.1 \pm 0.1	0.1 \pm 0.1
<i>Cephalodella</i> _sp1.	0.1 \pm 0.1	0	0.1 \pm 0.1	0.1 \pm 0.1
<i>Cephalodella</i> _sp2.	14.1 \pm 3.9	38.4 \pm 14.1	42.9 \pm 12	48.4 \pm 18
<i>Keratella quadrata</i>	4.5 \pm 1.8	10.3 \pm 4	11.5 \pm 3.3	10.7 \pm 3.2
<i>K. cochlearis/cruciformis</i>	29.1 \pm 16.8	96.4 \pm 32	217 \pm 116	319 \pm 210
<i>Synchaeta</i> spp.	0.1 \pm 0.1	0.1 \pm 0.1	0.1 \pm 0.1	0.1 \pm 0.1
<i>Lecane</i> spp.	0	0.1 \pm 0.1	0	0.1 \pm 0.1
<i>Colurella dicentra</i>	0	0.1 \pm 0.1	0.1 \pm 0.1	0.6 \pm 0.2
<i>Notholca</i> spp.	3.9 \pm 2.3	0	2.4 \pm 0.9	4.3 \pm 1.8
Unidentified rotifers				
Tot Rotifera	52\pm21	146\pm38	275\pm119	383\pm227
Gastropoda				
<i>Hydrobia</i> spp. (larvae)	6.1 \pm 3.0	6.5 \pm 3.4	14.9 \pm 10.9	3.3 \pm 1.6
Total zooplankton	556\pm73	375\pm50	496\pm85	398\pm228

^a Including adults of *Acartia bifilosa* and *Eurythemora affinis*

Figure captions

Figure 1. Experiment 1. (a) Copepoda and (b) Rotatoria abundance (individuals per litre, mean \pm SE) in the microcosms on day (D) 3, 7, 16 and 20. CT = 0 μ g/L, LT = 15 μ g/L, HT = 60 μ g/L (nominal azoxystrobin application), n = 6. Asterisks denote significant differences from control at each sampling occasion (Dunnett's post hoc test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), n.a. = not analysed due to variance heterogeneity

Figure 2. Effects of azoxystrobin exposure on biomass (mean \pm SE) of three phytoplankton taxa, (a) Chrysochromulina spp. (b) dinoflagellates and (c) diatoms (Pennales), after 7 and 20 days, in microcosms (CT, LT, HT; for explanations see Fig. 1). Abundance data were converted to biomass (pg/L) from estimated biovolume assuming a specific gravity of 1.

Figure 3. Phytoplankton primary production (μ g C/L*h) (mean \pm SE) in microcosms at day (D), 1, 2, 4, 8, 11, 14 and 17 (n = 6) and day 0 (start, n = 18). #: marginal significant differences ANOVA ($p = 0.056$), Dunnett's post hoc test $p = 0.061$ and $p = 0.075$, for low and high treatments, respectively, compared to control. n.a. = not analysed due to variance heterogeneity. For treatment descriptions, see Fig. 1.

Figure 4. Chlorophyll a in the water (μ g/L) (mean \pm SE) in microcosms at day (D) 3, 7, 11, 15 and 20 (n = 6). For description of treatments and asterisks, see Fig. 1.

Figure 5. Bacterial ¹⁴C-leucin incorporation (dmp $\times 10^3$ per incubation minute, mean \pm SE) in experimental microcosms on day (D) 0, 2, 4, 8 and 14. On day 0, n = 3 (randomly selected), at remaining sample occasions n = 6. For description of treatments and asterisks, see Fig. 1.

Figure 6. Experiment 2. The effect on zooplankton community structure after 12 days of azoxystrobin exposure at the nominal concentrations 0, 3, 7.5 and 15 μ g azoxystrobin/L (n = 5). a) Abundance of Copepoda and Rotifera (individuals per litre, mean \pm SE). Asterisks denote significant differences from controls at each sampling occasion (Dunnett's post hoc test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

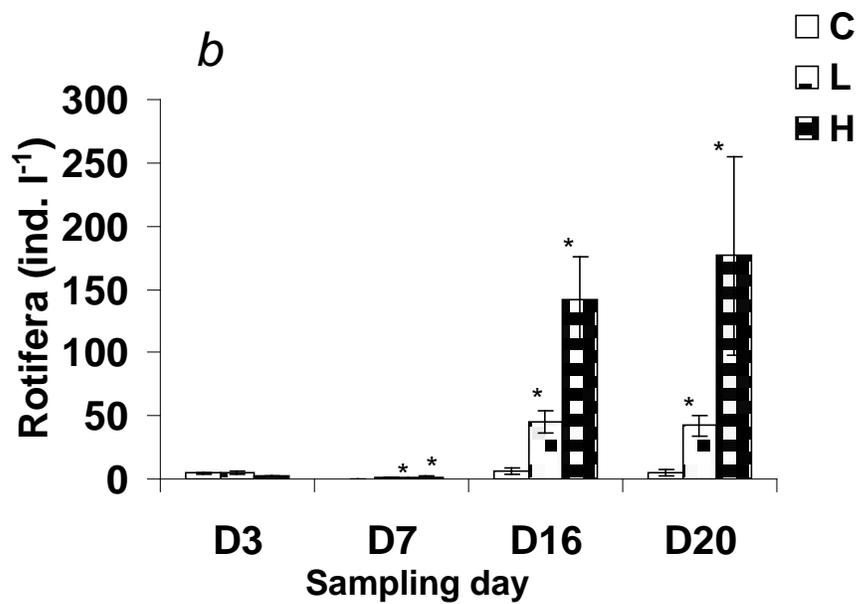
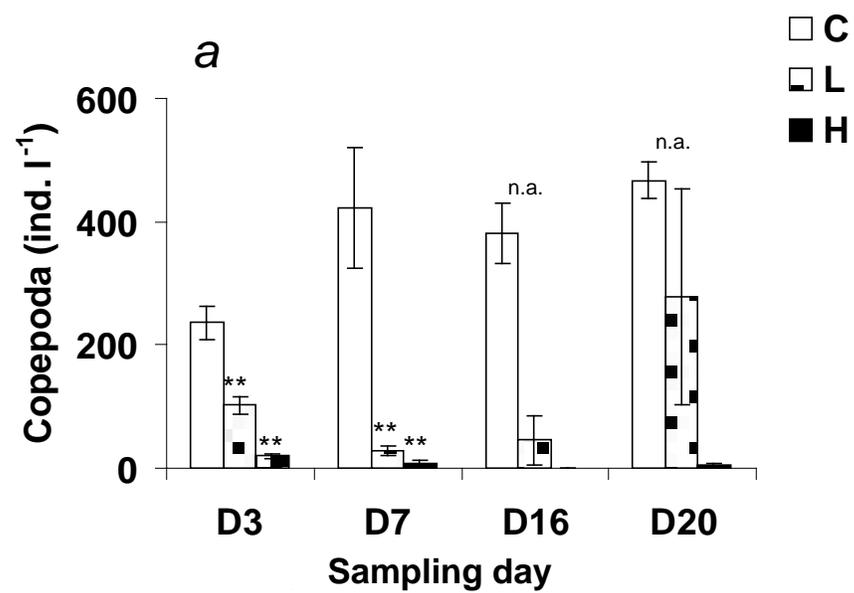


Figure 1a and b.

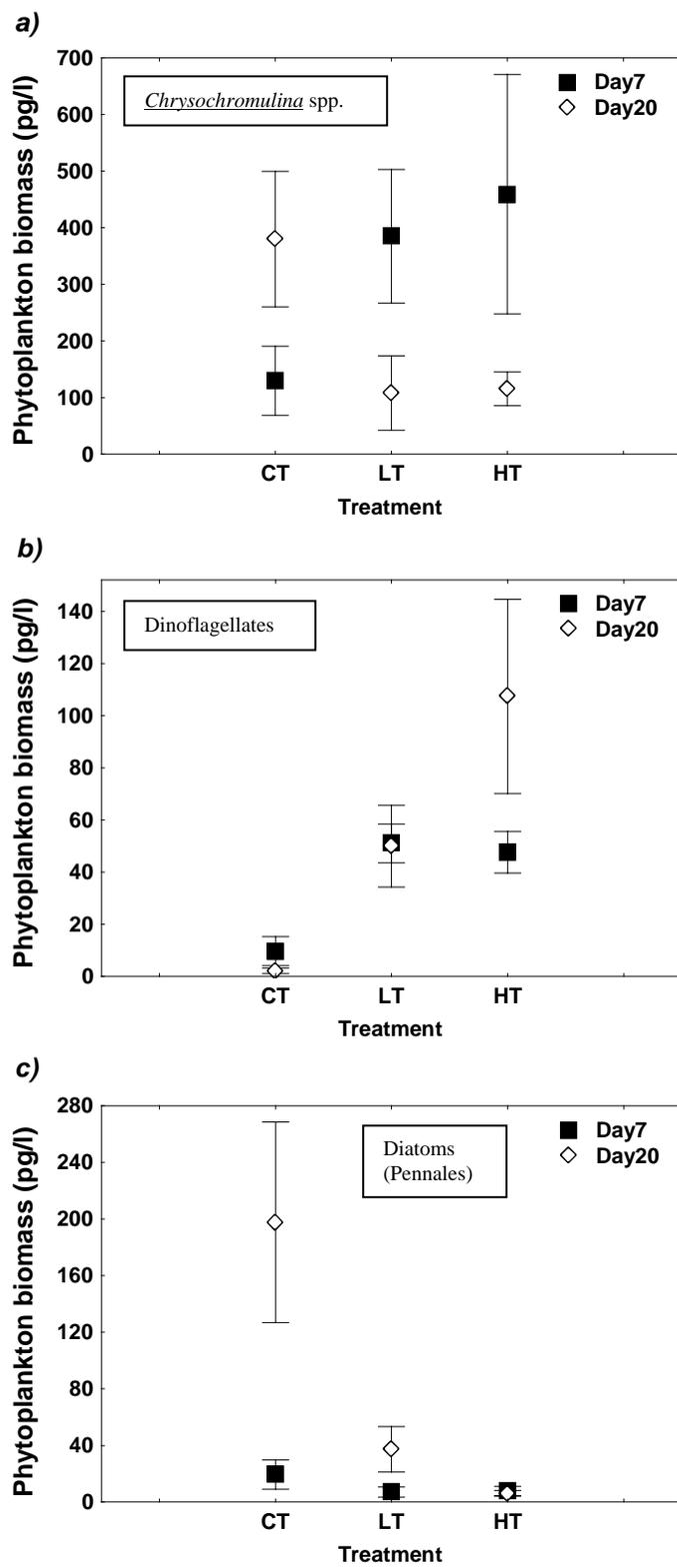


Figure 2a, b, c.

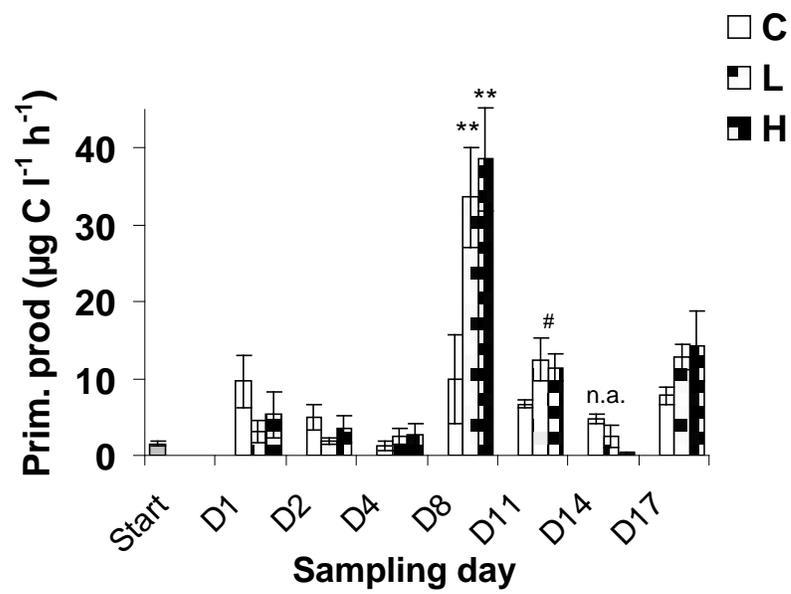


Figure 3.

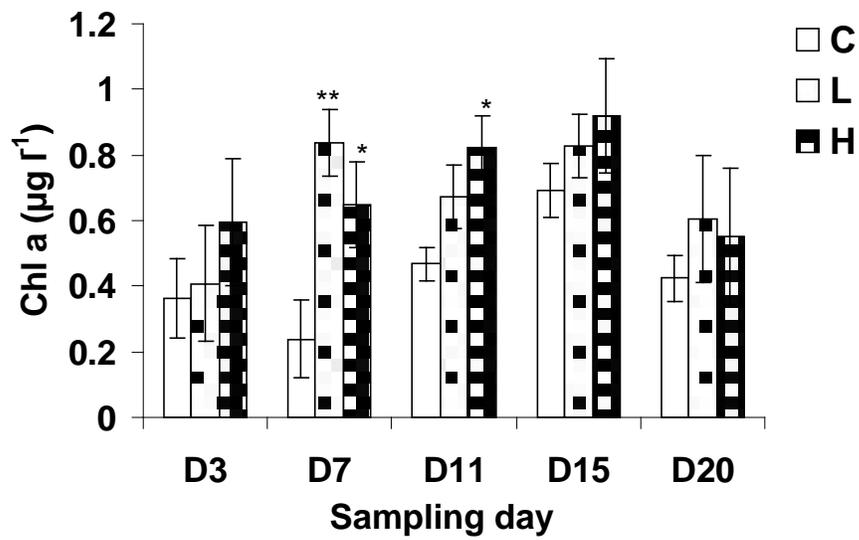


Figure 4.

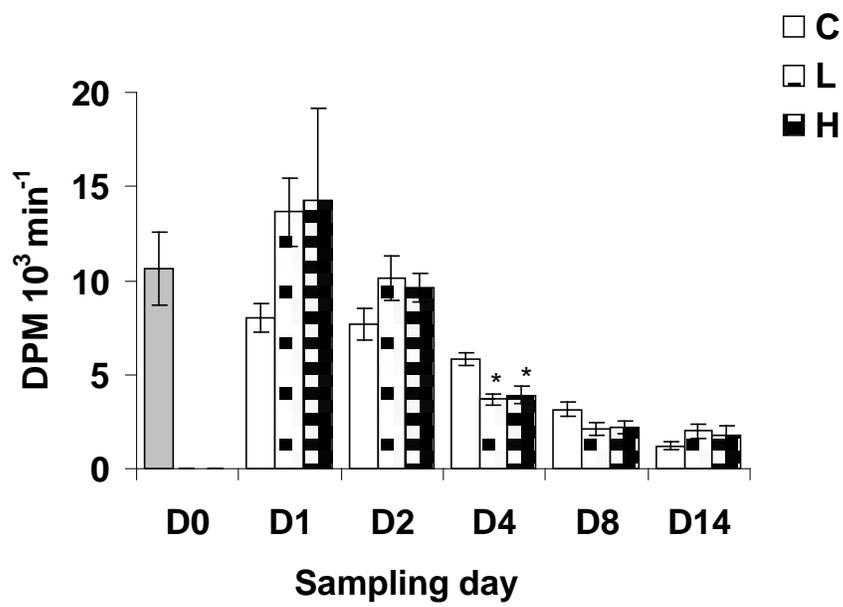


Figure 5.

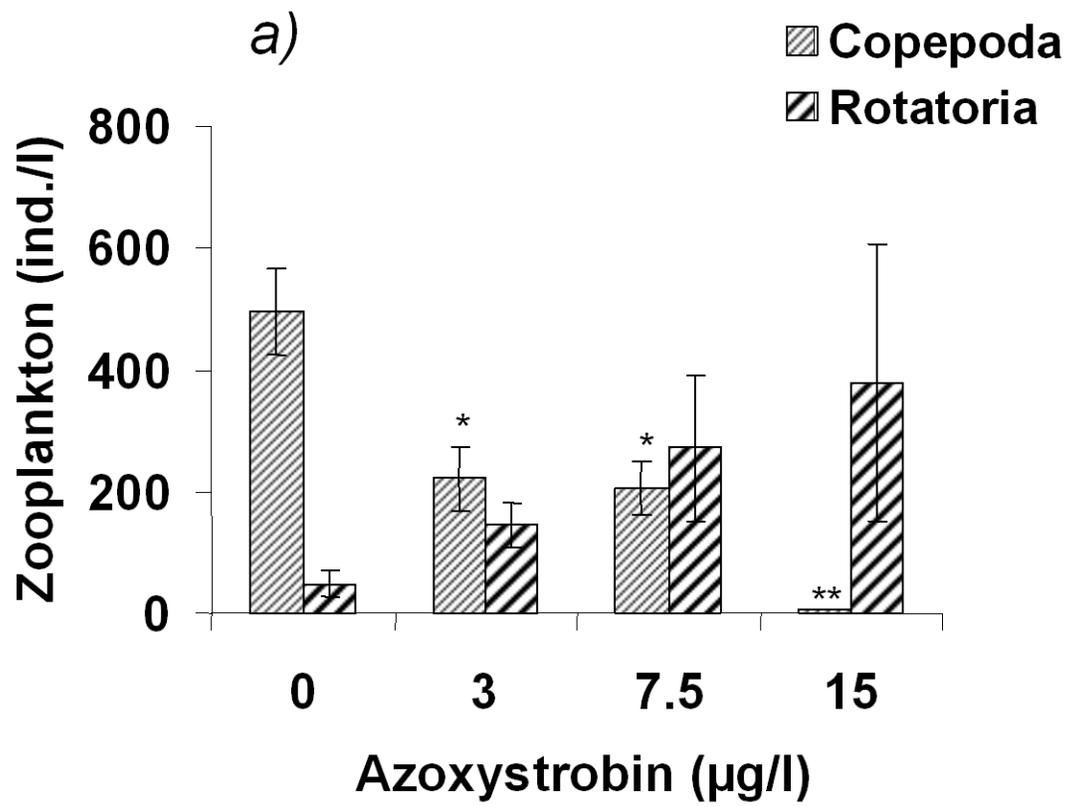


Figure 6.