Experimental Assessment of the Environmental Fate and Effects of Triazoles and Benzotriazole

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Summary — The environmental fate and effects of triazoles and benzotriazoles are of concern within the context of chemical regulation. As part of an intelligent testing strategy, experimental tests were performed on endpoints that are relevant for risk assessment. The experimental tests included the assessment of ecotoxicity to an alga, a daphnid and zebrafish embryos, and the assessment of ready biodegradability. Triazole and benzotriazole compounds were selected for testing, based on existing toxicity data for vertebrate and invertebrate species, as well as on the principal component analysis of molecular descriptors aimed at selecting the minimum number of test compounds in order to maximise the chemical domain spanned for both compound classes. The experimental results show that variation in the toxicities of triazoles and benzotriazole across species was relatively minor; in general, the largest factor was approximately 20. The study conducted indicated that triazoles are not readily biodegradable.

Key words: *algae, aquatic toxicity, benzotriazoles, daphnids, ready biodegradability, triazoles, zebrafish embryos.*

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Introduction

Benzotriazole and its derivatives are commonly used in many applications, including, for example, the inhibition of copper and other 'yellow metal' corrosion, but they are also present in numerous domestic and industrial products, such as de-icing fluids, automotive coolants, anti-freezes, cutting fluids, hydraulic brake fluids, and coating materials (1). In addition, they are used as brightening agents in the metal plating industry (1). Benzotriazoles may persist in the environment for a very long time, due to their resistance to oxidation under environmental conditions, the UV stability of the compounds (2), and their resistance to biodegradation (3). Benzotriazoles are also known to be fairly soluble in water and have a limited sorption tendency. If released into the environment, these substances have the potential to cause damage to aquatic ecosystems (4–6), as they are known to be toxic to the fathead minnow, *Pimephales promelas,* and the crustacean, *Daphnia magna* (4). They are also known to induce toxic effects in plants (2).

Triazoles were introduced in the 1980s, and are used as fungicides for plant protection. They are important tools against diseases of turf grasses, vegetables, citrus fruits, field crops and ornamental plants (7). In addition, they are some of the most widely-used active substances in the biocides available on the global market. Triazoles are also known to be fairly soluble in water, not to be readily degradable, and to have a limited sorption tendency. Triazoles are toxic to fish and other aquatic organisms, but are practically non-toxic to birds and bees (7).

The success of chemical regulation benefits from the availability of appropriate experimental tests with environmental endpoints relevant for risk assessment. However, given the scarcely available resources for testing and risk assessment, it is crucial that the chemicals and endpoints to be tested are carefully selected. An intelligent testing strategy uses all the available chemical information to guide further experimental testing, whereas quantitative structure–activity relationships (QSARs) use chemical analogies to quantify missing experimental data on endpoints of concern. The purpose of this study was to generate reliable data on the environmental fate and effects of triazoles and benzotriazole ([B]TAZs), on the endpoints relevant for risk assessment, in order to generate and/or validate QSAR models. Therefore, a selected set of tria-

zoles and benzotriazole were tested, according to Organisation for Economic Co-operation and Development (OECD) Test Guidelines (TGs) for biodegradability and aquatic toxicity to a freshwater alga, a daphnid and to fish embryos. To this end, an experimental design that was mainly based on structural similarity, was used to prioritise (B)TAZs for experimental testing.

Materials and Methods

Toxicity testing of substituted triazoles and benzotriazole was performed with the green alga, *Pseudokirchneriella subcapitata*, the daphnid, *D. magna*, and embryos of the zebrafish, *Danio rerio*. In addition, ready biodegradability testing was also carried out.

The chemicals to be tested, for which there were little or no experimental data on the toxicological endpoints measured, were selected based on the criteria that they were evenly distributed with respect to the first two principal components derived by principal component analysis (PCA) on DRAGON descriptors (8). The final outcome of the experimental design consisted of a list of priority (B)TAZs representative of the entire structural space. This selection also included chemicals with a structure similar to (B)TAZs, and that were recognised as active in the available literature data on vertebrate and invertebrate toxicity (9). Thereupon, a further selection was applied based on the commercial availability of the chemicals to be tested.

The triazoles selected for testing on the alga and ready biodegradability are shown in Table 1.

The toxicity of triazoles to the freshwater alga, *P. subcapitata*

A 72-hour toxicity test was performed with selected triazoles on *P. subcapitata* (formerly known as *Selenastrum capricornutum*). The purpose of this test was to determine the effects of a substance on the growth of freshwater microalgae, and it was performed according to OECD TG 201: Freshwater Algae and Cyanobacteria, Growth Inhibition Test (10).

Preparation of the test solutions

The test chemicals were purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). The medium for the test was prepared according to OECD TG 201. The reference substances 3,5-dichlorophenol and potassium dichromate were tested as a means of verifying the test procedure.

Chemical measurements

The concentrations of triazoles in the test cultures were determined at the beginning and at the end of the test, by gas chromatography with mass spectrometry (GC/MS). GC/MS was performed with a HP 6890 GC coupled to a HP 5973 mass spectro-

Table 1: The triazoles and benzotriazole selected for testing

a) On the alga and ready biodegradability	
	CAS number
Cyproconazole	94361-06-5
Diclobutrazol	75736-33-3
Difenoconazole	119446-68-3
Diniconazole	083657-24-3
Epoxiconazole	106325-08-0
Hexaconazole	79983-71-4
Myclobutanil	88671-89-0
Paclobutrazol	76738-62-0
Penconazole	066246-88-6
Propiconazole	060207-90-1
Triadimefon	43121-43-3
Triazophos	024017-47-8
Uniconazole-P	083657-17-4
b) On *Daphnia*	
	CAS number
Benzotriazole	95-14-7
Cyproconazole	94361-06-5
Diclobutrazol	75736-33-3
Fenchlorazol-ethyl	103112-35-2
Flusilazole	85509-19-9
Guanazole	1455-77-2
Hexaconazole	79983-71-4
Myclobutanil	88671-89-0
Paclobutrazol	76738-62-0
Ribavirin	36791-04-5
Triadimefon	43121-43-3
Triticonazole	131983-72-7
c) On zebrafish embryos	
	CAS number
Benzotriazole	95-14-7
Cyproconazole	94361-06-5
Fenchlorazol-ethyl	103112-35-2
Flusilazole	85509-19-9
Guanazole	1455-77-2
Hexaconazole	79983-71-4
Myclobutanil	88671-89-0
Paclobutrazol	76738-62-0
Ribavirin	36791-04-5
Triadimefon	43121-43-3
Triticonazole	131983-72-7

meter (Hewlett-Packard, Palo Alto, CA, USA). The GC separation was performed on a DB-5MS column, 40m × 0.18mm internal diameter (ID), with a film thickness of 0.18µm (Agilent Technologies, Wilmington, DE, USA). To prepare the samples, test water samples (2ml) were transferred to glass tubes and 3ml of hexane were added. Ultrasound-assisted liquid–liquid extraction was performed (15 minutes). Phase separation was achieved by centrifugation at 3005*g* for 5 minutes at room temperature. The hexane phase was transferred into a vial and diluted with ethyl acetate. The extracts were analysed with GC/MS in selected ion monitoring (SIM) mode. Quantification of the selected triazoles was performed according to external standard calibration.

Algae

The algae were obtained from a culture collection of SAMS Research Services Ltd (Argyll, Scotland, UK). The initial cell concentration in the test culture was approximately 10^4 cells/ml, as determined by manual cell counting under the microscope, according to OECD TG 201. The concentration range at which effects are likely to occur was determined on the basis of results from range-finding experiments. For the test, at least five concentrations, arranged in a geometric series, were selected. It was ensured that the lowest concentration selected did not have any observed effect on the growth of the algae.

The test

Algae growing exponentially were exposed to the test substance in batch cultures over a period of 72 hours. The test endpoint was inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth-rate) during the exposure period. From the average specific growth-rates recorded in a series of test solutions, the concentration inducing 50% inhibition of the growth rate was determined and expressed as the E_rC50. In addition, the No Observed Effect Concentration (NOEC) was statistically determined. Test cultures containing the desired concentration of test substance and the desired quantity of algal inoculum were prepared by diluting aliquots of stock solutions of the test substance and of algal suspension with filtered algal medium. The culture flasks were shaken and placed in the culture apparatus; the cultures were maintained at a temperature of 20 ± 2°C. The cell concentration in each flask was determined 24, 48 and 72 hours after the start of the test by using a Perkin Elmer Victor 3, 1420 Multilabel Counter (Perkin Elmer, Singapore, Republic of Singapore). The pH was measured at the beginning of the test and after 72 hours of exposure. The area where the cultures were incubated received continuous, uniform fluorescent illumination with a light intensity of approximately 5000lux.

Statistical analysis

The average specific growth rate and the percent inhibition of growth rate for each replicate treatment were calculated by using ToxCalc™ — Toxicity Data Analysis Software, Version 5.0.32 (McKinleyville, CA, USA). The percentage reduction in average growth rate at each concentration of the test substance as compared to the control value, was plotted against the logarithm of the concentration. The E_rC50 can be read from the resulting graph. Analysis of variance (ANOVA) techniques were also employed for estimating the Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) values. To this end, the mean response for each concentration was compared with the control mean by using an appropriate multiple comparison, or trend test method. The basic ANOVA assumption of homogeneity of variance was assessed. The assessment was performed by using the ToxCalc Bonferroni *t*-test.

The toxicity of triazoles and benzotriazole to the aquatic invertebrate, *D. magna*

The purpose of this test was to determine the effects of a substance on the mobility of daphnids. The test was performed according to OECD TG 202: *Daphnia* sp., Acute Immobilisation Test (11).

Preparation of the test solutions

The test chemicals were purchased from Fluka, except benzotriazole, which was purchased from Sigma-Aldrich (St Louis, MO, USA). The test medium that was used was M4, which was prepared according to OECD TG 202 (11). Most of the substances were dissolved in dimethyl sulphoxide (DMSO), and 100, 500, 750 and 1000µl of the stock solutions were diluted into 100ml of M4 medium. The dissolved oxygen levels did not change significantly during the experiments, nor did the pH.

Daphnids

The culture conditions of the daphnids were as follows: 25–30 adults were maintained in 4 litres of M4 medium, aerated with slowly bubbling air (few bubbles/min), and were fed algae (*P. subcapitata*) and yeast extract (20mg/L) daily, except during the

weekends. Juveniles were taken from adults that were 2–4 weeks old. The culture and test conditions were similar: 16-hour light/8-hour dark cycle, temperature 19 ± 1°C.

The test

Young daphnids, fewer than 24 hours old at the start of the test, were exposed to the test substances at a range of concentrations for a period of 48 hours. Per 100ml glass beaker, five juveniles were used with 20ml of test solution. Four beakers were used for each test concentration, with a minimum of 5 test concentrations per chemical, excluding the controls/blanks. Daphnid immobilisation was recorded at 48 hours and compared with the control values. The results were analysed in order to calculate the EC50 at 48 hours.

Statistical analysis

GraphPad Prism 4.01 was used to calculate EC50 values, by applying the method of non-linear regression curve fitting and assuming a sigmoid dose–response curve with a top of 100% and a bottom of 0%.

The toxicity of triazoles and benzotriazole to *D. rerio* embryos

The test was performed according to the Draft Proposal for a New OECD Guideline for the Fish Embryo Toxicity (FET) Test (12).

Preparation of the test solutions

The test chemicals were purchased from Fluka, except benzotriazole, which was purchased from Sigma-Aldrich. Stock solutions were prepared in DMSO, with a final test concentration of 0.2% carrier (2ml/L) in the dilution series. Per compound, six test concentrations were prepared in Dutch Standard Water (DSW; demineralised water supplemented with 100mg/L $NHCO_3$, 20mg/L $KHCO_3$, 200mg/L $CaCl_2 \cdot 2H_2O$ and 180mg/L $MgSO_4 \cdot 7H_2O$ and then aerated for 24 hours at 27°C). For fenchlorazol-ethyl, benzotriazole and paclobutrazol, the maximum solubility was reached below the highest nominal test concentration. In these cases, crystals were observed on the bottom of the well, or floating on the surface of the test medium. These observations in over-saturated solutions were not taken into account during the final calculations. Potassium dichromate was used as a negative control substance, and 3,4-dichloroaniline as a positive control substance, at concentrations of 1mg/L and 8mg/L, respectively. The results showed that the background sensitivity of the egg clutches tested were within the expected ranges.

Maintenance of fish and egg spawning

For details on fish maintenance and egg production, see Hermsen *et al.* (13).

The test

Each test was repeated three times. If necessary, the concentration range was adjusted between different experiments. To start the exposure simultaneously, 40 fertilised eggs were introduced into 50ml of the test solution. Viable eggs were selected and incubated individually in 2ml of medium in 24-well flat-bottomed plates (Falcon, Omnilabo, Breda, The Netherlands). Four wells per plate were used for the controls and 10 wells for each test concentration. The temperature during the exposure was kept at 26.5 ± 0.5°C. The pH of the dilution medium (DSW) was on average 8.1 (range 7.4–8.3). The oxygen level during the exposure was ≥ 6.6mg/L and water hardness was 214mg/L $CaCO_3$, which was within the prescribed range of 10–250mg/L. The embryos were exposed to the test chemicals under static conditions, and microscopic observations were carried out 24, 48 and 72 hours after the start of the experiments, with 10–60× magnification (Wild binocular, Heerbrugg, Switzerland). The lethal endpoints were: coagulation of the embryo, no tail detachment, absence of heartbeat, and no somite formation.

Statistical analysis

All the LC50 values were calculated by using the trimmed Spearman-Kärber model (14). Since the eggs were all obtained from the same batch, the controls for all the compounds were pooled.

Ready biodegradability test on triazoles

Ready biodegradability (RB) is an indication of the ease with which a chemical is oxidatively degraded by microbial action under conditions typically found in a polluted river or in sewage treatment plants (15). The 28-day test for ready biodegradability in an aerobic aqueous medium was performed according to OECD TG 301 D: Ready Biodegradability (16).

Preparation of the test solutions

The chemicals tested were purchased from Dr Ehrenstorfer GmbH. Mineral medium was pre-

pared according to OECD TG 301 (16). In order to verify the procedure, a reference compound that met the criteria for ready biodegradability was tested, by setting up an appropriate vessel in parallel to the normal test runs. The reference compound was sodium acetate, and the degradation was tested by determining the removal of dissolved organic carbon (DOC).

Chemical measurements

The concentrations of triazoles in the test samples were determined at the beginning of the test, day 1, day 14 and day 28, by online solid phase extraction–liquid chromatography-electrospray-tandem mass spectrometry (online SPE Spark, LC-ESI-MS/MS API 2000). Online SPE pre-concentration was performed by loading 20ml of the sample onto cartridges of PLRP-s (cross-linked styrene-divinylbenzene polymer, 15–25µm particle size). The analytes were eluted through a LC Zorbax SB-C18 column (Agilent Technologies, 3.0 × 150mm, with a particle size of 3.5µm), by using a gradient of 5mM NH_4OH buffer/acetonitrile as the mobile phase at a flow rate of 0.25ml/min. Further LC-MS/MS determination was performed in the multiple reaction monitoring (MRM) mode by recording two MRM transitions per compound. The quantification of the selected pesticides was performed according to external standard calibration.

The inoculum and abiotic degradation

The inoculum for the test was obtained from water in an aquarium. Abiotic degradation was confirmed by inactivating the microorganisms with mercury chloride.

Ready biodegradability test

A solution of the test substance in a mineral medium was inoculated and incubated under aerobic conditions, in the dark at 20 ± 2°C. The test medium contained a relatively low concentration of biomass. A reference compound was run in a parallel test to verify the execution of the procedures. Possible abiotic degradation was investigated separately. The degradation of the test substances was determined by DOC measurement. Samples were taken at sufficiently frequent intervals (on days 0, 1, 14 and 28) to allow the identification of the beginning and end of biodegradation. Degradation was followed by specific chemical analysis at frequent intervals over a 28-day period to assess the degradation of triazoles. The degree of biodegradation was calculated by expressing the concentration of the test substance removed (corrected for that in the blank inoculum control) as a percentage of the concentration initially present (Equation 1). A test was considered valid, if the difference of extremes of replicate values of the removal of the test chemical at the end of the test was less than 20%, and if the percentage degradation of the reference compound had reached the pass levels by day 14.

Statistical analysis

The mean values of the duplicate measurement of the parameter in both test vessels and inoculum blank were used in the calculation of D_t, the percentage degradation after 28 days. With specific chemical analytical data available, degradation at day t of the test was calculated from:

$$D_t = \left[1 - \frac{C_t - C_{blt}}{C_0 - C_{bl0}}\right] * 100 \qquad \text{[Equation 1]}$$

where D_t = % degradation at day t of the test; C_0 = the mean starting concentration of the test substance in the inoculated culture medium containing the test substance in mg/L; C_t = the mean concentration of the test substance in the inoculated culture medium containing test substance at day t of the test in mg/L; C_{bl0} = the mean starting concentration of the test substance in blank inoculated mineral medium in mg/L; and C_{blt} = the mean concentration of the test substance blank inoculated mineral medium at day t of the test in mg/L.

Results and Discussion

Toxicity studies with *D. magna* and *D. rerio* were conducted on selected triazoles and benzotriazole. The toxicity studies with *P. subcapitata* and the biodegradation studies were conducted on selected triazoles.

Toxicity of triazoles to the freshwater alga, *P. subcapitata*

In the laboratory toxicity study with the freshwater alga, *P. subcapitata*, E_rC50 values and NOEC values were determined for 13 triazoles. The results of the toxicity testing on *P. subcapitata* are based on actual measured concentrations. We found that, according to the Directive on Classification, Packaging and Labelling of Dangerous Substances (17), all 13 substances were toxic to *P. subcapitata*, and that toxicities did not vary widely among the 13 triazoles studied. Table 2 provides the experimentally-derived E_rC50 values after 72 hours of exposure, whereas Table 3

Table 2: Overview of E_rC50 values for a set of 13 triazoles after a 72-hour exposure

Substance	CAS number	E_rC50 72 hours (mg/L)	95% CI	R^2
Cyproconazole	94361-06-5	8.84	5.17–12.9	0.981
Diclobutrazol	075736-33-3	4.40	4.20–4.57	0.916
Difenoconazole	119446-68-3	1.44	1.19–1.51	0.908
Diniconazole	083657-24-3	1.81	1.74–1.87	0.952
Epoxiconazole	106325-08-0	8.65	n.a.	0.974
Hexaconazole	79983-71-4	3.81	3.36–4.27	0.906
Myclobutanil	88671-89-0	14.2	13.3–15.2	0.896
Paclobutrazol	076738-62-0	12.05	9.00–15.1	0.960
Penconazole	066246-88-6	3.62	3.40–3.85	0.829
Propiconazole	060207-90-1	4.17	3.75–4.63	0.964
Triadimefon	43121-43-3	7.51	5.31–9.77	0.947
Triazophos	024017-47-8	7.59	6.65–8.57	0.946
Uniconazole-P	083657-17-4	> 6.9[a]	n.a.	n.a.

The endpoint of toxicity assessment was inhibition of growth of Pseudokirchneriella subcapitata. *Toxicity endpoints were calculated based on measured concentrations. CI = confidence interval; n.a. = not applicable.*

[a]*The toxicity exceeded the water solubility (95% CI could not be calculated).*

contains the NOEC values found for each of the chemicals tested. Difenoconazole was found to induce the highest level of toxicity to the alga, considering its E_rC50 of 1.44mg/L; the lowest level of toxicity was found for myclobutanil, with an E_rC50 value of 14.2mg/L.

A limited amount of information is available on the toxic effects of triazoles in freshwater algae, especially with regard to a 72-hour testing period.

Table 3: Overview of the NOEC values for a set of 13 triazoles after a 72-hour exposure

Substance	CAS number	NOEC 72 hours (mg/L)
Cyproconazole	94361-06-5	0.170
Diclobutrazol	075736-33-3	0.190
Difenoconazole	119446-68-3	0.100
Diniconazole	083657-24-3	0.570
Epoxiconazole	106325-08-0	< 0.098
Hexaconazole	79983-71-4	< 0.190
Myclobutanil	88671-89-0	< 0.240
Paclobutrazol	076738-62-0	< 0.630
Penconazole	066246-88-6	0.200
Propiconazole	060207-90-1	0.065
Triadimefon	43121-43-3	1.900
Triazophos	024017-47-8	1.700
Uniconazole-P	083657-17-4	< 0.830

The endpoint of toxicity assessment was inhibition of growth of Pseudokirchneriella subcapitata. *Toxicity endpoints were calculated based on measured concentrations.*

A study on *P. subcapitata* exposure to propiconazole found that this substance was toxic to this organism, with a 72-hour E_rC50 value of 5.0mg/L (18). More recently, another study found that propiconazole was highly toxic to *P. subcapitata*, with a 72-hour IC50 of 390µg/L (19), but it should be noted that this value is based on nominal concentrations, instead of actually determined concentrations, as was the case in our study. These data on the toxic effects of propiconazole in freshwater algae do not differ significantly from the data in our study (72-hour E_rC50 value of 4.17mg/L). Limited data on the toxic effects of triazoles in *P. subcapitata* are also available in the Pesticide Properties Database (PPDB; 9). A study conducted on *P. subcapitata* exposed to paclobutrazol and penconazole found that these substances were toxic to the alga, with 72-hour E_rC50 values of 7.2mg/L for paclobutrazol and 4.9mg/L for penconazole (20). Similarly, these data on the toxicity of paclobutrazol and penconazole to freshwater algae did not differ significantly from the data obtained in our study (72-hour E_rC50 values of 12.1mg/L for paclobutrazol and 3.6mg/L for penconazole).

Toxicity of triazoles and benzotriazole to the aquatic invertebrate, *D. magna*

In the laboratory toxicity study with the aquatic invertebrate, *D. magna,* EC50 values were determined for 11 triazoles and for benzotriazole. The results for the acute toxicity assessment are shown in Table 4. We found that toxic concentrations of the substances tested varied over two orders of magnitude among the 11 triazoles and

Table 4: Overview of EC50 values obtained for a set of 11 triazoles and benzotriazole after a 48-hour exposure

Substance	CAS number	EC50 48 hours (mg/L)	95% CI	R^2
Benzotriazole	95-14-7	155.40	154.4–156.5	1.000
Cyproconazole	94361-06-5	30.90	21.4–44.8	0.955
Diclobutrazol	75736-33-3	12.90	10.4–16.1	0.857
Fenchlorazol-ethyl	103112-35-2	> 2.50[a]	n.a.	n.a.
Flusilazole	85509-19-9	3.17	1.66–6.05	0.902
Guanazole	1455-77-2	4.13	3.53–4.84	0.990
Hexaconazole	79983-71-4	4.90	4.43–5.41	0.990
Myclobutanil	88671-89-0	12.40	10.3–14.9	0.984
Paclobutrazol	76738-62-0	45.20	35.4–57.7	0.717
Ribavirin	36791-04-5	684.00	588–796	0.928
Triadimefon	43121-43-3	29.10	28.3–29.8	0.998
Triticonazole	131983-72-7	9.56	5.70–16.0	0.930

The endpoint of toxicity assessment was immobility of Daphnia magna. *Toxicity endpoints were calculated based on nominal concentrations. CI = confidence interval; n.a. = not applicable.*

[a]*The toxicity exceeded the water solubility (95% CI could not be calculated).*

benzotriazole. Fenchlorazol, flusilazole, guanazole and hexaconazole were much more toxic than ribavirin and benzotriazole. The 48-hour EC50 values varied from 3.17mg/L for flusilazole, to 684mg/L for ribavirin. According to the Directive on Classification, Packaging and Labelling of Dangerous Substances (17), flusilazole, guanazole, hexaconazole and triticonazole are toxic to *D. magna*; cyproconazole, diclobutrazol, myclobutanil, paclobutrazol and triadimefon are harmful to *D. magna*; and benzotriazole and ribavirin are non-toxic to *D. magna* (17).

Some data are publicly available on the toxic effects of triazoles and benzotriazole in daphnids (20). A 48-hour EC50 study conducted with *D. magna* exposed to cyproconazole found that the substance was harmful, with an EC50 value of 26mg/L (20). Another investigation confirmed that cyproconazole is harmful to *D. magna*, with an EC50 of 22mg/L (20). These data on the toxic effects of cyproconazole to daphnids do not differ significantly from the data obtained in our study. Further data on the toxic effects of triazoles in *D. magna* are available in the Pesticide Properties Database (PPDB; 9). A

Table 5: Overview of LC50 values obtained for a set of 10 triazoles and benzotriazole after a 72-hour exposure

Substance	CAS number	Test 1 LC50	Test 1 95% CI	Test 2 LC50	Test 2 95% CI	Test 3 LC50	Test 3 95% CI
Benzotriazole	95-14-7	16.00	8.61–29.70	2.63	1.68–4.11	0.67	n.a.
Cyproconazole	94361-06-5	40.20	29.40–54.90	45.00	36.20–56.00	40.70	28.6–57.9
Fenchlorazol	103112-35-2	2.52	1.31–4.85	3.33	2.90–3.81	1.78	n.a.
Flusilazole	85509-19-9	6.12	4.92–7.62	5.46	n.a.	8.10	5.72–12.40
Guanazole	1455772	≥ 29.70	n.a.	≥ 79.30	n.a.	17,320.00	n.a.
Hexaconazole	79983-71-4	9.68	6.72–13.90	5.30	4.02–7.00	7.60	5.31–10.90
Myclobutanil	88671-89-0	14.10	11.30–17.70	14.10	11.30–17.50	15.82	11.6–21.7
Paclobutrazol	76738-62-0	14.50	3.69–57.30	≥ 88.10	n.a.	54.26	44.9–65.5
Ribavirin	36791-04-5	≥ 73.30	n.a.	≥ 147.00	n.a.	≥ 1000.00	n.a.
Triadimefon	43121-43-3	36.00	25.00–54.90	50.90	n.a.	45.40	36.2–56.8
Triticonazole	131983-72-7	≥ 31.80	n.a.	≥ 95.30	n.a.	≥ 95.30	n.a.

The zebrafish embryo toxicity test lethal endpoints were: mortality/no heartbeat/no somite formation/no detachment of tail. Toxicity endpoints were calculated based on nominal concentrations, LC50 (mg/L) calculated with the trimmed Spearman-Kärber method. CI = confidence interval; n.a. = not applicable.

study conducted on *D. magna* exposed to cyproconazole, flusilazole, hexaconazole, myclobutanil, paclobutrazol and triticonazole found that these substances were harmful, or toxic, to these organisms with 48-hour EC50 values of > 22mg/L for cyproconazole, 3.4mg/L for flusilazole, 2.9mg/L for hexaconazole, 17mg/L for myclobutanil, 33.2mg/L for paclobutrazol and 9mg/L for triticonazole (9). Similarly, these results on the toxic effects of triazoles in daphnids do not differ significantly from the values obtained in our study (48-hour EC50 values of 30mg/L for cyproconazole, 3.2mg/L for flusilazole, 4.9mg/L for hexaconazole, 12.4mg/L for myclobutanil, 45.2mg/L for paclobutrazol and 9.6mg/L for triticonazole).

Toxicity of triazoles and benzotriazole to *D. rerio* embryos

In the laboratory toxicity study with embryos of *D. rerio*, LC50 values were determined for 10 triazoles and benzotriazole. The results of the acute toxicity testing of these substances to *D. rerio* are shown in Table 5. We found that toxicity to the fish embryos varied among the tested substances, from 0.67mg/L for benzotriazole to 17,320mg/L for guanazole (72-hour LC50). According to the Directive on Classification, Packaging and Labelling of Dangerous Substances (17), benzotriazole, fenchlorazol, flusilazole and hexaconazole are toxic to *D. rerio*; cyproconazole, myclobutanil, paclobutrazol triadimefon are harmful to *D. rerio*; and guanazole, ribavirin and triticonazole are non-toxic to fish embryos.

Some information is available in the literature on the toxic effects of triazoles and benzotriazole in vertebrates, but not in *D. rerio* embryos. A study conducted on fathead minnow (*Pimephales promelas)* exposed to benzotriazole found that the 96-hour LC50 was 65mg/L (1). The fathead minnow is a freshwater fish that belongs to the minnow family (Cyprinidae), just like the zebrafish (*D. rerio*). Overall, these data on the toxic effects of benzotriazole in fish do not differ significantly from the data in our study.

Variation in toxicity across species

The variation in toxicity across the species tested — an alga (*P. subcapitata*), a daphnid (*D. magna*) and a fish (*D. rerio* embryos) — is shown in Figure 1. The test results show that, for most substances, sensitivity across species does not vary over a wide range (in general, the largest factor is about 20). Furthermore, no single species was consistently the most sensitive to all of the substances tested. For cyproconazole, hexaconazole and triadimefon the toxicity of triazoles decreased in the following order: freshwater alga > daphnid > fish. In these cases, *D. rerio* was five times less sensitive than *P. subcapitata*. In the acute toxicity tests with myclobutanil, *D. rerio*, *D. magna* and *P. subcapitata* showed the same range of sensitivity. Most notably, however, is the observation that *D. magna*

Table 6: Overview of mean values of measured concentrations for a set of 13 triazoles at days 0, 1, 14 and 28 in a ready biodegradability test

Substance	C_0	C_1	C_{14}	C_{28}	C_{blt}
Cyproconazole	1.0	1.0	0.80	0.95	0.025
Diclobutrazol	5.0	5.0	4.90	4.85	0.025
Difenoconazole	5.0	5.0	4.80	4.65	0.025
Diniconazole	10.0	10.0	9.05	9.00	0.025
Epoxiconazole	5.0	5.0	4.65	4.90	0.025
Hexaconazole	10.0	10.0	10.00	9.30	0.025
Myclobutanil	10.0	10.0	9.95	8.35	0.025
Paclobutrazol	10.0	10.0	8.85	8.65	0.025
Penconazole	5.0	5.0	4.90	4.85	0.025
Propiconazole	1.0	1.0	0.90	1.00	0.025
Triadimefon	10.0	10.0	10.00	8.85	0.025
Triazophos	0.1	0.1	0.09	0.085	0.025
Uniconazole-P	50.0	50.0	50.00	43.90	0.025

C_{blt} represents concentration in blank at days 0, 1, 14 and 28. All concentrations were below the detection limit of the method (< LOD) and are calculated as LOD/2. The values are µg/L.

Table 7: Overview of mean values of biodegradation, D_{28} (%), for a set of 13 triazoles at day 28 in a ready biodegradability test

Substance	D_{28}	95% CI
Cyproconazole	5.1	0.36
Diclobutrazol	3.0	0.08
Difenoconazole	7.0	0.31
Diniconazole	10.0	0.36
Epoxiconazole	2.0	0.04
Hexaconazole	7.0	0.10
Myclobutanil	16.5	1.32
Paclobutrazol	13.5	0.28
Penconazole	3.0	0.12
Propiconazole	< 3.0	n.a.
Triadimefon	11.5	0.63
Triazophos	20.0	3.67
Uniconazole-P	12.2	0.73

The percentage of biodegradation is calculated from the data presented in Table 5 by using Equation 1. The values correspond to %. CI = confidence interval; n.a. = not applicable.

was substantially less sensitive to benzotriazole than were *D. rerio* and *P. subcapitata*, with differences in toxicity of about two orders of magnitude.

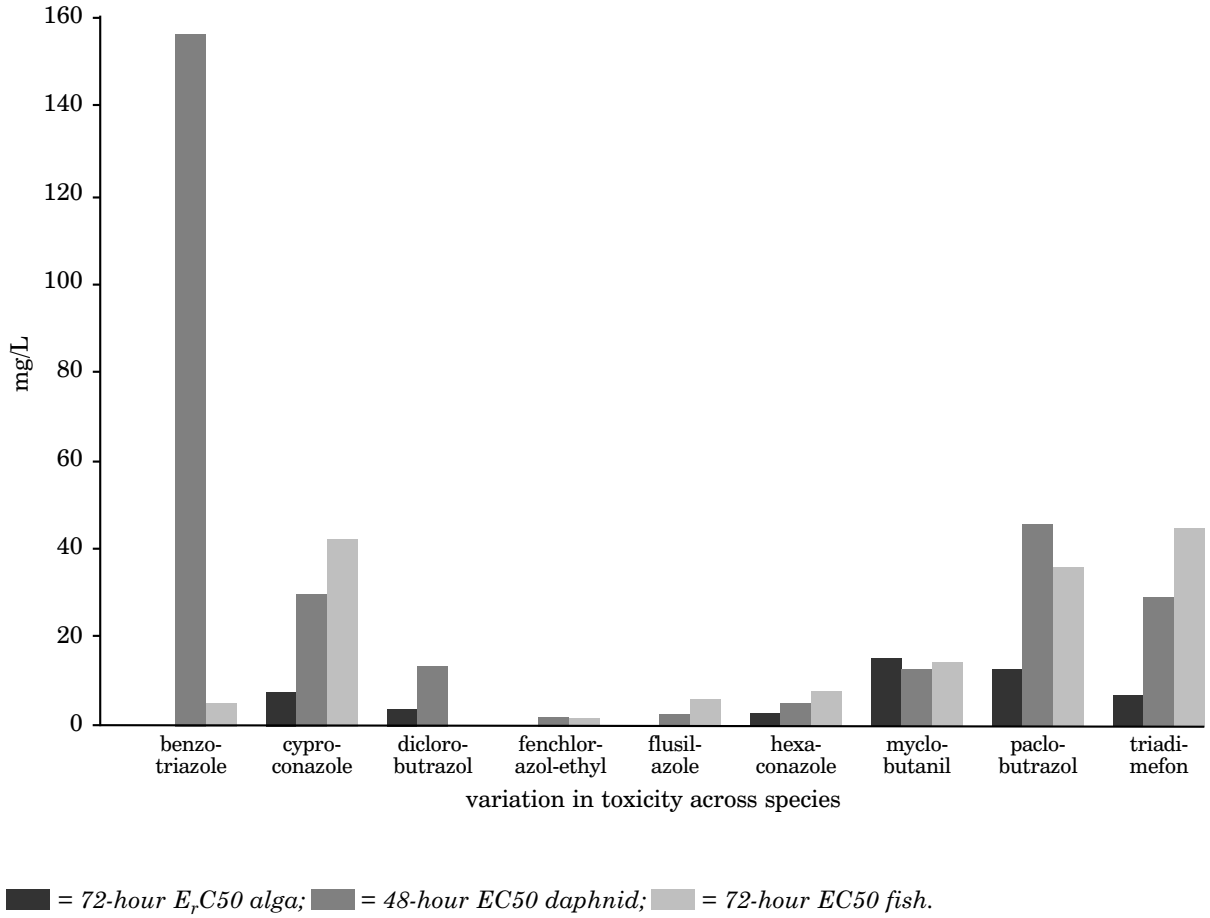
Ready biodegradability testing of triazoles

In the laboratory study on ready biodegradability, the values for primary degradation at day 28, were determined for 13 triazoles. Table 6 presents the concentrations tested, as measured on days 0, 1, 14 and 28 of the test. In the overview of biodegradation test results shown in Table 7, D_{28} is expressed as the percentage of degradation on day 28 of the test. The pass level for ready biodegradability is 70% removal of the substance. This pass level has to be reached in a 10-day window within the 28-

Figure 1: Variation in toxicity across species

Substance	72-hour E_rC50 alga	48-hour EC50 daphnid	72-hour EC50 fish
Benzotriazole	—	155.40	6.43
Cyproconazole	8.84	30.92	42.0
Diclobutrazol	4.40	12.93	—
Fenchlorazol-ethyl	—	2.50	2.54
Flusilazole	—	3.17	6.56
Hexaconazole	3.81	4.90	7.53
Myclobutanil	14.17	12.36	14.7
Paclobutrazol	12.05	45.20	34.4
Triadimefon	7.51	29.06	44.1

Variation in toxicity across the alga Pseudokirchneriella subcapitata*, the daphnid* Daphnia magna *and fish embryos of* Danio rerio; — *not tested.*



= *72-hour E_rC50 alga;* = *48-hour EC50 daphnid;* = *72-hour EC50 fish.*

day period of the test. As expected, these substances are not readily biodegradable.

Only limited experimental data are available on the ready biodegradability of triazoles. A study conducted on cyproconazole, epoxiconazole and paclobutrazol (17) indicated negligible biodegradation. These results are fully in line with the results from this study.

Conclusions

This study reported on the acute aquatic toxicities of selected triazoles to the green alga, *P. subcapitata*, the acute aquatic toxicity of selected triazoles and a benzotriazole to the daphnid, *D. magna*, and to embryos of the fish *D. rerio*. Besides toxicity testing, the ready biodegradability of selected triazoles was also investigated. The experimental tests presented here are part of an intelligent testing strategy, in which a set of compounds were selected, based on their representativeness of the whole class of triazoles and benzotriazoles. The selection of compounds was based on experimental design, but was also influenced by the availability and production costs of the compounds. The data on toxic effects obtained in this study have been used for the evaluation of QSAR predictions generated within the CADASTER project. The comparison between experimental and predicted data is described by Cassani *et al.* (21).

We have shown that triazoles are toxic to freshwater algae. The toxicity of triazoles and benzotriazole to daphnids and to fish embryos varied, depending on the substance. Triazoles are not ready biodegradable. All the results reported here are in line with the scarce literature data on the toxicity and degradability of triazoles and benzotriazole.

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