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Short Communication

Bisazir as a chemosterilant to control invasive vertebrates: ecotoxicity and efficacy to induce male sterility in *Lithobates catesbeianus*

Sarah Descamps[1,2,*] and Alain De Vocht[1,2]

[1]*PXL- Bio Research, Agoralaan Building H , BE-3590 Diepenbeek, Belgium*
[2]*Hasselt University, Centre for Environmental Sciences, Agoralaan Building D, BE-3590 Diepenbeek, Belgium*
**Corresponding author*
E-mail: *sarah.descamps@uhasselt.be*

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Abstract

Chemical sterilisation is a way to control populations of invasive exotic species. To investigate the potential to control populations of invasive American bullfrog (*Lithobates catesbeianus*), 26 adult male individuals were caught and injected with a dose of 0, 12.5, 25, 50, 100 mg/kg bisazir in order to induce DNA fragmentation in sperm cells and subsequent induce sterility. The results indicate that injecting 50 mg/kg bisazir causes significant fragmentation in the sperm of *Lithobates catesbeianus*. Before using chemicals *in situ* their potential risk for the environment should be documented. As a first step the inherent ecotoxic properties of bisazir were evaluated in both acute and chronic aquatic tests: microtox (*Aliivibrio fischeri*), microalga (*Raphidocelis subcapitata*), duckweed (*Lemna minor*), waterflea (*Daphnia magna*). The no effect concentration (NOEC) was 1 mg/l for *Daphnia* reproduction. Based on these results the predicted no effect concentration (PNEC) value was 20 µg/l. These results show the inherent ecotoxic properties of the compound and raise questions on the safe applicability in aquatic habitats.

Key words: *Rana catesbeiana*, American bullfrog, sterile-male-release technique, DNA fragmentation, chemical control, environmental risk

Introduction

Invasive species are a worldwide threat for native biodiversity, and are a leading cause of the extinction of species (Bellard et al. 2021; Clavero and Garcia-Berthou 2005; Duenas et al. 2021). The American bullfrog, *Lithobates catesbeianus* (Shaw, 1802), is listed as one of the world's 100 most invasive species, based on its invasive character and ecological impact (Lowe et al. 2000). This species is considered to be responsible for the decline of numerous populations of native species (Adams and Pearl 2007; Bellard et al. 2016). The importance of this issue is recognized in the published EU regulation (EU 1143/2014) on invasive alien species (IAS) where the bullfrog is present on the first edition of the list of invasive species of Union concern in 2016 (Commission Implementing Regulation 2016; European parliament 2014).

Free-ranging bullfrog populations are present in six countries in Europe; Belgium, France, Germany, Greece, Spain and Italy (Ficetola et al. 2007). They form regionally a threat to native biodiversity through predation, habitat and food competition (Batista 2002; Cross 2002; Wang and Li 2009) and as a vector of the Chytrid-fungus (*Batrachochytrium dendrobatidis* Loncore, 1999) (Blaustein and Kiesecker 2002; Hanselmann et al. 2004; Pasmans and Martel 2012). This fungus is the agent of chytridiomycosis, an infectious disease considered as one of the main causes of global amphibian decline and extinction (Bellard et al. 2016; Garner et al. 2006; Pounds et al. 2006).

Currently control methods for the American bullfrog are limited to the removal of larvae and adults by the use of fyke netting, traps, shooting or draining the ponds they inhabit (Adriaens et al. 2013; Berroneau et al. 2008; Mandin 2015; Rosen and Schwalbe 1995). These measures only, however, are not effective to stop the further expansion of the species. Eradications in Europe have only been successful in an early stage of invasion (Banks et al. 2000; Devisscher et al. 2012; Ficetola, et al. 2007; Theismeier et al. 1994). New control methods for well-established populations are urgently needed to stop the further dispersion of this invasive species in Europe. Total eradication or permanent population control of these populations are long or everlasting and consequently very costly.

The sterile-male-release technique (SMRT) can be an efficient method to control widespread populations but implies the sterilisation of individuals through chemical or physical actions. The theory behind this technique of population control consists of the introduction of a high amount of sexually sterile males for several successive generations. Subsequently most wild females will mate with sterile males which will decline the population towards extinction (Knipling 1959). The breeding of American bullfrog takes place in May to July in the northern, and from February to October in the southern hemisphere. Their fertilization is external, with the females depositing as many as 20 000 eggs in a clutch of jelly in warm, still, shallow waters (Bee 2002; Knipling 1959; Ryan 1980). In most cases the fertilisation of the female is carried out by one male which makes this species suitable for the SMRT technique (Knipling 1959).

The SMRT through chemical sterilisation was at first successfully implemented on the screwworm fly (*Cochliomyia hominivorax* Coquerel, 1858) in 1966 (Baumhover 1966). Then several other species of mosquitoes who are a vector of diseases followed (Klassen and Curtis 2005). Several methods have been applied in an attempt to create sterile individuals in sea lamprey (*Petromyzon marinus* Linnaeus, 1758*)* and rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792*)*. Intraperitoneal injection of gossypol-acetic acid (Rinchard et al. 2000), H_2O_2 (Ciereszko et al. 2005), UV-radiation (Dietrich et al. 2005), radiation with cobalt-60 and cesium-137 (Hanson 1990) have been tested but do not show the same effect or efficiency as the

injection of the chemical bisazir in sea lamprey (Great Lakes Fishery Commission 1995). Most potential alternatives for bisazir are less toxic and only reduce the fertilisation rate of the eggs by diminishing the sperm mobility, leading to a higher probability that the eggs will be fertilised by other fertile males (Ciereszko et al. 2004).

Bisazir, trademark of P,P-bis(1-aziridinyl)-N-methylphosphinothioic amide, is an aziridinyl compound with a genotoxic effect related to its alkylating properties. Consequently, it can alkylate DNA and more specific the purine base, guanine, in which the 7-nitrogen is highly nucleophile. Furthermore it is known to cause the selective inhibition of cell division in rapidly proliferating cells such as haemopoietic cells and germ cells in mice (Rudrama and Reddy 1985). The use of bisazir on cotton spotted bollworm (*Earias fabia* Cramer, 1781*)* resulted in significant changes in DNA, RNA, protein and free amino acid levels in testes (Srivastava and Kumar 1984). The spermatozoa were unable to fully activate the metabolic activities of the eggs and lead to a reduced protein synthesis. When eggs do hatch, there is no detection of viable larvae; bisazir induces chromosomal breakage which leads to a genetic imbalance in the cleavage nuclei and ultimately ends in embryonic death (Bergstedt and Twohey 2007; Srivastava and Kumar 1984).

Experimental implementation of bisazir as an agent to induce sterility was used in the Great Lakes (US) from 1991–1999 to control invasive sea lamprey. The lampreys were injected with 100 mg/kg bisazir, which gave 0–0.1% viable embryos. Doses of 25 and 50 mg/kg gave a maximum of 0.7% viable embryo's (Bergstedt and Twohey 2007). Application of the sterile-male-release technique with this species showed that when the number of the sterile individuals is high enough (20–30% catch/year) and applied during three generations the population density dropped with 90–98% (Klassen et al. 2005). Moreover, it was shown that the release of male sterile lampreys was more effective for the population decline than the release of sterile females (Klassen et al. 2004). In fish the sterility, achieved by chemical sterilisation, does not affect the mating and competitive behaviour. Sperm concentration, mobility and fertilisation rate is not different in comparison with non-sterile males. The male sterility is linked with damage in the DNA of sperm and the lack of repair mechanisms which makes DNA damage in the sperm irreversible (Ciereszko et al. 2005). So far, no reports on the effect of bisazir on the sperm of amphibians have been published.

Toxicity studies of bisazir are very limited and fragmented. Oral administration of respectively 2.12, 4.25 and 8.25 mg/kg bisazir in mice revealed a significant increase of chromosomal aberrations and sperm head abnormalities at all doses tested (Rudrama and Reddy 1985). Toxicity studies with oral administration to albino rats showed that the median lethal dose LD_{50} was 25.2 mg/kg, which implicate that bisazir is highly toxic (U.S. Fish and Wildlife Service 1995).

Using bisazir in the field requires an ecological risk assessment. Data on the inherent ecotoxicity of the product and – when inherent ecotoxicity is the case – data on exposure concentrations (fate and behaviour in the environment) are needed to evaluate the potential risk. Only few data are available in literature. Data for the freshwater flea (*Daphnia magna* Straus, 1820) (48h median effective concentration EC_{50} = 70 mg/l) and rainbow trout (96h median lethal concentration LC_{50} > 100 mg/l) indicate that bisazir is only slightly toxic to aquatic organisms. The octanol/water partition coefficient of bisazir, K_{ow} = 2.3, indicates that this chemical is not bioaccumulating in the food chain (U.S. Fish and Wildlife Service 1995).

Acute and chronic ecotoxicological data are generated in this study to define the inherent ecotoxicity properties of the product, and to evaluate the need for exposure assessment. The injection of bisazir, as used to control sea lamprey in the Great Lakes USA, is used in this pilot study to evaluate its effect on the DNA of the sperm from the American bullfrog.

The findings of this research can support a possible application of the sterile-male-release technique to control the large and wide-ranging populations of this invasive aquatic species.

Materials and methods

Amphibians

Twenty-six sexual mature adult male American bullfrogs were caught by electric fishing (Deka 3000 Lord) and fykes (0.8 m diameter, 1 cm mesh) in May 2011 in a pond in the valley of the Grote Nete (51.147037; 5.137958). Sexual maturity was assessed by defining secondary sex characteristics such as the presence of a vibrant yellow throat and nuptial pads. The animals had a mean length (snout-vent) ± SD of 12.53 ± 1.55 cm and mean weight ± SD of 200.44 ± 71.50 g. During the experiment each frog was held in a plastic tank of 84 l with half land and half water biotope at a temperature of 20 °C according to the international, national, and institutional ethical standards. The frogs were fed daily with topmouth gudgeons *(Pseudorasbora parva* Temminck & Schlegel, 1846*)* and bullfrog larvae.

Bisazir injection and DNA fragmentation

Bisazir, $C_5H_{12}N_3PS$ (LKT Lab., USA), was injected intraperitoneal at a concentration of 12.5 (n = 6), 25 (n = 7), 50 (n = 4), 100 mg/kg (n = 5) in NaCl 0.9% (Table 1). Selection of the concentrations was based on the results of bisazir injections in sea lamprey (Bergstedt and Twohey 2007). A control group (n = 4) was treated with injections of NaCl 0.9%. The wastewater from the tanks was collected and filtered by a carbon filter so no chemical compounds due to excretion were released in the environment. After 10 days the adult males were injected with 8.6 IU hCG (Chorulon, Intervet) + 0.6 µg LHRH/g ((Des-Gly^{10}, D-His(Bzl)^6, Pro-NHEt^9)-LHRH,

Table 1. Overview on the amount of *Lithobates catesbeianus* individuals used for the injection of different concentrations of bisazir, their survival and the number used for comet assay tests.

Concentration bisazir (mg/kg)	# injected	# dead	# comet assay
0	4	0	4
12.5	6	1	5
25	7	4	3
50	4	0	4
100	5	5	0

Bachem) and urinary sperm was collected after three hours by pressing the lateral abdomen of the frog gently while protracting the legs to the head. The sperm was directly transferred in ice cold 50 µl 1X PBS (Ca^{2+}, Mg^{2+} -free) and used for comet assay.

To determine the amount of DNA-fragmentation in the collected sperm through single cell gel electrophoresis a CometAssay-kit (Trevigen Inc., USA) was used. The samples were examined under a fluorescence microscope (Nikon Eclipse 80i) with a ccd camera (DFK 41AF02 FC, Imaging Source) at excitation 494 nm and emission 52 nm.

Hundred random comets per organism were scored and % DNA in their head and tail was measured using the software program OpenComet (Gyori et al. 2014) and ImageJ 1.46 (National Institutes of Health, USA). Statistics were performed using SPSS statistics 22 (IBM) and R (R Development Core Team). A mixed model was fitted on the log-transformed outcome, such that variability due to dependency in the data could be captured by using a subject-specific random effect term. Group was used as a categorical fixed effect. P-values for pairwise comparisons between groups were adjusted through a Tukey-Kramer post-hoc test. A global significance level of 5% was applied.

Ecotoxicology

A ½ dilution series of bisazir was prepared in dimethyl sulfoxide (DMSO) with 100 mg/ml being the highest concentration. These were used in the tests at 1% in the Microtox® test (final test concentrations: 1000 mg/l + 9 serial ½ dilutions) and 0.1% in the other tests (final test concentrations: 100 mg/l + up to 5 serial ½ dilutions). In the *Daphnia* reproduction test the final test concentrations were 4 – 3 – 2 – 1 – 0.5 mg/l.

Microtox® (Microlan)

The effect on the bioluminescent signal of the autoluminescent bacteria *Aliivibrio fischeri* (Beijerinck, 1889) after 30 minutes of exposure is used as an indicator for toxic effects. The test protocol is based on ISO 11348-3 (International Organization for Standardization (ISO) 2011), using 3 replicates. Decrease in bioluminescent signal was compared to the decrease in solvent control (1% DMSO).

Algal growth inhibition test

The effect on the growth rate of unicellular green algae (*Raphidocelis subcapitata* Korshikov, 1990) when exposed for 72 hours is used as an indicator for toxic effects. The test protocol is based on the OECD guideline for testing chemicals 201 (OECD 2014), using own culture organisms and OECD medium as dilution medium. The specific growth rate for 0 to 72 hours is compared to the specific growth rate in solvent control conditions (0.1% DMSO). Respectively 6 and 3 replicates were used for the controls and test solutions.

Daphnia immobility test

The number of mobile/immobile water fleas (*Daphnia magna*) after 48 h of exposure was measured. An increase in the number of immobile organisms (% of number of organisms that are exposed) is an indication for toxicity. The test protocol is based on the OECD guideline for testing chemicals 202 (OECD 2014), using own culture organisms and JP4 medium as dilution medium. Respectively 6 and 4 replicates were used for the controls and test solutions.

Daphnia reproduction test

The number of juveniles produced by individual water fleas during 21 days of exposure was measured and compared to the number in solvent control conditions (0.1% DMSO). The test protocol is based on the OECD guideline for testing chemicals 211 (OECD 2014), using own culture organisms and JP4 medium as dilution medium. Ten replicates were used for all conditions.

Duckweed growth inhibition test

The number of fronds developed by the individual plants (*Lemna minor* Linnaeus, 1753) during 7 days of exposure are counted and compared to the number in solvent control conditions. The test protocol is based on the OECD guideline 221 for testing chemicals (OECD 2014), using own culture organisms and Steinberg medium as dilution medium. Three replicates were used for each condition.

Results

During the 10 days between the initial injection of bisazir and the screening of the spermatozoa 10 individuals died (Table 1). Major lethality was present in the group who received an injection of 25 mg/kg (57%) and 100 mg/kg (100%). Comets were subsequently scored on a total of 16 individuals. DNA fragmentation in the spermatozoa was visualized by oval heads and visible tails under the fluorescence microscope (Figure 1).

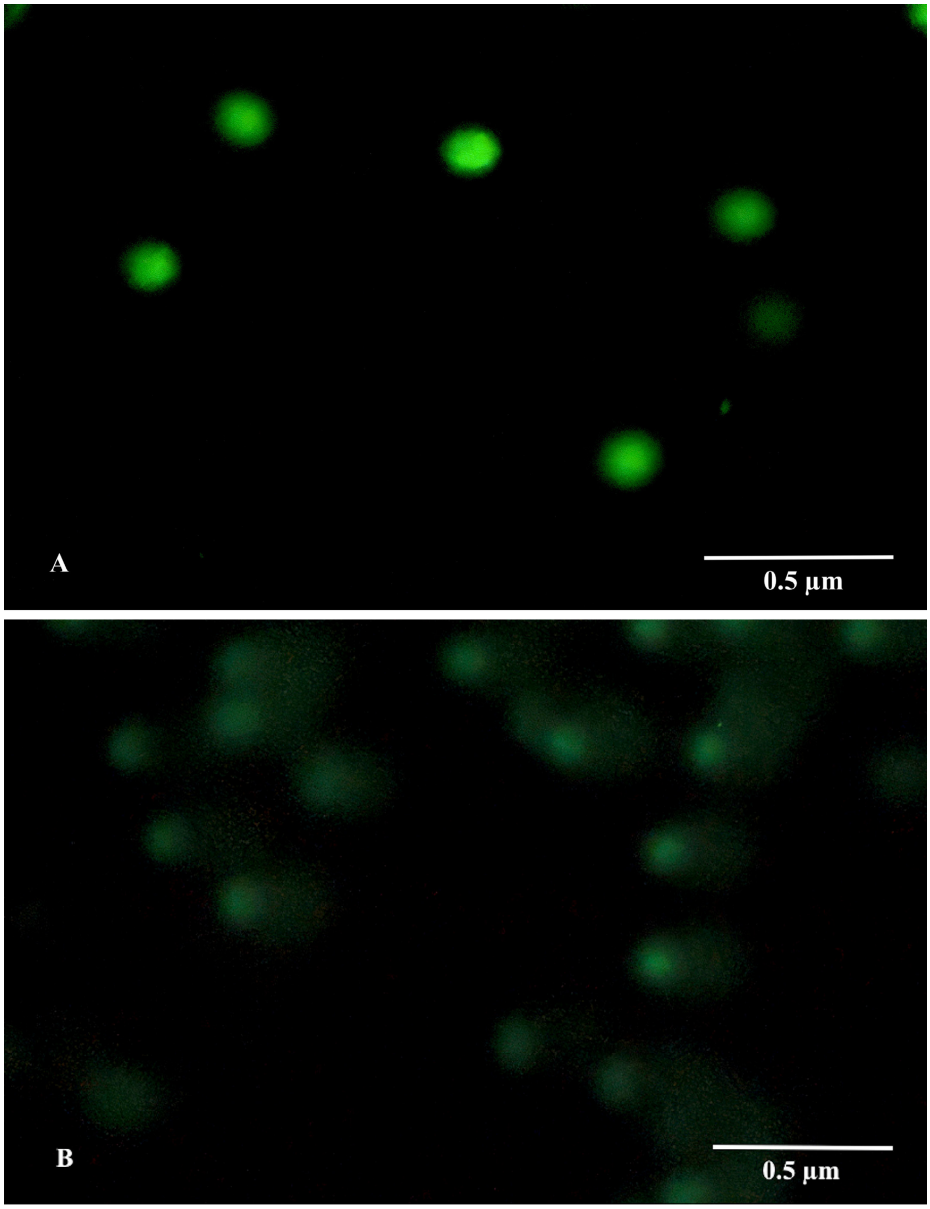

Figure 1. Comet assay of the spermatozoa of *Lithobates catesbeianus* from the control group (A) injected with 0.9% NaCl and the group (B) injected with 50 mg/kg bisazir (200x).

The control group injected with 0.9% NaCl had a mean score ± SD of % DNA in the sperm head of 93.58 ± 1.10%. American bullfrogs injected with 12.5, 25 and 50 mg/kg showed a mean score ± SD of 90.79 ± 4.34%, 89.89 ± 3.41% and 78.19 ± 1.02% respectively (Figure 2).

Statistical analysis of the data revealed an overall significant difference between the groups ($p < 0.0001$). Moreover, a significant difference was found in the % DNA in the head of the control group versus the 50 mg/kg bisazir treated group ($p < 0.0001$), between 12.5 mg/kg and 50 mg/kg ($p < 0.0001$) and 25 mg/kg versus 50 mg/kg ($p = 0.0001$).

The ecotoxicity effect values are resumed in Table 2. The sensitivity of the organisms for bisazir is variable: algae were not affected by concentrations up to 100 mg/l, acute effects were seen in Microtox®, *Daphnia* and *Lemna* with EC_{50} values of respectively 40, 74 and 89 mg/l. The lowest effect value

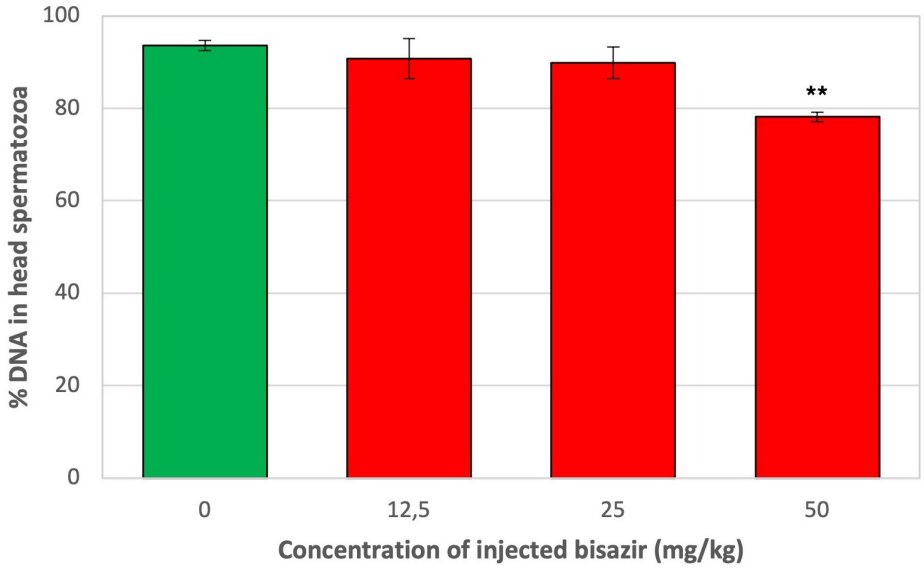

Figure 2. Mean percentage DNA in the head of the spermatozoa at different concentrations of injected bisazir (** $p < 0.0001$). The error bars display the ± SD. Zero mg/kg bisazir (n = 4), 12.5 mg/kg (n = 5), 25 mg/kg (n = 3), 50 mg/kg (n = 4).

Table 2. Summary of the ecotoxicity effect values: EC_{50} (median effective concentration), NOEC (no observed effect concentration), LOEC (lowest observed effect concentration).

Test	Effect value (mg/l)
Microtox (30')	EC_{50} = 40
Algae growth rate inhibition (72 h)	EC_{50} > 100
Lemna growth inhibition (7 d)	EC_{50} = 89 NOEC = 25 LOEC = 50
Daphnia immobility test (48 h)	EC_{50} = 74
Daphnia reproduction test (21 d)	NOEC = 1 LOEC = 2

was the no observed effect concentration (NOEC) value in the *Daphnia* reproduction test (1 mg/l). According to the EU risk assessment methodology (European Commission 2003) the predicted no effect concentration (PNEC; safe environmental concentration for lifetime exposure) based on this data set (assessment factor 50) is 20 µg/l.

Discussion

Due to a high stress level, adult wild caught bullfrogs are hard to keep in captivity. Ten of the 26 animals died before the comet assays tests could be performed, among them all who were injected with 100 mg/kg. The question rises if the deaths were caused by stress or the high concentration of injected bisazir. The response is inconclusive because all the animals that were injected with 100 mg/kg bisazir died, but none who got 50 mg/kg and then again four who were treated with 25 mg/kg (Table 1). Since none of the animals injected with 100 mg/kg bisazir survived this concentration is probably too high and consequently too toxic for American bullfrogs.

Although only a limited number of individuals could be used, this experiment shows that American bullfrogs injected with 50 mg/kg bisazir

have a statistically significant difference in percentage of DNA in the head and tail of the spermatozoa compared to the control group and American bullfrogs injected with 12.5 or 25 mg/kg. These preliminary results imply that the sperm is fragmented, and sterility is induced with an injection of 50 mg/kg bisazir.

The question rises if this DNA damage is sufficient to induce 100% sterility in bullfrogs in order to use the organisms in a sterile-male-release program to control the widely spread exotic invasive populations. In humans, DNA fragmentation detected through an alkalic comet assay is reported to be responsible for a decrease in fertilisation rate and it showed a strong negative relationship with embryo quality (Simon et al. 2011). Future research on amphibians in this matter is needed.

The results in the ecotoxicity test battery show that bisazir has inherent ecotoxic properties. The compound is genotoxic and the ecotoxicity data presented here show that bisazir is also toxic for the aquatic environment with long term effects at rather low concentrations (1–2 mg/l) with derived PNEC value as low as 20 µg/l. This implies that the environmental risk for bisazir must be defined before it can be used in the field.

Data are needed to model the fate and behaviour in the environment of bisazir. Toxicokinetic data on bisazir are however scarce and it is not clear how residual bisazir and/or its metabolites are released from treated organisms to the aquatic ecosystem during their life and during decomposition after death. It is yet unsure if bisazir is metabolised in the living animal and is excreted as metabolites or as original molecule. The elimination of ^{14}C-residues in adult sea lamprey was investigated and showed that most radioactive residues that remained after 48 h in the injected animals were tissue-bound and evenly distributed in subcellular fractions of the liver. It was not clear if it was bisazir or its metabolites that were found in the liver (Allen and Dawson 1987). Some preliminary results indicate that the compound is not really biodegradable. K_{ow} values predict low bioaccumulating potential.

The ecological risk of this chemical needs to be further documented by investigating the exposure (toxicokinetics, the biodegradation pathways and bioaccumulation potential in the aquatic environment, adsorption behaviour…). When relevant the acute and chronic toxicity of possible residues and metabolites need to be established and the safe values for unwanted genotoxic effects need to be documented.

Taking into account the inherent properties of bisazir it is important to assess extensively the long-term implications of metabolites and residues to prevent unexpected and unwanted environmental risks when the product is used in the field. Furthermore, Europe has set out a legislation (European parliament and Council of the European Union 2009) on the sustainable use of pesticides where the use in aquatic ecosystems must be reduced and possibly banned. Therefore, alternatives should be investigated to avoid chemical control of invasive species, especially in aquatic habitats.

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Authors' contribution

S.D. designed and performed the experiments and analysed the data. S.D. wrote the manuscript in consultation with A.D.V.

Ethics and permits

All applicable international, national and institutional guidelines for the care and use of laboratory animals were followed (approval number 201024). Supervision was performed by the Ethical Commission of Hasselt University and the Flemish Authority on Animal welfare.

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