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# Current status and future prognosis of malaria vector-control pesticide ecotoxicology and *Xenopus* sp.

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# Table of contents

## Problem statement

Whilst frogs are generally considered as good indicators of environmental change, they are not widely utilised as indicators in ecological studies. With much of current research relying on model organisms as proxies for other animals or even humans, toxicology data on these model organisms is important for higher tier risk assessment studies and ecological modelling. The issue at hand is that for some topics, even when looking at model organisms, this empirical data does not seem to be as common as expected when doing literature research. Malaria vector control (MVC) is often a sensitive subject due to the paradox surrounding it (Bouwman et al. 2011). The lack of toxicological data mentioned feeds into the issues complicating debates on the chemical vector control interventions. Although being in use for decades, there are still many unknowns regarding the environmental impact of these pesticides. Informed decision making and policies require that this information first be available. The main research problem investigated in this review is this lack of toxicological and biomonitoring data available for one of the model organisms (*Xenopus laevis*) representative of aquatic ecosystems, and to a lesser extent, anurans. Species from the *Xenopus* genus are endemic to Africa and their distribution includes many areas where malaria vector control is applied. Reliable toxicology data on these frogs regarding malaria vector control pesticides (MVCPs) would strengthen the use of these animals as bio-indicators.

## Introduction

The global fight against malaria, nature's most prolific killer, is largely structured around vector control (i.e. controlling mosquito populations). This entails the use of various methods including biological control through the use of larvivorous fish, the efficacy of which has not yet been validated (Walshe et al. 2017). But, for the most part, vector control globally relies on chemical control through the use of various insecticides. These MVCPs include persistent and non-persistent compounds from various pesticide classes. Pesticides are used individually or in combination and through different application methods. The most common and effective chemical methods of intervention are indoor residual spraying (IRS) and insecticide treated nets (ITNs) (Glunt et al. 2013). The World Health Organisation (WHO) Global Malaria Programme lists 12 recommended insecticides for vector control (Table 1). These insecticides belong to four chemical groups: organochlorines, pyrethroids, organophosphates, and carbamates (WHO 2006). The use of these pesticides also varies between countries based on policy. Recently there has been a global decline in the use of pesticides for IRS, partly as efforts to reduce selection resistance in mosquitoes (Alonso and Noor 2017). Efforts to find alternative

insecticides started in the late 1960's to early 1970's. Driven mainly by resistance in mosquitoes and subsequently by the secondary environmental effects of some pesticides such as DDT. The first official effort by the WHO to explore alternative insecticides was at the International Conference on Alternative Insecticides for Vector Control in 1971 (WHO, 1971). Here it was stated that ideal insecticides should be of low toxicity to mammals, and high toxicity to insects, however not much focus was drawn to the effects on lower vertebrates. Acetylcholinesterase acting insecticides such as carbamates and organophosphates, received major attention as the first alternatives, along with pyrethroids. The development of new synthetic pyrethroids introduced this group as a promising alternative. The pyrethroids currently recommended by the WHO for malaria vector control are considered the more advanced synthetic pyrethroids able to show better photo-stability and potency towards mosquitoes than the natural pyrethroids (pyrethrin I and II; CAS: 121-21-1 and CAS: 121-29-9). The chirality of pyrethroids play a crucial role in their activity and stability. The ability to separate single isomers allowed for more targeted efficacy such as with bioresmethrin (CAS: 28434-01-7), the 1R-*trans* isomer of resmethrin (CAS: 10453-86-8), showing very low toxicity to mammals, yet equal efficacy against insects to the parent resmethrin (Knaak et al. 2012). The addition of the a-cyano group in the development of cypermethrin (CAS: 67375-30-8) and deltamethrin (CAS: 52918-63-5) greatly increased photo-stability of these insecticides (Knaak et al. 2012).

Regardless of efforts made to improve on vector control insecticides since the1970's, a "golden bullet" that is highly toxic to mosquitoes, is persistent in its effect on insects, has no resistance build-up in mosquito populations, and with zero to minimal non-target effects, has not been discovered. The nature of pesticides and some application processes used, such as spraying a water-based solution, causes relatively easy transfer to surrounding environments, especially aquatic environments that tend to act as sinks for accumulation of chemical pollution (Van der Oost et al. 2003). Lambert (2001) reported on amphibian deaths in Africa with regard to pesticide usage. Although pesticides may not be the only factors involved, some of the reported deaths occurred even after lower application rates than those recommended for MVC by the WHO (Table 1). The principle of introducing neurotoxic chemical agents to any ecosystem (aquatic or terrestrial) on a regular basis inevitably results in a potential risk to non-target organisms in that ecosystem. To assess these potential effects, model organisms are often used. This review discusses anurans from the genus *Xenopus*, with particular focus on *Xenopus laevis* (African clawed frog) and its use as a model organism in research regarding MVCPs around the world.

## History of *Xenopus* use in research

*Xenopus laevis*, the frog species most commonly used in research, is endemic to southern Africa. Starting in the late 1930s, *X. laevis* was distributed to laboratories worldwide, since they served as the most reliable pregnancy test until the 1960s (Gurdon and Hopwood 2000). With laboratory housing conditions becoming refined, and *X. laevis* being distributed to laboratories around the world, it quickly became a model for scientific teaching and research. Its use in pregnancy testing relied on ovulation being induced in the female frogs, which paved the way for harvesting and artificial insemination of the ova for regulated breeding. Nieuwkoop and Faber (1994) studied the development of *X. laevis* in great detail. They produced a normal development table for 66 stages of *X. laevis* development from fertilization to adulthood, first published in 1956, with a final refined version in 1994. This opened the door for the use of *X. laevis* as a model for frog development studies.

The immune system of *Xenopus* changes drastically as it develops (Robert and Ohta 2009), which may cause sensitivity to the teratogenic effects of compounds they are exposed to, or even increase susceptibility to secondary infection (Mann et al. 2009). *Xenopus laevis* tadpoles are filter feeders, which increases their uptake of chemicals in the water column. Dumont et al. (1983) used the Nieuwkoop-Faber developmental staging model and developed a standardised test called the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) (updated document: ASTM 1998), which allows for the measurement of developmental toxicity at different degrees through mortality, morphological changes, and growth rate. All the measured morphological changes, and the severity thereof, were categorized by Hu et al. (2015). Most of the available data produced on amphibian ecotoxicology regarding this species is based on FETAX.

The validity of *X. laevis* as a model frog can be questioned, besides its use in FETAX. Two arguments against its use as a model are, firstly, that it is a fully aquatic species and therefore is not necessarily representative of anurans as a whole. Secondly, adult *X. laevis* are known as being hardy and can tolerate a wider range of physicochemical water parameters and, in some cases, toxicants as well, compared to other anurans. The basis of FETAX, however, is the sensitivity of the embryos and tadpoles. This indicates that the life-stage examined should be considered carefully when *X. laevis* is used as test model. The context in which a study is done can also validate the use of the species, as members of the *Xenopus* genus can be seen as good representatives of freshwater organisms in general. This is based on the mid-level trophic position they hold in aquatic ecosystems (Lindholm et al. 2007; Wolmarans et al. 2015; Dalu et al. 2016). Their permeable skin can lead to increased dermal uptake, which also makes them good models for bioaccumulation studies. Increased uptake plays a large role when investigating toxicants with high affinity to sediments. These frogs tend to dwell in the sediment with their highly permeable belly skin exposed for extended periods. They also eat by stuffing food into their mouths

and ingesting it by means of a hyobranchial pump (Avila and Frye 1978), increasing the likelihood of sediment ingestion and subsequent chemical exposure. *Xenopus* laevis tadpoles are filter feeders, which also increases sediment ingestion throughout their development. Dalu et al. (2016) showed, through means of stable isotope ratio analysis, that sediments along with coarse particulate organic matter, contribute quite substantially to the diet of *X. laevis* tadpoles in ephemeral ponds, with sediment ranging between 1 and 33% and coarse particulate organic matter up to 40.1% of their diet.

The relative ease of housing, artificially harvesting and fertilizing eggs, and reproducing these organisms in a laboratory setting, as described by ASTM (2012), has also played a role in the continued use of this species. The introduction of *X. tropicalis* (*Silurana tropicalis* in some literature) as a replacement for *X. laevis* in some research studies in the late 1990s came about largely due the tetraploidy of *X. laevis*, which hindered research in genetic and gene expression fields (Burggren and Warburton 2007). *Xenopus tropicalis* is a diploid and has a faster development timeframe (≈ six months to adulthood vs ≈ 12 months for *X. laevis*), and can also be used effectively with FETAX (Hu et al. 2015). Regardless of the use of *Xenopus* sp. in other types of studies, the FETAX model is still considered one of the primary assays to be used for testing teratogenic effects of compounds on lower vertebrate animals. Bearing this information in mind, it is interesting to note that the number of publications produced using *Xenopus* spp. as a model for pesticide research is quite low (Table 2). Although Boolean search systems (search system use is explained under availability of literature) for locating electronically-available publications in online databases do not necessarily provide an accurate number of publications available on a subject, it gives a good indication of whether the topic is popularly explored by scientists around the world.

## Scope of the review

In this review we summarize the literature available on *Xenopus* sp. in relation to the 12 WHO-recommended MVCPs in terms of acute, sub-lethal, and chronic toxicity. As supplementary information (Figure 1, and Figure 2), these data were (evaluated using used in meta-analyses for theoretical relative toxicity of the different pesticides to *Xenopus* sp. in order to pinpoint which pesticides might require more focus for future research. This review serves to bring to attention the lack of available literature in this field and the inconsistency of results between some of the existing records. We also identify gaps in the current knowledge, in order to promote more focussed future research in this field. Zippel and Mendelson (2008) proposed certain challenges to the global amphibian crisis, one of which was directly aimed at environmental contamination in generating

data on sub-lethal and chronic effects. Now, a decade later, we hope that this review can stress the lack of progress made in this regard in the context of the pesticides in question.

## Data inclusion methods and criteria

Search queries were performed on Scopus, Web of Science, and other online databases using selected keywords (Scopus search outcomes given in Table 2) pertaining to MCVPs and *Xenopus*. The US-EPA ECOTOX database was also queried, with Pauli et al. (2000) as secondary sources. Some criteria were necessary in the filtering of data used for this review. To be acceptable for inclusion, a study had to report on *Xenopus* sp. in relation to one or more of the 12 WHO-recommended malaria vector control pesticides listed in Table 1. Studies reporting toxicological effects without any corresponding reference as to exposure amount or biological load were excluded. Laboratory and field-generated data were included. As dichlorodiphenyltrichloroethane (DDT; CAS: 8017-34-3), one of the pesticides included, is considered a persistent organic pollutant (POP), studies reporting only bioaccumulation data were also included. The main breakdown products of DDT, Dichlorodiphenyldichloroethane (DDD; CAS: 72-54-8) and Dichlorodiphenyldichloroethylene (DDE; CAS:72-55-9), can be even more persistent in the environment and are therefore indirectly introduced via DDT introduction. Studies reporting sub-lethal effects of these compounds were also included in the study. The majority of studies within search parameters that were excluded from this review, involved transgenic *Xenopus* oocytes used merely as a medium in which to analyse the reaction of transplanted genes to pesticides. If such studies did not report effects relatable to *Xenopus* sp. itself, they were excluded from this review.

## Availability of literature

Amphibian ecotoxicology is a research topic that has increased in popularity over the last decade, a trend also seen in literature included in this review. A Scopus database query performed in August 2018 using the search phrase “anura AND pesticide” in the field “Abstract, title and keywords” returned 395 entries. When the more refined search term “*Xenopus* AND insecticide” was used 267 entries were given. Replacing “insecticide” with the names of the 12 WHO-recommended MVCPs, the highest output was for “*Xenopus* AND deltamethrin” with 47 entries. Whilst this number may seem high, only eight of these publications fitted the criteria for inclusion in this review (See search results provided in Table 2). Through individual pesticide and *Xenopus* combination searches, 135 entries were given in total, of which only 46 entries fitted the criteria for inclusion in this review.

Globally, amphibians are widely distributed, with their highest species density being found in tropical and sub-tropical areas (FEOW 2008). These are also the regions with temperatures suitable for transmission of *Plasmodium* sp. throughout the world (MAP 2018), yet there seems to be a lack of literature on amphibians — especially from tropical and sub-tropical regions — and on ecotoxicology with regard to MVCPs. With overlap in pesticide use between MVC, vector control for other tropical diseases and agriculture, the need for data from species relevant to these tropical and subtropical systems becomes clear. In the case of Africa, *Xenopus* sp. is a good starting point for collecting relevant anuran data.

## Summary and discussion of reviewed literature

### FETAX studies

With FETAX long being the most commonly used teratogenic toxicity model, it would be expected that most available literature on *Xenopus* and MVCP toxicity would originate from FETAX studies. Another advantage of this type of data is comparability, as FETAX allows for a standardized testing method with standardized endpoints. This is not really the case with MVCPs, though the literature available is much scarcer than expected, lowering the confidence of comparisons between data.

FETAX data relating to MVCPs available in literature are summarized in Table 3. Data relating to only three of the 12 WHO-recommended MVCPs could be found, namely DDT, deltamethrin, and malathion (CAS: 121-75-5).

#### Organochlorines

Saka (2004) analysed teratogenic effects through FETAX for three major DDT metabolites. For the *para-para*-isomers of DDT, DDD, and DDE assayed, the results showed sensitivity of *X. laevis* both in terms of mortality and malformations in the order of *p,p*-DDD > *p,p*-DDT > *p,p*-DDE, which happens to be the inverse of their expected half-lives in the environment. The LC50 of *p,p*-DDT was 35.8 mg/L with the EC50 at 14.71 mg/L. The LOEC for malformations was 6.24 mg/L, but the minimum concentration to inhibit growth (MCIG) was 1.5 mg/L. This places the MCIG/LC50 ratio at 0.042. The teratogenic index (TI = LC50/EC50) value for *p,p*-DDT was 2.4. As a standard in FETAX, any compound with a MCIG/LC50 ratio lower than 0.3 and a TI above 1.5 is considered teratogenic.

#### Pyrethroids

Teratogenicity of deltamethrin was analysed by Channing (1998), who reported an LC50 and EC50 of 0.19 mg/L and 0.006 mg/L, respectively, with a resulting TI of 31. Even though Channing (1998) did not report the MCIG or LOEC a TI value 20 times higher than the 1.5 cut-off means that deltamethrin is certainly regarded as teratogenic.

**Organophosphates**

Malathion was the only compound for which FETAX results were reported from multiple sources. Snawder and Chambers (1989) showed malathion to have an LC50 of 10.9 mg/L. They reported the EC50 in terms of specific malformations, the lowest of which was an abnormal pigmentation EC50 of 0.33 mg/L, and the highest an abnormal notochord EC50 of 2.16 mg/L. Bonfanti et al. (2004) did not expose tadpoles to a high enough range of malathion to calculate an LC50, with their highest concentration of 6 mg/L showing mortality of only 12%. They did, however, report an EC50 of 2.39 mg/L for total malformations. This value is similar to the notochord abnormality EC50 of Snawder and Chambers (1989), as Bofanti et al. (2004) reported the malformations observed to consist mostly of abnormal tail flexure, which is relatable to abnormalities of the notochord.

Neither Channing (1998) nor Snawder and Chambers (1989) reported a lowest-observed-effects concentration (LOEC) or minimum concentration to inhibit growth (MCIG) to compare with results from Saka (2004).

Technical DDT consists of about 85% *p,p*-DDT and about 15% *o,p*-DDT (ATSDR, 2002) with variable traces of other forms such as *p,p*- and *o,p*- DDD, and sometimes *o,o*-DDT. The majority of risk from direct exposure is therefore related to *p,p*-DDT. Hence, in analysing initial toxicity from MVC application, acute toxicity and teratogenic risk to *Xenopus* embryos, when exposed to the equal amounts of each MCVP, would be greatest from deltamethrin n, followed by malathion, and lastly DDT. However, these compounds are not necessarily used in equal amounts during MVC. With DDT being banned under the Stockholm convention (UNEP 2009) from other forms of use in most countries, it also carries the lowest risk of being used for other purposes in the environment. Malathion and deltamethrin are, however, actively used in agriculture, in turn increasing risk to non-target organisms, including *X. laevis*, in aquatic environments. Deltamethrin further has the highest potential to cause developmental malformations at concentrations that would not cause significant mortality, based on its high TI value.

Several other studies included in this review followed the FETAX protocol in terms of exposure, but reported on different endpoints to that of the standardized FETAX format, as in the previously-described studies. These

studies were therefore included in the acute toxicity or sub-lethal and chronic toxicity data collections (Tables 4 or 5), based on exposure duration and endpoints measured.

## Acute toxicity

Given greater variability in methods, age of animals and report structure outside the FETAX parameters, there were a few more acute toxicity studies that met the requirements for inclusion in this review. Acute toxicity records could be found only for deltamethrin, cyhalothrin (CAS: 91465-08-6), cypermethrin, fenitrothion (CAS: 122-14-5), and malathion (Table 4).

### Pyrethroids

Aydin-Sinan et al. (2012) used a modified FETAX protocol to expose *X. laevis* Nieuwkoop-Faber (NF) stage 46 tadpoles to deltamethrin in a commercial formulation. The concentration range of deltamethrin (1.1 – 38.4 µg AI/L) was too low to induce 50% mortality at 96 h. Maximum concentration induced 21.7% mortality, which is within reasonable expectations compared to the 190 µg/L LC50 (also 96 h) reported by Channing (1998) on embryos using the FETAX method. A 168 h LC50 reported by Aydin-Sinan et al. (2012) 6.26 µg AI/L.

Aydin-Sinan et al. (2012) also exposed *X. laevis* to $\lambda$-cyhalothrin in commercial formulation which showed greater toxicity than deltamethrin. The 96 h exposure also did not induce 50% mortality, but at 10 µg AI/L as the maximum concentration, 46.7% of the test population died. The 168 h LC50 was reported as 3.94 µg AI/L.

Yu et al. (2013) analysed the toxicity of $\alpha$-cypermethrin to *X. laevis*. Their study showed *X. laevis* to be one of the most sensitive anurans to $\alpha$–cypermethrin based on literature comparisons, and it was suggested as a useful species for studying the effects of pyrethroids. Very little literature on other anurans was available for comparison at the time of publication, which should be taken into account. The LC50 for *X. laevis* to $\alpha$-cypermethrin was recorded as 30.6 (CI 27.1-34.7) µg/L and 6.9 (CI 5.7-8.5) µg/L for embryos and free swimming larvae, respectively, making $\alpha$–cypermethrin more toxic to free-swimming larvae. This result brings into question the use of a single method such as FETAX as a definitive sensitivity test for these compounds to anurans. Incorporation of other life-stages seems necessary for a holistic approach to assess the effect these chemicals have on anuran populations and community structure. Publications by Yu et al. (2013, 2015b) were the only records of $\alpha$-cypermethrin acute toxicity testing on *Xenopus* available for the present review. Yu et al. (2015b) investigated combination effects of simultaneous exposure to $\alpha$-cypermethrin and ultraviolet-B radiation (UVB). The addition of UVB (intensity of 22-28 uW/cm2) showed increased mortality, but this was

mostly attributed to the toxic effects of UVB itself and no discernible relationship between pesticide-caused mortality and UVB-caused mortality could be distinguished. The results did however indicate a synergistic effect between the two in terms of sub-lethal effects such as growth and malformations.

**Organophosphates**

The only study on acute toxicity listed in Pauli et al. (2000) involving *Xenopus* and an MVCP was Elliot-Feeley and Armstrong (1982) involving *X. laevis* and fenitrothion. This was also the only existing acute toxicity record of fenitrothion on *Xenopus* we could find. The authors exposed *X. laevis* embryos at gastrulation phase to a range of concentrations (0.1-10 mg/L) of fenitrothion for 24h at various temperatures. The results showed an LC50 above 10 mg/L at 18°C (8% mortality at 10 mg/L) and 25°C, and an LC50 of 0.33 mg/L at 30°C. At 18°C, when exposed to a saturated solution (estimated at 30 mg/L), mortality increased to 89%, which places the theoretical LC50 at 18°C somewhere between 10 and 30 mg/L. The surviving embryos were raised to NF stage 37 and morphological changes recorded. The authors classed tadpoles based on the severity of morphological change, which showed increased severity corresponding to increased concentration. Significant differences in morphology were observed at 1 mg/L (at 18°C), while increased mortality from the control was measured at only 10 mg/L. Sensitivity tests were also conducted with 10 mg/L exposures at 25°C which indicated the highest mortality when exposed during stage 11-15, but with the highest incidence of morphological changes when exposed at stage 8-11. The sensitivity differences in terms of mortality at different developmental stages once again (as with $\alpha$-cypermethrin) seems to indicate that a single life-stage exposure is inadequate to determine realistic toxicity information when working with anurans.

Yu et al. (2013) also investigated the toxic effects of several pesticides, including malathion, following the FETAX method, except for a few modifications such as not de-jellying the eggs. This provided a more realistic exposure scenario, but excluded this study from outright comparison to other FETAX studies. Other parameters they adjusted were the mixing of egg clutches, larger test volumes, and tadpoles were considered dead when they did not respond to prodding rather than when they had no heartbeat (as described in the latest version of FETAX; ATSDR 2012). The results from Yu et al. (2013) showed malathion as having an LC50 of 5.4 (95%CI 4.9-6.0) mg/L and 6.7 (95%CI 6.1-7.5) mg/L for stage-8-11 and stage-46 larvae, respectively. This is lower than the values measured by Snawder and Chambers (1989) and Bonfanti et al. (2004), which the authors ascribed to their alterations on the FETAX protocol. In Yu et al. (2013), malathion showed less acute toxicity (endpoint: mortality) to free-swimming larvae (NF stage 46) than to embryos (NF stages 8-11) with similar sensitivity

indicated by growth and malformation endpoints. The authors also compared the sensitivity of *X. laevis* larvae to values from the literature on other amphibian species with *X. laevis* showing median sensitivity to malathion. Yu et al. (2015b) also investigated additive effects of UVB radiation to malathion toxicity, but with no discernible interaction in terms of mortality. However at lower concentrations the addition of UVB significantly increased the incidence of malformations such as abnormal tail tip and gut observed in conjunction with malathion exposure. Edema incidence, caused by UVB alone, was reduced in combined exposure with higher concentrations of malathion.

## Relative toxicity of MVCPs towards *Xenopus*

In order to evaluate the sensitivity of *Xenopus* sp. (data only available for *X. laevis* as representative of *Xenopus* sp.) to MVCPs, we analysed the acute toxicity data above to rank the 12 WHO MVCPs based on their relative potency towards *Xenopus* sp. We utilise the term potency in a generic manner to indicate comparative toxicity towards *Xenopus* sp. rather than terms such as hazard or risk since in the context of risk assessment these terms would imply so form of known relationship between exposure and effect. By plotting the available LC50 values for each pesticide vs the recommended manufacturer's application rate ($m^2$) we derived course relative MVCP potency categories (Figure 1). High potency towards *Xenopus* sp. relates to a high toxicity (i.e. low LC50 value) and a high application rate, and conversely the lowest application rate and highest LC50 value would result in the lowest potency. The boundary between high and low potency was derived by the combination of highest application rate between all compounds and highest LC50 between all compounds as the best case for toxicity and worst case for application. This combination then provides a separation point for compounds having worse or better combination outcomes than the combination of worst and best case scenarios. Any points below this line are considered moderate to high risk, while any points above are considered low to very low risk. Zero risk cannot be used as a predicted value if application is a non-zero value. Based on these combinations, relative potency values were calculated using the following formula: Log((average dose x 1/mean LC50)+1) (Figure 2).

The use of cyhalothrin for MVC theoretically contributes the greatest acute toxicity potency to *Xenopus* sp. in a natural setting during MVC application out of all the MVCPs for which toxicity data is available. Fenotrothin, deltamethrin and malathion also have high relative potency while DDT has a moderate relative potency. *α*-Cypermethrin poses the lowest risk for acute toxicity to *Xenopus* sp. out of the possible MVCPs. The lack of acute toxicity data for bendiocarb (CAS: 22781-23-3), bifentrhin (CAS: 82657-04-3), cyfluthrin (CAS: 68359-

37-5), etofenprox (CAS: 80844-07-1), pirimiphos-methyl (CAS: 29232-93-7) and propoxur (CAS: 114-26-1) means that 50% of the WHO MVCPs could not be evaluated in this manner.

It should be stressed that there are specific limitations to the use of the relative potency values calculated in this manner. Firstly factors such as frequency of use, distance from spraying, temperature, wind, soil composition, runoff from precipitation, photolysis, and other forms of degradation will all affect the extent to which these animals are essentially exposed in the wild. These factors are not included as most of them are going to be scenario specific and using theoretical values can provide even more misleading results. The calculations performed in this review merely compare the potential to be introduced in a broad sense vs the potential to have a toxic effect on these animals. This provides comparative potency between the pesticides discussed in this review only. The lack of toxicity data on many of the discussed pesticides also limits the interpretation of these results in the wider context of all 12 WHO recommended MVCPs. In fact these limitations on the meta-analysis already provides insight into the issues regarding lack of data in this field. That being said, the comparisons provided between these pesticides from the relative potency values provides rationale as to prioritising pesticides for future research.

Pyrethroids are the compound group that contribute the greatest relative potency to *Xenopus* sp. in terms of having the potential to cause frog mortality in quantities used for MVC in Africa (Figure 2). This is due to the high sensitivity *Xenopus* has towards these compounds compared to other MVCPs. Even with DDT (organochlorine) application rate 26 times higher than the mean application rate of the pyrethroids, the mean relative potency of the three pyrethroids analysed is still higher than that of DDT. Based on application rates and sensitivity we can discern that $\lambda$-cyhalothrin holds the greatest potential for acute toxicity to *Xenopus* sp. followed by deltamethrin. However, all the toxicity tests included were not conducted under the exact same conditions, lowering the confidence of such a conclusion.

The use of multiple life-stages in anuran acute toxicity testing plays an important role in amphibian ecotoxicology as the most sensitive life-stage was not consistent throughout reviewed studies where both embryo and larval stages were used. The most sensitive life-stage appears to be compound-specific and may be related to the specific mechanism of action of each pesticide. Another issue already touched on with the available toxicity data is the lack of structure. Currently three internationally standardised tests for the *Xenopus* model are available. The Organisation for Economic Co-operation and Development (OECD) chemical safety testing protocol No. 231: Amphibian metamorphosis assay (AMA), No. 241: Larval amphibian growth, and

development assay (LAGDA), and FETAX. The AMA is to some extent a 21-day variation of FETAX and focuses only on the thyroid axis effects compounds can have that affect the metamorphoses of frogs. It can be applied to different anurans, but was also specifically designed around *X. laevis*, like FETAX. The LAGDA stemmed from the AMA and is considered a more comprehensive toxicity analysis enabling measurement of endocrine disruption through various pathways. The issue with using either of these assays for other species comes down to the developmental staging of the frogs. General frog development stages, as defined by Gosner (1960), has less detail than NF staging (Nieuwkoop and Faber 1991) for *X. laevis*, which is what makes the generalisation possible, but limits quantification during assays, and comparability to NF staged studies. The aquatic-terrestrial range of amphibians also provides complications as not all amphibians can be immersed continuously in exposure media, some species will die if they have prolonged contact with water due to unique osmoregulation adaptations. This is the same uniqueness that makes frogs so habitat-specific. Studying frog ecotoxicology in a laboratory is one thing, but relating those results back to real world exposure scenarios raises complications in itself between species and the respective habitats they inhabit.

The lack of acute toxicity data for WHO-recommended pesticides on a standard model organism is alarming. As *Xenopus* is not the only standard testing frog species in the world, it is fitting also to compare literature on other amphibian species. Species sensitivity distributions (SSDs) were compiled from the LC50 values discussed in this review and US-EPA ECOTOX database results on amphibians in relation to the 12 WHO MVCPs in question. Figure 3 shows an SSD of amphibians to malathion. LC50 data for studies ranging between 48 h and 96 h was included in the analysis. Data fitting these criteria were found for nine amphibian species. *Xenopus laevis* is shown to be the second least susceptible species in this regard. SSDs were also calculated for Deltamethrin, DDT and cypermethrin in the same manner (data not shown), however there were less than seven data points available for each of these pesticides, increasing the error margin severely. Both DDT and deltamethrin showed *X. laevis* to be the least susceptible species, but for cypermethrin *X. laevis* was the second most susceptible species, with only the Asian common toad *Duttaphrynus melanostictus* having a lower LC50. Interestingly, in terms of cypermethrin, *X. laevis* is thus a relatively sensitive anuran, but this pesticide was shown to have the lowest relative potency value to *X. laevis*. Conversely, malathion and deltamethrin had fairly high potency values compared to other MVCPs, but showed *X. laevis* as tolerant, compared to other frog species. This indicates an inflated potency towards most other frog species for these two compounds. Exposure scenarios for other species would differ based on their preferred habitats, which may lower the realistic risk posed by these compounds. The lack of data on other anuran species is also alarming as it indicates *Xenopus*

species aren't the only frogs neglected in this topic of research. Larger and more relevant exposure and sensitivity datasets are required in order to be able to properly investigate risks these pesticides pose to frogs. Realistic (or simulated-realism) exposure-response, and site specific application-risk investigations into all MVCPs, not only for *X.laevis* but also other frog species, seems to be a logical and crucial next step for amphibian ecotoxicology research and conservation in MVC regions. Of all the MVCPs with data available, the compound with the highest relative potency towards *X. laevis* was *λ*-cyhalothrin. The only other publication on acute toxicity to frogs for this compound in the ECOTOX database is for the Bog Frog *Fejervarya limnocharis*; previously *Rana limnocharis*, which showed a 48 h LC50 of 4 µg/L (Pan and Liang 1993), compared to the 3.97 µg/L LC50 for *X. laevis* only after 3.5 times longer exposure. The lack of comparable toxicity data with regard to MVCPs and frogs in general raises the question of how we can be sure that the current use of pesticides for MVC, excluding agricultural use, is not causing permanent damage to frog populations in malaria risk regions.

It is unlikely that mass mortalities of frog populations due to MVC may have gone unnoticed, but even if exposure does not result in mortality, it is conceivable that sub-lethal effects may occur. In the next section we summarize literature available on sub-lethal and chronic effects of MVCPs on *Xenopus* sp.

## Sub-lethal and chronic effects

### Organochlorines

DDT is a well-known endocrine disrupting compound (EDC) (Hoffmann and Kloas 2016). Palmer and Palmer (1995) investigated the use of plasma vittellogenin as a biomarker for oestrogenic activity in *X. laevis* males exposed to DDT. When exposed to 1 µg/g and 250 µg/g o,p-DDT through injection for 7 days, with blood analysed on day 14, both concentrations showed detectable vitellogenin levels significantly higher than control, and increasing in a dose-dependent manner. Hoffmann and Kloas (2016) studied the EDC effects of DDE on male *X. laevis* through analysing call behaviour. Significant reduction in advertising calls (female-attracting call) were seen at 3.18 ng/L exposure. This reduction, however, was not significant at 318.0 ng/L exposure, which did in turn show a significant increase in the amount of ticking (a type of release call). The manner in which calls were altered by the different concentrations of DDE is, according to the authors, suggestive that both estrogenic and antiandrogenic mode of action can be elicited at different concentrations with lower concentrations showing estrogenic activity and higher concentrations showing antiandrogenic activity.

### Pyrethroids

Apart from acute toxicity, Aydin-Sinan et al. (2012) also analysed a set of biomarker responses to analyse the sub-lethal effects of deltamethrin and $\lambda$-cyhalothrin on *X.laevis*. Both compounds significantly decreased ACP (acid phosphatase) and AST (aspartate aminotransferases) after 24h exposure. Cyhalothrin also significantly decreased CaE (carboxylesterase), and GST (glutathione-*S*-transferase), with LDH (lactate dehydrogenase) being inhibited by the highest concentration (10 µg AI/L). To our knowledge, this publication is the first report on the use of biomarker responses with *Xenopus* sp. in relation to MVCPs.

Rudek and Rozek (1992) exposed *X. laevis* to cypermethrin between 1 and 25 µg/L and found a dose-dependent increase in the occurrence of erythrocytes with micronuclei. The increase was, however, not as severe as in *Rana temporaria*. Martini et al. (2010) exposed *X. laevis* tadpoles to 0.24 µg/L $\alpha$-cypermethrin in order to measure immune responses. Cypermethrin was found to increase the messenger-ribonucleic acid (m-RNA) expression of heat shock protein 70 and interleukin-1β. Both of these play important roles in immune response and alterations to their normal expression could result in a compromised response to influences such as disease. In Yu et al. (2014) the behaviour of tadpoles exposed to pesticides were measured in terms of UVB radiation avoidance. A 0.5 µg/L cypermethrin exposure, however, showed no difference in time spent in or outside of the UVB-exposed area compared to control. A 1 µg/L cypermethrin exposure was excluded from the results as axial malformations interfered with swimming behaviour and subsequently light avoidance behaviour. Yu et al. (2015) exposed *X. laevis* embryos to cypermethrin and UVB simultaneously looking at sub-lethal effects in the form of Deoxyribonucleic acid (DNA) damage. Cyclobutane pyrimidine dimer accumulation, indicating DNA-adducts, increased significantly compared to both control and UVB-only exposure, at 5 µg/L cypermethrin exposure for 96 h with 63.0 mW/m$^2$ UVB introduced for the last seven hours of exposure. The m-RNA expression of genes indicative of DNA damage showed cypermethrin alone down-regulated the xeroderma pigmentosum group A (XPA) gene, and up-regulated the xeroderma pigmentosum group G (XPG) gene, and cockayne syndrome A (CSA) gene that play a role in playing a role in transcription coupled DNA excision repair, and cypermethrin also upregulated expression of the muir-torre syndrome (MUTL) gene which is involved in mismatch repair. In combination with UV, however, most of these effects were nullified, with only XPG up-regulation being enhanced even further. These results indicate that sub-lethal concentrations of cypermethrin can have stress response effects in *X. laevis* and that some of these effects may be enhanced in a natural environment with combined exposure to UVB radiation.

**Organophosphates**

In addition to mortality and developmental malformation, Snawder and Chambers (1989) also investigated the effects of malathion on nicotinamide adenine dinucleotide ($NAD^+$) in the exposed *X. laevis* embryos. They found significant reduction of $NAD^+$ showing a gradual almost linear decline on a log (dose) vs response curve. This dose-related reduction in $NAD^+$ is deemed independent from malformations observed, as other compounds tested showed variation in malformations observed with similar reduction in $NAD^+$. Following these results, Snawder and Chambers published two more studies (Snawder and Chambers 1990, 1993) in which they also exposed *X. laevis* embryos to malathion using the FETAX protocol. In Snawder and Chambers (1990) the effect of tryptophan combined with malathion on the $NAD^+$ levels was tested and, based on malformations observed, it was concluded that tryptophan could effectively combat the $NAD^+$ reduction effects of malathion, but with no resulting change in the malformations observed from malathion exposure. The authors also showed that 4-day old embryos exposed for one day showed similar malformations to those exposed for four days from zero days old, making the fourth day of development the most critical point in development in terms of malformation. Snawder and Chambers (1993) focused on the effects of malathion on collagen and subsequent notochord bending. Ultrastructural examination showed fewer collagen fibres in the notochords of exposed embryos, but quantification of collagen in homogenised embryos did not differ significantly from controls. Malathion was, however, shown to inhibit lysyl oxidase and collagen proline hydroxylase activity in *X. laevis* embryos in a dose-dependent manner, with IC50s of 0.7 nM and 0.58 uM, respectively. The inhibition prevents triple helix collagen from forming and in turn reduces the extracellular collagen fibres, even though total collagen content assays show no reduction.

Webb and Crain (2006) exposed *X. laevis* tadpoles to different concentrations of malathion over 30 days, with 1.0 mg/L showing a significant mortality rate compared to the control, and with more than 50% mortality at the end of the experiment. Webb and Crain (2006) exposed three-week-old tadpoles but the developmental stages were not reported, only that stages did not differ between treatments. This makes comparison difficult. Chemotti et al. (2006) investigated the teratogenic effects of malathion on *X. laevis* embryos using a modified 72 h FETAX method deigned for practical teaching applications. The tadpoles showed significant time-dependent increase in the notochord axis angle over 72 h at 1.0 and 2.5 mg/L malathion exposure, together with a significant reduction in length at 72 h exposure for both concentrations, compared to controls. Yu et al. (2014) also tested UVB avoidance behaviour in relation to malathion exposure in *X. laevis* tadpoles, but neither 1 nor 5 µg/L exposures showed significant differences in behaviour.

### ***In vitro* studies**

Due to *X. laevis*' extensive use in teaching and as a physiology model, many physiological tests have been performed on tissue preparations and cell cultures of these animals, and of their neuromuscular system in particular, in order better to understand the mechanistic action of neurotoxic compounds. Such studies provide useful information with regard to the mechanisms of action of pesticides. These studies are listed in Table 5.

**Organochlorines**

Van den Bercken et al. (1973b) investigated the action potential of muscle nerve fibres of *X. laevis* exposed to 25mg/kg *p,p*-DDT. The poisoning symptoms were described as restlessness and hyper-excitability, which increased in intensity until convulsions occurred, coupled with excessive skin mucus secretion. After euthanasia the action potential was measured on excised muscles. The treated animals showed an increase in the amplitude of negative after-potential. The duration of action potential was also increased in treated animals, attributed to a slower falling phase. Van Den Bercken et al. (1973) investigated the effects of DDT on prepared *X. laevis* lateral line organs at different temperatures. DDT exposure (2-4 mg/L) caused trains of impulse spikes instead of the normal single-impulse spontaneous activity. The number of spikes per train increased at lower temperatures, while the frequency of trains increased at high temperatures. Åhrem and Frankenhaeuser (1974) measured the permeability effects of DDT and its metabolites on *X. laevis* nerve fibres using potential clamping. Effects seen with *Lithobates pipiens* (previously *Rana pipiens*) from DDT on the permeability of $Na^+$ could not be replicated with *X. laevis*. Dichlorodiphenylacetic acid (DDA; CAS: 83-05-6), a minor metabolite of DDT was found to increase the permeability of $K^+$ through the nerve fibres at 4 mg/L. In a study by Åhrem et al. (1974) the acute effects of the same DDT metabolites were investigated on myelinated nerve fibres of *X. laevis*. This publication seems to be, to some extent, a detailed summary of significant results from Åhrem and Frankenhaeuser (1974) and merely confirms that the differences between *X. laevis* and *Lithobates pipiens*, in terms of DDT exposure mentioned in Åhrem and Frankenhaeuser (1974), are in fact due to differences in species response, along with a more detailed description of how DDA affects the $K^+$ membrane potential through interfering with inactivation of the potassium-gated channel. Repetitive firing in the lateral line organ of *X. laevis* caused by DDT exposure was also demonstrated by Akkermans et al. (1975). Vijverberg et al. (1982b) found the mechanism of action of DDT to be related to opening of the sodium channels in *Xenopus* myelinated nerve fibres, with DDT having no major effect on depolarisation of sodium channel, but causing sodium tailing after repolarization. Lutz and Kloas (1999) measured the oestrogen binding activity in primary liver cell cytosol from *X. laevis*. Between seven endogenous steroids and seven exogenous ligands known for oestrogen receptor binding analysed, *p,p*-

DDT had the lowest affinity for oestrogen receptor binding and was the only exogenous ligand with lower binding affinity than the endogenous steroids measured.

**Pyrethroids**

Vijverberg and Van den Bercken (1982) studied the effect of pyrethroids on the lateral line organs of *X. laevis*. Cypermethrin failed to induce repetitive activity within the first 5h in *in vitro* exposed studies. In *in vivo* exposed lateral line organs, however, long lasting trains of nerve impulses were induced after 4h when exposed to $5x10^{-6}$ M cypermethrin. In a study by Vijverberg et al. (1983) on myelinated nerve fibres $Na^{+}$ tail currents were caused by both cypermethrin and deltamethrin with deltamethrin showing the slowest decay of tail currents out of 11 pyrethroids tested. The rate of decay accounted for the differences in repetitive activity induced by the various pyrethroids. Ruigt and Van den Bercken (1986) tested the effects of pyrethroids on end-plate potentials and muscle action potentials in excised pectoralis nerve-muscle preparations of *X. laevis*. At 100 uM cypermethrin failed to induce repetitive end plate potentials. Cypermethrin was, however, very effective at producing repetitive action in muscle fibre.

In a study on the effects of deltamethrin (referred to as decamethrin in that study), Vijverberg and Van den Bercken (1979) noted that cypermethrin causes frequency-dependent reduction of action potential in *X laevis*. Deltamethrin was shown to induce this same depression at $10^{-6}$ M. Vijverberg and Van den Bercken (1982) also analysed the effect of deltamethrin on the lateral line organ. Long-lasting trains of nerve impulses were observed in *in vitro* experiments. The number of impulses per train was significantly higher in α-cyano containing pyrethroids (deltamethrin, cypermethrin etc.) than in non cyano containing pyrethroids (permethrin etc.). All pyrethroids analysed showed a temperature-dependent reduction in the length of impulse trains induced. Deltamethrin exposure also did not induce repetitive activity in excised sciatic nerves at $10^{-5}$ M concentrations after 24h. Ruigt and Van den Bercken (1986) also found no repetitive end plate potentials for deltamethrin exposure. End plate potentials were, however, induced by des-cyano-deltamethrin (Deltamethrin without its *α*-cyano group). Muscle action potentials were induced by both forms.

In general, mechanisms of action derived from the above studies showed activity between DDT and pyrethroids were shown to be very similar, with similar sodium channel activity suggested. Slight differences between different compounds can mostly be attributed to relaxation times after action (Vijverberg et al. 1982b).

### Organophosphates

Yamauchi et al. (2002) studied the effects of known EDCs on the binding of *X. laevis* plasma thyroid hormone-binding proteins. Malathion was analysed as one of the known EDCs, and showed weak inhibition of binding between triodothyronine and *X.laevis* transthyretin, with no observable binding effects on thyroid hormone receptor-β. The results indicated target sites other than thyroid hormone receptors as playing a larger role in the thyroid-disrupting action of malathion. Ji et al. (2016) investigated the effects of malathion on *Xenopus* oocyte development and found that 100 μg/L significantly reduced the time to maturation. This effect was greatly increased by both 50 μg/L and 100 μg/L 1:1 mixtures between malathion and atrazine. The mixture of the two pesticides also increased the mortality rate significantly in oocytes that were fertilized post-exposure. However, de-jellying of eggs was observed to influence post-fertilisation death rate which may have pronounced such effects.

## Bioaccumulation studies

## Laboratory studies

### Pyrethroids

For the purposes of in vitro testing with transgenic *Xenopus* oocytes, Harril et al. (2005), investigated the link between dose and accumulation in non-transfected oocytes (normal *Xenopus* oocytes) to determine the active concentration of deltamethrin in *in vitro* studies. Their study showed deltamethrin a having the ability to accumulate actively in cell tissue in both a time-dependent and dose-dependent manner. In exposed oocytes deltamethrin reached an accumulated concentration equivalent to exposure dose at 55.5 min. At 180 min the accumulated dose reached approximately double the media dose. The results from Harril et al. (2005) were further investigated by Watkins et al. (2007) who also showed a dose-dependent accumulation of deltamethrin in *X. laevis* oocytes after 1h of exposure. Interestingly, voltage-sensitive sodium channel expressing ($Na_v$ 1.2 + $\beta_1$)-transfected oocytes showed lower accumulation than non-transfected or sham-transfected oocytes. These sham-transfected oocytes underwent the transfection process as blanks being transfected only with water and thus having no genetic alteration. This was done in order to determine if the transfection method affected the oocyte response.

Oocytes, however, do not have the same enzymatic defences against xenobiotics as fully developed frogs do, thus this type of accumulation may not be seen in developed embryos. Although, the ability of deltamethrin to

accumulate in *Xenopus* oocytes proposes a possible risk to freshly laid frog eggs in the wild experiencing exposure events. It is also unknown how the stability of deltamethrin could be altered when accumulated in tissue, perhaps extending the chemical's half-life in the process. Results by Wolmarans (unpublished data) assessing accumulation in adult *Xenopus muelleri* (n=12) from a MVC sprayed area in South Africa collected in May 2016 (outside the MVC spraying season) showed no detectable accumulation of any MVCP (LOQ=0.01 ppm) other than DDT in adult frogs, indicating that deltamethrin or other pyrethroid accumulation in wild *Xenopus*, if at all possible, is most likely temporary, with a quick cessation period, which reduces the risk of chronic exposure effects emerging.

## Field studies

### Organochlorines

Only DDT is expected to show bioaccumulation in a field setting, as it is the only MVCP considered to be a persistent organic pollutant (POP). Three studies could be found on DDT accumulation in *Xenopus* sp. in the field. Hothem et al. (2006) investigated the effects of pollutants on water birds from the Mojave Desert in North America (no active DDT use mentioned), incorporating the accumulation of pollutants in *X. laevis* into the study as a potential food source for birds. None of the frogs analysed had accumulated DDT or metabolite concentrations above limit of detection (0.01 µg/g), except one individual which had 0.054 µg/g wm *p,p*-DDE. Viljoen et al. (2016) measured DDT in *X. laevis* and *X. muelleri* fat bodies in an area sprayed with DDT for MVC through means of IRS in the Limpopo Province of South Africa. All DDT metabolites detected showed higher accumulation at sprayed sites compared to reference sites. No parent DDT (neither *p,p*-DDT nor *o,p*-DDT) was however detected in the frog fat bodies. The authors measured various morphometric factors which indicated observed testicular asymmetry, but with no significant statistical differences. Total DDT levels measured in *Xenopus* sp. in this study ranged from 41 to 391 ng/g wet mass. The authors compared these values to literature on other frog species and values for this study are at the lower end, but within comparable range to concentrations measured in fat bodies of *Rhinella marina* in Mexico (Gonzalez-Mille et al. 2013) and of *Rana clamitans* in Canada (Harris et al. 1998). Wolmarans et al. (2018) measured DDT accumulation in whole body *X. muelleri* from another MVC-sprayed region in South Africa across four surveys over a two year period. The survey with the highest total DDT accumulation in this study showed a mean of 2,062 ng/g lipid. The study showed no discernible correlations between spraying periods and DDT accumulation. However, unlike the results of Viljoen et al. (2016), parent DDT was detected in two of the four surveys, with one survey showing a

ratio of DDT:∑DDD/E (sum of metabolites) greater than one, indicative of exposure to new DDT. In Wolmarans et al. (2018) biomarker responses were also measured in liver and muscle tissue. The authors showed significant relationship between some of the measured biomarkers and both *p,p*-DDT and *p,p*-DDE, indicating that the accumulation of these two forms of DDT could cause changes in the energy dynamics of *X. muelleri*. The *p,p*-DDE accumulation showed a reduction in acetylcholinesterase and malondialdehyde in the liver and energy availability in the form of carbohydrates and proteins in muscle tissue. The *p,p*-DDT accumulation indicated an increase in lipid energy availability and cellular energy consumption in muscle tissue.

Lambert et al. (1997, 2001) investigated frog deaths related to pesticide use and bioaccumulation in sub-Saharan Africa extensively, but made no mention of *Xenopus* with regard to accumulation, or deaths due to DDT or any of the other MVCPs. In this sense, the ecological resilience of *Xenopus* sp. is apparent, as population declines are rarely seen in areas where agricultural pesticides are regularly used. In this regard we hypothesise that some form of generational adaptation may result in the development of resistance to pesticides by wild populations. This is a factor that further brings into doubt the relevance of laboratory based toxicity testing without some form of ecological context.

## Environmental concentrations

To put into perspective the data discussed on *Xenopus*' sensitivity towards, and accumulation of MVCPs, comparisons need to be made to environmental levels globally, with a focus on Africa as being the natural distribution of *Xenopus*.

### Organochlorines

The highest environmental level of DDT measured in water in Africa was by Sibali et al. (2008) with total DDTs of 3.0 mg/L in Hartbeespoort Dam, South Africa (*p,p*-DDT was 1.5mg/L). The highest recording previous to that was by Henry and Kashimba (2003) in Lake Victoria (Kenya) at 1.6 mg/L. Other African studies reported levels well below these levels with most values below 1 µg/L. Van Dyk et al. (2010) measured DDT in in potable water from the Limpopo Province in South Africa (in an MVC area) at 7.6 µg/L of with the maximum *p,p*-DDT at 1 µg/L and maximum o,p-DDT at 0.8 µg/L. This is just below the *o,p*-DDT levels at which significant vittellogenin induction was shown (Palmer and Palmer 1995). However all of these levels are well above the sub-lethal threshold where changes in calling behaviour of *Xenopus laevis* was found (Hoffmann and Kloas 2016). The high water levels reported by Henri and Kashimba (2003) and Sibali et al. (2008) are also

still well below acute toxicity levels and lower than the LOEC reported through FETAX studies showing no threat to *Xenopus* in their natural environment in terms of mortality. Most other African studies on DDT focused on sediment. The most recent study in Africa reporting water DDT concentration was Ogbeide et al. (2018) who reported levels <0.001 ng/L total DDTs in surface waters from Edo State, Nigeria.

**Pyrethroids**

A recent review by Tang et al. (2018) summarizes global environmental levels of pyrethroids who attributed their environmental distribution to agricultural use. Of the pyrethroids mentioned in the water dataset of Tang et al. (2018), cypermethrin is most frequently detected in water samples from around the world with the highest concentration reported in Bangladesh at 80.50 µg/L in lake water. Studies from China, USA and Czech Republic all showed levels close to or above 30 µg/L. These are levels that would lead to acute toxicity in *Xenopus* embryos and larvae according to the data previously discussed under acute toxicity. No African data are available on current pyrethroid levels in water. This makes comparison between *Xenopus*' sensitivity to pyrethroids less relevant as the environmental data are for regions outside of *Xenopus*' natural distribution. Historical levels of pesticides recorded in South African aquatic ecosystems were summarised by Ansara-Ross et al. (2012). Pyrethroids historically measured in South African waters include cyfluthrin (<0.0006 µg/L) and cypermethrin (up to 40.7 µg/L) in northern KwaZulu-Natal province (an MVC region) (Sereda and Meinhardt, 2003), and deltamethrin (1.43 µg/L) in runoff water (Dabrowski et al. 2002) in the Western Cape (not a MVC region). River water from the same Western Cape area (Louwrens River) where runoff was measured by Dabrowksi et al. (2002), was analysed by Bollmohr et al. (2007) who reported cypermethrin at 0.1 µg/L. Even though these are historical values from more than a decade ago, the maximum concentration of cypermethrin found in a MVC area by Sereda and Meinhardt (2003) was high enough to have theoretically caused acute toxicity to *Xenopus*. This region is host to both *X. laevis* and *X. muelleri*, but the latter is far more commonly found in this region today. Sensitivity differences between these species have never been assessed, but may hypothetically be a contributing factor. In terms of sediment concentrations of pyrethroids, there are two studies from African countries outside of South Africa reporting pyrethroid concentrations in sediments. Daka et al. (2006) sampled sediment from different habitats in an area of the Okavango Delta in Botswana aerially sprayed with 0.26 g/ha deltamethrin for Tsetse fly control. Deltamethrin in pool sediment had the highest levels which reached a maximum five days after spraying at 0.291 µg/g dry mass. This value dropped slightly towards 0.221 µg/g at 17 days after spraying. Otulona et al. (2016) found cyhalothrin at 77.75 µg/g dry mass in sediments from the Aiba stream in Iwo, Nigeria. This is extremely high considering Bollmohr et al. (2007) reported a maximum of 2.78 ng/g dry mass in sediments from

the Western Cape in South Africa. Other historical sediment data from South Africa are reported in terms of wet mass concentrations making comparison difficult. Sereda and Meinhardt (2003) measured sediment Deltamethrin levels up to 90 ng/g wet mass and cyfluthrin levels up to 467 ng/g wet mass in the MVC region of northern KwaZulu-Natal.

**Organophosphates**

The only historical record of organophosphates in South African waters is malathion found at 20 ng/L in runoff at the Lourens River in the Western Cape Province (Thiere and Schulz 2004). Although the organic content from these agricultural runoff is higher and would be more likely to transport pesticides, pesticide concentrations are in the same range as levels found in headwaters of the Donjiang River in China (malathion: 14.94-33.11 ng/L) by Chen et al. (2018). This is lower than the 0.7 nM (approximately 231.25 ng/L) IC50 for lysyl oxidase by malathion shown by Snawder and Chambers (1993).

Based on recorded levels of pesticides in African waters, pyrethroids (specifically cypermethrin) seem to pose the greatest risk to *Xenopus* in their natural environment. The data are, however, mostly outdated and unfortunately there is no consistent ongoing monitoring of pesticide levels in African waters. The fact that both environmental levels and the combination of pesticide application use and dose data point to pyrethroids as a possible threat to *Xenopus* wellbeing in Africa; warrants further investigation into actual environmental pyrethroid and pesticide mixtures levels of exposure to all frogs and not *Xenopus*).

## Future prognosis for amphibian ecotoxicology:

Based on the data reviewed, it is clear that gaps exist within amphibian, specifically anuran, ecotoxicology literature. Acute toxicity data for one of the standard test species was available for only half of the WHO-recommended pesticides, and not enough data on different species exists for species sensitivity distribution analyses. In addition, most of the available acute toxicity data can be considered old literature. Ethics in vertebrate toxicological research may well be a contributing reason behind this lack of data. However, considering comments by Lillicrap et al. (2016) on the future of vertebrate ecotoxicology and steps forward, standardised tests such as FETAX, AMA and LAGDA — mentioned specifically as the “go to” current methods for amphibian ecotoxicology — should have minimal issues associated with them in terms of ethical approval as these are globally accepted standardised tests that are also used as alternatives to other animal tests. A recent publication by Brod et al. (2018) does suggest that amphibian research is at a crossroads in terms of animal

welfare in research. If questions posed by Brod et al. (2018) on amphibian welfare (such as appropriate euthanasia methods and measurable biomarkers of welfare applicable to amphibians) aren't sufficiently answered in the near future, along with renewed interest in amphibian research by funders, it may greatly hinder progress made in generating relevant and usable ecotoxicology data on amphibians.

Another possible reason for the lack of data already existing in this field is a lack of interest amongst researchers. Even though the amphibian ecotoxicology research community around the world is quite small, amphibians are one of the fastest-vanishing groups in the world, which should be an incentive for more detailed research. Zippel and Mendselson (2008) wrote a call-to-action on the global amphibian crisis (i.e. the rapid loss of amphibian species globally). The authors state that a valuable lesson from this crisis is the way in which the, then, 20 years of monitoring provided increasing knowledge on amphibian declines, but did little to prevent extinctions globally, and the term "business as usual" is deemed no longer sufficient by itself for resolving environmental issues. In our opinion, a decade later, many of the questions posed have still not been answered adequately. There is a clear need, not only for toxicity or sensitivity data of anurans towards MVCPs, but also linkages between application rates and exposure, and how these relate to accumulation, or effects, as well as linkages between laboratory and real-world scenarios, and monitoring of current environmental exposure levels. The exploration of mixture effects is something that has only started to progress recently in amphibian ecotoxicology, and could prove useful in amphibian health risk assessment studies in future. There is a need for comprehensive datasets on amphibian ecotoxicology, which can be achieved only through inter-laboratory collaboration and data-sharing in this regard.

## Abstract

Anurans from the genus *Xenopus* have long been used as standard testing organisms, and occur naturally in tropical and sub-tropical areas where malaria vector control pesticides are actively used. However, literature on the toxic effects of these pesticides is limited. This review analyses the available data pertaining to both *Xenopus* and the pesticides used for malaria vector control in order to determine the pesticides that have the greatest potential to influence amphibian health, while also identifying gaps in literature that need to be addressed. Amphibian diversity has shown the fastest decline of any group, yet there are still voids in our understanding of how this is happening. The lack of basic toxicity data on amphibians with regard to pesticides, is an issue that needs to be addressed in order to improve effectiveness of amphibian conservation strategies. Meta-analyses performed in this review, show that, at current usage, with the available acute toxicity literature, the pyrethroid pesticide group could hold the highest potential to cause acute toxicity to *Xenopus* sp. in relation

to the other MVCPs discussed, but lack the of data cripples the efficacy with which meta-analyses can be performed and conclusions made from such analyses. Several studies have shown that DDT accumulates in *Xenopus* sp. from malaria vector control areas, but accumulation of other MVCPs in frogs is still largely unknown. Through this review we hope to encourage future research into the field of amphibian ecotoxicology, and to promote the use of the *Xenopus* standard model in order to build comprehensive datasets that may be used in amphibian conservation.

Keywords: *Xenopus*, anura, malaria, vector control, organochlorine, pyrethroid, organophosphate, DDT, deltamethrin, cyhalothrin, malathion, cypermethrin, fenitrothion, amphibian ecotoxicology, pesticide, FETAX, acute toxicity, chronic effects, species sensitivity distribution, sub-lethal effects, frogs, amphibian well-being, conservation, model organism

## Conclusion

The clearest issue with anuran ecotoxicology regarding pesticides is consistency between reports, mostly due to method-related differences. This is partly accounted for by the fairly common use of FETAX. But the second limiting factor, availability of data, then comes into play. Very few records exist regarding the standard test species, *X. laevis*, and, casting an even wider net by including the whole genus *Xenopus*, does not increase the availability by any significant means. It is of concern, since most of these pesticides are still being used in agriculture and sectors other than MVC around the world, that for the twelve WHO-recommended MVCPs so little data is available. Biomonitoring data of these pesticides in Africa is also outdated.

Based on the available literature, of the four pesticide groups, pyrethroids may hold the greatest acute toxicity potential to *Xenopus*. This does not mean that at current usage *Xenopus* are at risk of acute toxicity, but merely that, if any of the MVCPs were to result in acute toxicity, based on the sensitivity of *Xenopus* towards pyrethroids and current and recommended usage, pyrethroids would be the most likely compound group to show such effects in the field, however, these potentials are based solely on the data available and are not definitive values in a broader context than how they are used in this study. Based on sub-lethal activity, it is clear that very little is known about how these pesticides affect anurans, in particular at sub-lethal concentrations. The field data available speaks to the persistence of DDT in the environment. As DDT is the only one of the MVCPs known to bioaccumulate in frogs, of the twelve MVCPs, it can be considered to carry the greatest chronic risk to all anurans for this reason. The EDC effects of *p,p*-DDE on *Xenopus* calling behaviour raises concern, as *p,p*-DDE is the most persistent metabolite of DDT and shows the greatest bioaccumulation potential. If more sub-

lethal effects linking with accumulation, such as those measured by Wolmarans et al. (2018), can be confirmed through laboratory and field testing it will increase the certainty of risk values that can be calculated from field accumulation data for amphibian populations.

## Recommendations

There is a definite need for further monitoring of anurans in Africa with regard to the effects of pesticides, specifically within the genus *Xenopus*. Lambert (2001) stated the need for using African amphibians, such as *Xenopus* in particular, more extensively as a monitoring tool in Africa. Increased use of *Xenopus* in monitoring is not reflected by literature as of yet. Based on the aim of this review, the authors encourage new research on anuran ecotoxicology, specifically in terms of insecticides used in vector control and agriculture, in order to fill the gaps in the current knowledge base. In future studies, *Xenopus* sp. could be used as a starting point around which to build a comprehensive toxicological dataset in order better to understand the impact that human activity has on anuran well-being. The use of adverse outcome pathways may serve as a useful tool to understand sub-lethal effects observed, and is yet to be used in anuran studies. However, the basic empirical toxicity of these pesticides towards anurans still requires attention.

## Summary

This review concerns the genus *Xenopus* and literature relating it to malaria vector control pesticides. Criteria for literature to be included for review include that dealing with *Xenopus* sp. and exposure to any one of the twelve WHO-recommended MVCPs. The data analysed showed that, with the available acute toxicity literature, the pyrethroid pesticide group holds the greatest potential to cause acute toxicity when used for malaria vector control, but acute toxicity cannot be interpreted as a likely outcome based on the data available and limitations to the analyses performed. This potency is increased by the concurrent use of these pesticides in agriculture at even greater doses. The only persistent pollutant in the list of pesticides analysed is DDT. Field literature show that DDT accumulates (actively, in some cases) in *Xenopus* sp. in a natural setting both inside and outside MVC spraying areas, with possible sub-lethal effects associated with the accumulated concentrations. There is a lack of published research in terms of both acute toxicity and sub-lethal effects with regard to anurans in general and MVCPs. Some of the recommended pesticides have no associated toxicity literature with regard to any anuran standard test species, let alone to *Xenopus*. This review serves to indicate the need for more detailed information regarding pesticides recommended for vector control, and for anurans in general, both in a laboratory and field

setting. With amphibians declining rapidly globally, and with *Xenopus* being a well-established standard test species, this species may serve as a good starting point for building a comprehensive dataset in this regard.

## List of abbreviations

MVC – Malaria Vector Control

MVCP – Malaria Vector Control Pesticide

ITN – Insecticide Treated Net

IRS – Indoor Residual Spraying

WHO – World Health Organisation

FETAX –Frog Embryo Teratogenesis Assay – *Xenopus*

DDT – Dichlorodiphenyltrichloroethane

DDD – Dichlorodiphenyldichloroethane

DDE – Dichlorodiphenyldichloroethylene

DDA – Dichlorodiphenylacetic acid

LAGDA – Larval Amphibian Growth and Development Assay

AMA – Amphibian Metamorphosis Assay

OECD – Organisation for Economic Co-operation and Development

LC50 – lethal concentration where 50% of the test population died

EC50 – Effective concentration where 50% of the test population are affected

IC50 – Inhibition concentration where 50% of the test population show inhibition of a measured aspect

AI – Active ingredient

LOEC – Lowest observed effects concentration

MCIG – Minimum concentration to inhibit growth

TI – Teratogenic index

NF – Nieuwkoop-Faber

SSD – Species sensitivity distribution

POP – Persistent organic pollutant

EDC – Endocrine disrupting compound

XPA – Xeroderma pigmentosum group A gene

XPG – Xeroderma pigmentosum group G gene

CSA – Cockayne syndrome A gene

MUTL – Muir-torre syndrome gene

ACP – acid phosphatase

CaE – carboxylesterase

GST – glutathione-S-transferase

LDH – lactate dehydrogenase

$NAD^+$ – nicotinamide adenine dinucleotide

m-RNA messenger Ribonucleic Acid

DNA Deoxyribonucleic acid

UVB – Ultraviolet B

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