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Investigation of potential endocrine disrupting effects of mosquito larvicidal *Bacillus thuringiensis israelensis* (*Bti*) formulations

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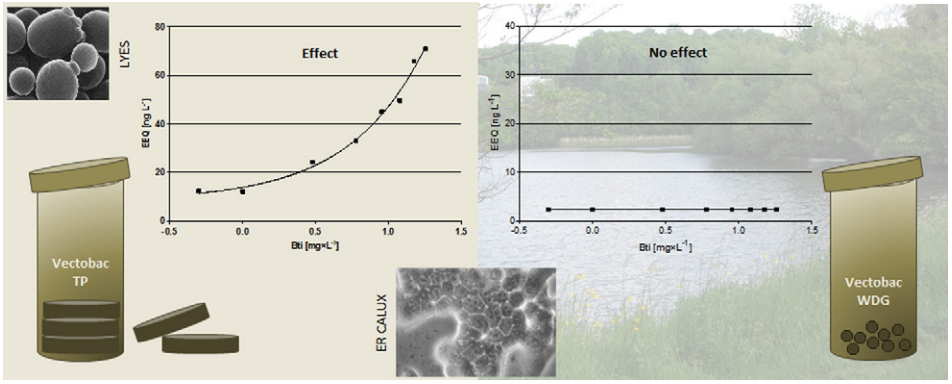
HIGHLIGHTS

- Some *Bti* formulations show significant dose-dependent estrogenic activity in vitro.
- Estrogenicity was found in high concentrations of Vectobac TP and solid formulations.
- There was no clear effect on the steroidogenesis in human H295R cells.
- *Bti* treated surface water as well as ground water showed no estrogenic activity.
- Our data supports previous studies on the safe use of *Bti* even in sensitive habitats.

GRAPHICAL ABSTRACT



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ABSTRACT

Bti is successfully used as a biological control agent for mosquito control. It has proven to be ecological friendly, and thus, is used in ecologically sensitive habitats. Recent investigations of groundwater in Germany have detected estrogenic activity in five consecutive groundwater wells in a region where *Bti* is applied. Therefore, it was suspected that this compound can act as an environmental xenoestrogen.
In the present study, five *Bti* formulations as well as the active ingredient, VectoBac® TP (TP), were investigated regarding their estrogenic activity using the LYES and ER CALUX® assays. Furthermore, their steroidogenesis

Abbreviations: 17-OHP, 17-hydroxyprogesterone; 21-OHP, 21-hydroxyprogesterone; ASD, androstenedione; *Bti*, *Bacillus thuringiensis var. israelensis*; COR, corticosterone; CPRG, chlorophenol red-β-D-galactopyranoside; DCM, dichloromethane; DMSO, dimethyl sulfoxide; E2, 17ß-estradiol; ED, endocrine disruptor; EEQ, estradiol equivalents; ER CALUX®, Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression; H295R, H295R Steroidogenesis assay; LOEC, lowest observed effect concentration; LYES, lyticase assisted yeast estrogen screen; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PRO, progesterone; PTFE, polytetrafluoroethylene; TP, VectoBac® technical powder; WDG, VectoBac® water dispersible granules; WS, water sample.

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disruption properties were studied using the H295R Steroidogenesis Assay. Additionally, field samples from a *Bti* application area as well as samples from an artificial pond were examined.
Three of the *Bti* formulations and the active ingredient TP showed significant estrogenic activity in the LYES (up to 52 ng·l^{-1} estradiol equivalents (EEQ) in the 18-fold concentration) and/or the ER CALUX® (up to 1 ng·EEQ·l^{-1} in the 18-fold concentration). In the H295R significant but weak effects with no dose–response-relationship on the production of estradiol, and 21-hydroxyprogesterone (WDG) as well as testosterone (TP) by H295R cells could be observed. The field samples as well as the samples from the artificial pond showed no significant increase of estrogenic activity after application of TP or WDG in the ER CALUX®. With the exception of the controlled laboratory experiments with direct application of *Bti* to the utilized in vitro test systems the present study did not reveal any significant effects of *Bti* on endocrine functions that would indicate that the application of *Bti* could cause adverse endocrine effects to organisms in aquatic ecosystems. Instead, our results support previous studies that the use of *Bti* products against mosquitos would be safe even for sensitive habitats such as conservation areas.

1. Introduction

As a biological alternative to synthetic insecticides, such as the organophosphates Fenitrothion (O,O-Dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate) and Temefos (*O,O,O′,O′*-Tetramethyl *O,O′*-sulfanediylbis(1,4-phenylene) diphosphorothioate), *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) toxins are increasingly used for control of mosquitoes since the 1980s (Becker, 1997; Boisvert et al., 2007). The larvicidal activity is due to δ-endotoxins, which are synthesized during sporulation. Based on a large number of toxicity studies with non-target organisms the environmental safety of *Bti* has been suggested (Boisvert and Boisvert, 2000). It was therefore recommended for use in aquatic environments and drinking water reservoirs by the World Health Organization (WHO, 1999). Nevertheless, screening for endocrine activity was not included in the previous studies (El-Bendary, 2006; Russell and Kay, 2008; Tilquin et al., 2008).

During a study that investigated the toxic properties of groundwater in the Upper Rhine area (Germany) (Wölz et al., 2011) estrogenic activities were detected for five consecutive groundwater wells (data not published). In this area intensive *Bti* application is carried out. Because no suspected or confirmed estrogen receptor agonists were detected in the samples, it was hypothesized that *Bti* could have contributed to these activities. Consequently, a spiked water sample, containing a commercial available *Bti* formulation, was tested in the Yeast Estrogen Screen (YES) (Routledge and Sumpter, 1996). Again, estrogenic activity was detected (data not shown). To confirm the suspected estrogenic activity of *Bti*, the present study that investigated the estrogenic activity of the active substance VectoBac® TP, five formulations and the ingredients of one of the formulations was performed.

Attempts to explain the overall estrogenic activity of water samples by mass-balance studies are not conclusive in many cases and detection of known or suspected estrogenic active substances underestimated the response in the bioassays. Therefore, it is important to explore the potential contribution of other, so far unknown, compounds to the measured biological effects (Schulte-Oehlmann et al., 2006). In addition to natural and synthetic hormones, endocrine disruptors (EDs) include a range of other classes of chemicals such as phthalates, organotin compounds and hydroxylated polychlorinated biphenyls (PCBs) as well as several pharmaceuticals and personal care products. Due to their different physicochemical properties initial identification of hormonally active substances is not straightforward (Heberer, 2002; Van der Linden et al., 2008). The concentration of most potent EDs in the environment is near or below the detection limit of current chemical analysis, i.e. the lower ng·l^{-1} range. This becomes an issue as some of these compounds – particularly hormones – exert their effects in this range. Additionally, chemical interactions of EDs have to be taken into account to estimate the potential impacts of EDs in aquatic ecosystems.

To examine estrogenic activity of *Bti* two receptor mediated assays, the Lyticase Yeast Estrogen Screen (LYES) (Routledge and Sumpter, 1996; Wagner and Oehlmann, 2009) and the Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression (ER CALUX®) assay (Legler et al., 1999), were applied. Furthermore, the H295R Steroidogenesis assay (H295R) (Hecker et al., 2011), that enables detection of effects on the steroid synthesis pathway, was used to identify potential disruptors of sex steroid production. Previous studies reported the applicability of a combination of receptor-mediated and non-receptor-mediated assays for a holistic evaluation of potential endocrine activity in complex (waste)water and sediment samples (Grund et al., 2011; Hecker and Hollert, 2009; Kase et al., 2009; Leusch, 2008; Leusch et al., 2010; Maletz et al., 2013). As previously reviewed by Hecker and Hollert (2011) the combined use of receptor-mediated and non-receptor-mediated-methods is necessary to enable objective assessment of endocrine disrupting potentials of complex samples.

The aim of the present study was to investigate the potential estrogenic activity as well as possible impacts on steroidogenesis of *Bti* and its formulations usually applied in aquatic ecosystems and private water bodies. Furthermore, it should be examined if the detected activity was caused by the active ingredient or the adjuvants or the fermentation slurry, respectively.

2. Material & methods

2.1. Samples

2.1.1. Bti formulations for the public market

In an initial experiment, four different commercially available *Bti* formulations for the public market were tested in the LYES regarding their estrogenic activity: Three solid products (tablets) and one liquid suspension. The tablets as well as the liquid formulation were obtained from local stores, online stores as well as a registered association to cover the whole range of purchase possibilities of the public market. The tablets were homogenized using a porcelain mortar and weighed. A 30-fold concentration in mg·l^{-1} was calculated depending on the dosage recommendations of the manufacturers (Table 1). This stock concentration resulted in a maximum concentration of 18-fold after dilution within the LYES.

The homogenized tablets as well as the liquid formulation were diluted in 250 ml tap water and samples were stirred overnight. Samples were stored at 4 °C in amber glass bottles with polytetrafluoroethylene (PTFE) lids.

2.1.1.1. Ingredients of product B. As one manufacturer kindly provided the composition of the tablet, screening of the individual substances contained in this product in the LYES was possible. The tablets contain the active substance as well as six adjuvants. According to the highest tested concentration of the investigated formulas, the main adjuvants were diluted in tap water and tested in an 18-fold concentration. As the composition of the screened product is proprietary single substances are not disclosed in this study. For further investigations the