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RESEARCH ARTICLE

Non-avoidance behaviour in enchytraeids to boric acid is related to the GABAergic mechanism

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Abstract Soil invertebrates, e.g. enchytraeids, are known to be able to avoid unfavourable conditions, which gives them an important ecological advantage. These organisms possess chemoreceptors that can detect stressors, which in turn activate responses such as avoidance behaviour. We studied the avoidance behaviour in response to boric acid (BA) using enchytraeids. Results showed not only no avoidance, but that increasing concentrations seemed to have an "attraction" effect. To study the underlying mechanism, a selection of genes targeting for neurotransmission pathways (acetylcholinesterase (AChE) and gamma-aminobutyric acid receptor (GABAr)) were quantified via quantitative real-time polymerase chain reaction (qPCR). Evidences were that BA is neurotoxic via the GABAergic system mechanism where it acts as a GABA-associated protein receptor ($GABA_AR$) antagonist possibly causing anaesthetic effects. This is the first time that (non)avoidance behaviour in invertebrates was studied in relation with the GABAergic system. We strongly recommend the combination of such gene and/or functional assay studies with the avoidance behaviour test as it can bring many advantages and important interpretation lines for ecotoxicity with minor effort.

Keywords Mechanism of response · Soil invertebrates · Avoidance behaviour · Neurotransmitter targets · Enchytraeids · Boric acid

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Introduction

Boric acid (BA) affects survival and reproduction of several soil invertebrate species, e.g. earthworms, enchytraeids, collembolans and mites in a concentration-dependent manner (Becker et al. 2011; Owojori et al. 2014). Moreover, BA is the reference substance for the avoidance test for earthworms (ISO 2008a) and the reproduction test for mites and collembolans (OECD 2009; 2008). The pros and cons of BA as a reference substance were discussed by Amorim et al. (2012), with one of the main criticisms being the lack of avoidance behaviour to BA as shown by some species, e.g. *Enchytraeus albidus* and *Folsomia candida* (Amorim et al. 2012, 2008).

Avoidance behaviour is an end point with important ecological relevance as it may prevent the population decline when organisms can detect toxicants and escape to non-toxic patches in the field (Amorim et al. 2008). Avoidance tests are standardized (ISO 2008a; b) and can be used to assess the hazardous potential of stressors (Amorim et al. 2008; Novais et al. 2010). These tests are based on the fact that organisms have chemoreceptors that detect the presence of unfavourable conditions, e.g. chemicals (Amorim et al. 2008; Edwards and Bohlen 1996; Römbke and Schmidt 1999). If a chemical is detected, the information is processed via the nervous system and a response to stimuli can be initiated and translated as avoidance behaviour. It is known that chemicals can affect neurotransmission mechanisms, for example, Pereira et al. (2013) showed that dimethoate negatively affected reflexes and locomotion in *F. candida* due to inhibition of acetylcholinesterase (AChE), and hence avoidance was not possible.

Acetylcholine (ACh) and gamma-aminobutyric acid (GABA) are among the group of neurotransmitters described in vertebrate and invertebrate nervous system. ACh is a major excitatory neurotransmitter (Lauder and Schambra 1999), while GABA is the major inhibitory transmitter at neuromuscular synapses and central nervous system (Lunt 1991). In

fact, several pesticides/insecticides were specially designed to bind the receptors and affect these pathways, e.g. dimethoate and chlorpyriphos are known inhibitors of AChE (Pope 1999), and phenylpyrazole insecticides are GABA receptor antagonists (Cole et al. 1993). Moreover, studies show that chemicals can affect GABAergic and cholinergic pathways (Garcia-Reyero et al. 2011; Gong et al. 2012), even when not designed to target the nervous system. BA (and borate salts) is commonly used in pesticide products such as insecticides, acaricides and fungicides, among others. BA action as insecticide has been attributed to the abrasive effects on the cuticle and starvation due to degeneration of gut cells (Cochran 1995; Ebeling et al. 1975); however, neurotoxic effects were described in the cockroach *Blattella germanica* (Habes et al. 2006).

In the present study, we aimed to investigate the (non)avoidance behaviour of enchytraeids to BA and understand the underlying mechanisms. Hence, the cholinergic and GABAergic pathways were studied via quantitative gene expression (quantitative real-time polymerase chain reaction (qPCR)).

Material and methods

Test organisms

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. *E. crypticus* cultures were kept in agar plates prepared with a salt solution of $CaCl_2$, $MgSO_4$, KCl and $NaHCO_3$ and fed ad libitum with oatmeal. Cultures were maintained in laboratory under controlled conditions at 18±2 °C and a photoperiod of 16:8 h (light–dark).

Test soil

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The main characteristics can be described as follows: pH (0.01 M $CaCl_2$)=5.5, organic matter=1.77 %, cation exchange capacity (CEC)=10.1 %, water holding capacity (WHC)=41.8 %, grain size distribution of 7.3 % clay, 13.8 % silt and 78.9 % sand.

Test chemical and spiking

Boric acid (BA; H_3BO_3) (Merck, 99.8 % purity) was spiked as aqueous solutions onto pre-moistened soil. Stock solution was prepared and serially diluted. Soil batches per concentration were homogeneously mixed, and the soil moisture was adjusted to 50 % of the WHC_{max}.

Test procedures

Avoidance test

The avoidance test was performed using *E. crypticus* following the earthworm avoidance test guideline (ISO 2008a) with adaptations as described in, e.g. Amorim et al. (2008) and using the two chamber test. Soil was spiked in the following concentrations: 0-200-350-630-1125-2000 mg/kg soil dry weight (DW). Five replicates per treatment were used. In short, plastic containers (7.8×4.3ø cm) with one removable plastic divider were used; each replicate contained 50 g of soil (25 g each side), this being control and spiked soil. After this, the wall was gently removed and ten adult organisms (with clitellum) were placed on the contact line of the soils. Boxes were covered with a lid (containing small holes) and kept, for 48 h, at 20±2 °C and a photoperiod of 16:8 h (light–dark). At the end of the test period, the divider was again inserted in the separation line between the two soils and each side of the box was independently searched for worms.

Gene expression—quantitative real-time PCR

Organisms were exposed to 0-200-630-1000 mg BA/kg soil (DW) in three replicates. Each replicate consisted of a glass vessel (8×4ø cm) containing 20 g of soil, and ten adult organisms per replicate were used. After 48 h, organisms were carefully removed from soil, rinsed in distilled water, frozen in liquid nitrogen and stored at −80 °C until further analysis. RNA was extracted from the pool of organisms per replicate, using the SV Total RNA Isolation System (Promega). The quantity and purity of the isolated RNA were measured with nanodrop (NanoDrop ND-1000 Spectrophotometer), and its integrity was checked on a denaturing formaldehyde agarose gel electrophoresis.

Gene targets included the homologous to AChE and GABA-associated protein receptor ($GABA_AR$) as retrieved from the *E. crypticus* library (Castro-Ferreira et al. 2014) (Table 1). Actin was used as housekeeping gene being its suitability as housekeeping tested in all the RNA samples used to perform the qPCR. Primers were designed with the software Oligo ExplorerTM (version 1.1.2). Efficiency and specificity of each primer was determined by observing the obtained standard and melting curves, respectively, for all primer sets.

To perform qPCR, the total RNA (0.5 μg) from samples was converted into cDNA through a reverse transcription reaction using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Each replicate was applied in triplicate on a 96-well optical plate (GeneAmp®, Applied Biosystems). Amplification was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on the 7500 Real-Time PCR System (Applied Biosystems). Reaction

Table 1 Primer sequences used for the qPCR gene quantification in *Enchytraeus crypticus*

Sequence homology description	5′–3′ forward primer	5′-3′ reverse primer
Actin	GCTGTCCTTCTGTCCCAT	GCTGTCTTCCCCTCAATC
Acetylcholinesterase (AChE)	AATAAACACCCTCGTCAGT	ATCCTCCATCAGAAGCAAT
Gamma-aminobutyric acid receptor-associated protein ($GABA_AR$)	AGAAGTACTTGGTGCCGG	AGGAATGACGTTGTTGATG

conditions consisted of one initial cycle at 50 °C for 2 min, followed by a denaturation step at 95 °C for 2 min, 40 cycles at 95 °C for 32 s and 1 cycle at 60 °C for 1 min. Finally, a dissociation step was made consisting of 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C.

Data analysis

Avoidance was calculated as the percentage of worms that avoided the treated soil in the test container from the total number of worms in that container. The mean percentages of net responses (NR) were calculated as follows: $NR=((C-T)/N)\times 100$, where C is the number of organisms observed in the control soil, T is the number of organisms observed in test soil and N is the total number of organisms per replicate. A positive (+) NR indicates avoidance and a negative (−) NR indicates a non-response (or attraction) to the chemical. Effect concentrations (EC_x) calculations were performed by modelling data to threshold sigmoid two parameters regression model, using the Toxicity Relationship Analysis Program (TRAP) software.

For qPCR data, a mean normalized expression value was calculated from the obtained Ct values of the test genes using the Relative Expression Software Tool (REST-MSC). This software was used to assess differences between treated groups versus control ($p<0.05$ based on pairwise fixed reallocation randomization test). EC_x calculations were performed by modelling qPCR data, used as fold change ($\log_2$ relative expression), relative to the control (considered zero expression) using the logistic two parameters model in TRAP software.

Results

Results of the avoidance behaviour test are presented in Fig. 1a.

The validity criteria for avoidance test were fulfilled (≥90 % organisms' survival). Organisms were showing a decrease in avoidance behaviour, i.e. there was an increasing number of organisms present on the spiked side of the container with increase in BA concentration. The EC_x estimated through model fitting to experimental observations are shown in Table 2.

Gene expression results (qPCR) showed that $GABA_AR$ was significantly up-regulated ($p<0.05$) when exposed to BA showing an increase with the dose from 200 to 630 mg BA/kg (Fig. 1b). The organisms exposed to 1000 mg/kg died. Results showed that, e.g. 20 and 50 % increase in gene expression occurs at ca. 16 and 270 mg BA/kg (Table 2). No major changes occurred for AChE gene expression.

Discussion

Results showed that *E. crypticus* does not avoid BA (up to 2000 mg/kg), similarly to previous observations using the alternative species *E. albidus* (Amorim et al. 2008).

The inability to avoid certain chemicals has been reported previously, e.g. for *E. albidus* exposed to linear alkylbenzene sulfonates (LAS) and tributyl-tin-oxide (TBTO) (Amorim et al. 2008), or for *Lumbricus terrestris* exposed to chlorpyriphos (Martinez Morcillo et al. 2013). Further, for BA testing using *E. crypticus*, not only the organisms were not able to avoid BA but the response indicated what appeared to be an "attraction" which increased with the increase in concentration. Such attraction pattern has been observed before for *F. candida* (Collembola) when exposed to dimethoate (Pereira et al. 2013). These authors confirmed that the organisms were paralysed with increasing concentrations of dimethoate, this being correlated with a decrease in the AChE activity. Hence, the so-called attraction was actually a reflection of being trapped.

The link between neurotransmission pathways and avoidance behaviour has been investigated by other authors (Hodge 2000; Jordaan et al. 2012; Martinez Morcillo et al. 2013; Pereira et al. 2010). Among other examples included is the effect of azinphos-methyl (Jordaan et al. 2012) on *Eisenia andrei* juveniles. In both cases, the chemicals were organophosphates pesticides—cholinesterase (ChE) inhibitors—hence, the results were in agreement with the chemical mode of toxic action.

Our results for enchytraeids and BA indicate no effect on AChE gene expression, i.e. this non-avoidance behaviour is not related with cholinergic pathways. Furthermore, results on the AChE enzymatic activity performed on the species of the same genus *E. albidus* showed no effect (see Supplementary

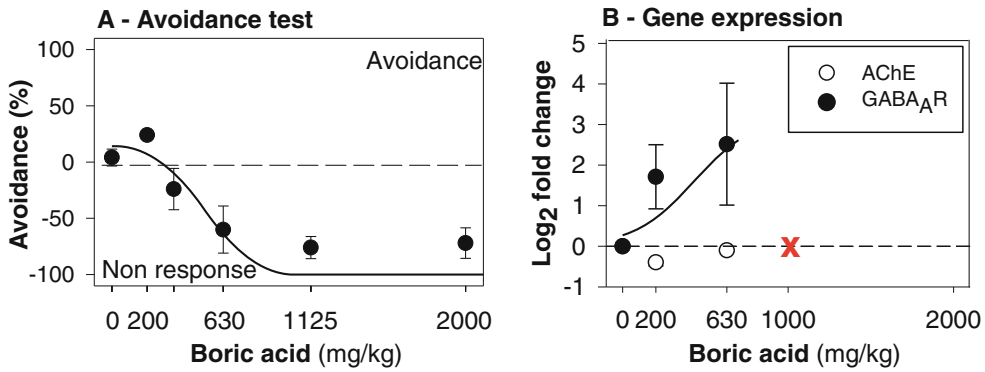

Fig. 1 Results from the exposure of *Enchytraeus crypticus* to boric acid in LUFA 2.2 soil during 48 h. **a** Avoidance behaviour test. **b** Quantitative gene expression using qPCR for acetylcholinesterase (AChE) and gamma-aminobutyric acid receptor-associated protein ($GABA_AR$) codifying genes. Results are expressed as mean±standard error. *Solid line* represents model fitted to data. *X*, organisms died

material for details). Interestingly, our results indicated that it could be related with the GABAergic system, another neurophysiological mechanism. GABA is known to function as a major inhibitory neurotransmitter (Lunt 1991); the up-regulation of $GABA_AR$ results in the increase of GABA binding eventually causing anaesthetic effects. The gene transcribing for $GABA_AR$ was up-regulated, corresponding to 20 and 50 % increase when exposed to 16 and 270 mg BA/kg, respectively. This is in agreement with the increase in the attraction behaviour, which in fact coincides with the paralysis/anaesthetic effect induced by BA, hence the inability to escape. The up-regulation of the $GABA_AR$ and the paralysis are linked, and the weight of evidence is reasonable for the non-avoidance rational. This anaesthetic effect would explain the higher number of organisms present on the spiked soil (compared to the control): above a certain concentration of BA, the worms are too late to escape to the control side. The present results can also point to an attraction towards the chemical, since more organisms were found in the spiked soil above 630 mg BA/kg.

The $GABA_AR$ gene regulation has been studied in mice where the long-term effect of the anaesthetic ketamine is related to the up-regulation of $GABA_AR$, which inhibits the control of the neurotransmission in the central nervous system (Tan et al. 2011). A study by Gong et al. (2012) showed the neurotoxicity of the compound CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,1,0,12-hexaazaisowurtzitane) to *Eisenia fetida* by reduction in the nerve impulse conduction velocity. This effect was explained by a series of events triggered by $GABA_AR$ up-regulation. According to the authors (Gong et al. 2012), the up-regulation of $GABA_AR$ is explained as a consequence of initial non-competitively blocking of the ligand-gated $GABA_AR$ ion channels. This mode of neurotoxic action was also described for RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) which is a recognized $GABA_AR$ antagonist (Garcia-Reyero et al. 2011). Possibly, BA initially acts as a $GABA_AR$ antagonist in *E. crypticus*, and the up-regulation of $GABA_AR$ observed is the response of the organism to compensate for that effect.

The fact that, for gene expression analysis, organisms died during the exposure to 1000 mg BA/kg whereas in the avoidance organisms were tested up to 2000 mg BA/kg, may seem contradictory. Although, in an avoidance test, organisms have control versus spiked in the same test vessel, meaning that the organisms can spend time in both sides and reduce their total exposure compared to the exposure for gene expression. The overall results can indicate that organisms in the avoidance test must go through spiked and control soil before the paralysis effect occurs and organisms become immobilised on the spiked side.

Given that enchytraeids' avoidance to BA is impaired and that effects on reproduction EC_{50} are in the order of 100–200 mg BA/kg (Amorim et al. 2012; Becker et al. 2011), such

Table 2 Effect concentrations (EC_x) for *Enchytraeus crypticus* when exposed to boric acid in LUFA 2.2 soil, in terms of avoidance behaviour and gene expression (GE) of the $GABA_AR$ and AChE

Endpoint	EC_{10} (95 % CI)	EC_{20} (95 % CI)	EC_{50} (95 % CI)	EC_{90} (95 % CI)	Model parameters
Non-avoidance	249 (24–474)	342 (173–511)	527 (386–656)	699 (419–978)	$S=1.99\times10^{-3}$, $Y_0=14$
$GABA_AR$ GE	n.d.	16 (−28–68)	268 (234–321)	666 (577–756)	$S=0.14\times10^{-2}$, $Y_0=3.21$
AChE GE	n.e.	n.e.	n.e.	n.e.	–

Logistic 2 parameters model was used

CI confidence interval, *S* slope, Y_0 top point, *n.d.* not determined, *n.e.* no effect

exposed populations will be at higher risk to decline compared to those populations that are able to avoid. Soil boron concentrations range from 20 to 300 mg/kg in the environment (U.S. Department for Health and Human Services 2010); hence, this is very important on a more ecological level and we would like to highlight the implications and additional impact of BA on terrestrial ecosystem.

Conclusions

Indications are that BA causes anaesthetic effect on *E. crypticus* measured as non-avoidance since organisms are not able to escape the spiked soil. BA is neurotoxic via the GABAergic system mechanism where it acts as a $GABA_AR$ antagonist causing anaesthetic effects.

To our knowledge, this is the first time that non-avoidance behaviour in invertebrates was studied in relation with the GABAergic system. In the future, if non-avoidance behaviour is observed, the study of $GABA_AR$ gene expression (and ChE) could be easily included and provide important interpretation lines, e.g. towards improvement of the ecological relevance of the conclusions.

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Conflict of interests The authors have nothing to declare.

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