# Benzotriazole is anti-estrogenic in vitro but not in vivo

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23

**Abstract**

Benzotriazole (BT) is an anti-corrosive agent, which is well known for its use in aircraft deicing and antifreeze fluids (ADAFs), but is also used in dishwasher detergents. It is highly persistent in the environment, and BT is therefore frequently found in runoff emanating from large airports, as well as in the surrounding groundwater. In addition, BT has recently been found to be ubiquitous in Swiss waste water treatment plant (WWTP) effluents and their receiving waters. However, there is very little in the way of chronic toxicity data available on which to base a sound ecological risk assessment of this chemical. In vitro assays conducted using a recombinant yeast (anti-)estrogen assay indicated that BT possessed clear anti-estrogenic properties. The potency of this chemical was approximately one-hundred times less potent than Tamoxifen, which was used as a positive control. However, a subsequent in vivo study, involving the analysis of vitellogenin induction and somatic indices in adult fathead minnows exposed to BT at concentrations of 10, 100 and 1000 µg/L for two weeks, showed no evidence of anti-estrogenic activity of this compound. The possibility exists that higher concentrations of BT may yet induce the type of activity observed in vitro, although the concentrations used here already far exceed those reported in surface water samples. Further, adverse effects may be observed in fish or other organisms exposed to BT for a longer period than that employed here, although such studies are costly and are unlikely to be included in standard risk assessment procedures. A rigorous investigation of the chronic toxicity of BT is imperative.

Key Words: Benzotriazole, Anti-estrogenic, Fathead minnow, Yeast screen, Anti-corrosive

## Introduction

Benzotriazole (BT) is a commonly used industrial chemical, which was recently found to be ubiquitously occurring in municipal effluents in Switzerland, as well as in their receiving waters, at microgram per litre concentrations [1, 2]. Despite this, surprisingly little is known about the environmental contamination of surface waters by benzotriazole, or indeed about any chronic toxicity which may result from the exposure of aquatic organisms to this chemical.

Benzotriazole is an anticorrosive agent, used in aircraft deicing and antifreeze fluids (ADAFs), which are critical to the safety and smooth running of airports in cold climates. As such, ADAFs are used in extremely large quantities; it is estimated that 3,785 L is used each time a large passenger jet is deiced, and 8 million litres of these chemicals are used per year in Canada alone [3]. For this reason, much of the attention on environmental concentrations of BT has focused on runoff from airports, as well as water bodies and ground water systems receiving such runoff [4-6]. Benzotriazole is also used in machine dishwashing detergents for silver protection [7], and it is this aspect of its application that was considered to be responsible for the presence of relatively high concentrations of the chemical in the Swiss study [1]. In addition, elimination of BT during the wastewater treatment process appears to be poor. Consequently, concentrations of BT measured in sewage effluents are reasonably high. For example, concentrations of up to 100 µg/L were detected in secondary effluents emanating from Swiss waste water treatment plants (WWTP) [1], and an average concentration of 9.6 µg/L was observed in WWTP effluents around Berlin,

Germany [8]. Subsequent studies have corroborated these findings, with reports of mean concentrations of benzotriazoles of 1 to10 µg/L measured in effluents across Europe [9]. Further, albeit limited, information is available concerning the occurrence of BT in surface waters. For example, a recent German study reported 0.9 µg/L in samples taken from Lake Tegel, 3.4 µg/L in those collected from the Landwehr Canal, and 0.2 µg/L in bank filtrate (used for drinking water production) [8]. These data are supported by those published by Reemtsma et al., where a mean concentration of approximately 0.5 µg/L benzotriazole was measured in surface waters sampled at five European locations [9].

Some of the first toxicity data produced on benzotriazoles and their derivatives were published by Cancilla et al.[3], who isolated a number of fractions of ADAFs to determine which of these fractions contributed to their microtox activity using the Microtox® bioassay. The first active fraction was found to be a mixture of benzotriazoles and tolyltriazoles (TTs). Since then, acute toxicity assays have revealed that, after 96 h, the LC50 (the concentration required to cause 50% mortality) was 65 mg/L BT for the fathead minnow (*Pimephales promelas*), with a corresponding NOAEC (no observable adverse effect concentration) of 46 mg/L. The water flea (*Ceriodaphnia dubia*) was found to be slightly less sensitive to BT, with an LC50 of 102 mg/L [10]. These concentrations are very high, and would suggest that these triazoles are unlikely to pose an acute threat to wildlife. However, there is little information that we are aware of concerning chronic toxicity of these chemicals. One study has tested the chronic toxicity of BT in a 21-day reproduction toxicity test using *Daphnia magna*, and reported a NOEC (no observed effect concentration) of 3 mg/l [11].

Based on these data, a predicted no effect concentration of 0.06 mg/L was calculated for BT [12]. Despite the apparently low acute toxicity of BT, the lack of chronic toxicity data available for this compound is somewhat surprising given that BT is highly persistent in the aquatic environment.

One of the major environmental concerns over BT is the widely acknowledged fact that it is highly resistant to biodegradation. For example, Breedveld et al. incubated BT for a five-month period with innoculum from airport soil matrices [6]. No evidence of biodegradation was observed, although removal of BT was observed under aerobic conditions. This was thought to be a result of evaporation, since a similar rate of removal was also observed in the abiotic control. Various other studies have also reported no evidence of microbial degradation of BT and its derivatives [12-15]. There have, however, been reports of photodegradation of this compound [16, 17], which may explain experimental losses in the absence of biodegradation, and prove useful in the consideration of remediation processes.

In addition to the persistent nature of BT, it is a highly soluble compound, with a water solubility of 28 g/L [2], and it is also weakly hydrophobic, possessing a log $K_{ow}$ of 1.23 [18]. A combination of these factors suggests that BT will be both available to aquatic organisms, as well as having the potential to be taken up by them. Two derivatives of BT have been detected in tissue extracts of fathead minnows downstream of ADAF-contaminated effluent outfall [19], demonstrating that this class of chemicals can be taken up by aquatic life-forms. This evidence, combined with the fact that BT has been found ubiquitously in those European

waters for which it has been tested, highlight the need for chronic toxicity tests to determine whether this compound poses a real risk to the aquatic ecosystem. The use of BT as a standard ingredient in dishwasher detergents across Europe indicates that the situation described in Switzerland by Voutsa et al. [1] will be reflected in other affluent European countries where dishwasher use has risen over recent years.

In the current study, BT was tested across a wide range of concentrations in the yeast estrogen screen for both estrogenic and anti-estrogenic activity. It was then assessed for anti-estrogenic activity in a fathead minnow vitellogenin assay to determine whether the in vitro message could be reproduced in an in vivo assay. Three concentrations were tested in vivo: the two lower test concentrations were within the range detected in the environment. A third, higher concentration of BT was also tested to increase the possibility of detecting a significant effect, thereby providing “proof of principle”.

## Materials and Methods

### *Reagents*

1H-Benzotriazole (≥99%), 17ß-estradiol (≥98%) and 4-OH-tamoxifen (98%) were obtained from Sigma Aldrich (Gillingham, Dorset, UK); 5,6-dimethyl-1H-benzotriazole monohydrate (5,6-dimethyl-BT) from Fluka (Gillingham, Dorset, UK). Oasis HLB (Hydrophilic-Lipophilic Balance) cartridges for solid phase extraction (SPE) were purchased from Waters (Elstree, Hertfordshire, UK).

**In vitro assays**

The yeast estrogen and anti-estrogen screens have been previously described [20-22]. Essentially, a gene for the human estrogen receptor (ERα) has been integrated into the main yeast genome, and is expressed in a form capable of binding to estrogen response elements and controlling the expression of the reporter gene, *lac-Z*. On activation of the receptor, the *lac-Z* gene is expressed, producing the enzyme β-galactosidase, which is secreted into the medium where it causes a colour change from yellow to red. The intensity of the red colour can be easily measured by absorbance.

The standard assay procedure, according to Routledge et al. [20], was followed for the estrogen screen. Estradiol (E2) was serially diluted from $2.72 \times 10^{-6}$ to $1.33 \times 10^{-9}$ g/L, and BT was serially diluted from 0.1 to $5 \times 10^{-5}$ g/L. For the anti-estrogen screen, The E2 was added to the medium, at a concentration of $6.8 \times 10^{-8}$ g/L. This was sufficient to induce a submaximal elevation in background absorbance in the absence of any other (chemical) influences. In the presence of a chemical which inhibits the activity of E2, a dose-dependent decrease in β-galactosidase expression (and therefore a reduction in intensity of colour change) can be observed. The positive control employed in this assay was the anti-estrogen 4-OH-tamoxifen, which was serially diluted from $4 \times 10^{-3}$ to $2 \times 10^{-6}$ g/L.

**In vivo assay**

*Test organisms*

Juvenile fathead minnows were obtained from Osage Catfisheries (Osage Beach, MO, USA). These were maintained in a recirculating aquarium system until they reached sexual maturity, when males and females could be distinguished from one another. Prior to the onset of the study, the fish were acclimatised in a flow-through tank system at equivalent conditions to those which they would experience during exposure to the test chemicals. Fish were fed twice daily throughout, once with frozen brine-shrimp, and once with flaked fish food.

*Test apparatus*

The flow-through system consisted of 30 L glass aquaria, to which carbon-filtered, heated (25˚C) water was supplied at a rate of 300 ml/min. Stock solutions of the test chemicals were applied using a multichannel peristaltic pump (205U; Watson -Marlow, Falmouth, Cornwall, UK), and were conveyed to mixing vessels together with the water supply to ensure as near as possible a homogeneous solution, prior to delivery to the tanks themselves. Estradiol was dissolved in dimethylformamide (DMF), and stocks were delivered at a rate of 0.02 ml/min, resulting in a final DMF concentration in the fish tanks of 67 µl/L, which is below the concentration recommended (100 µl/L) for solvent use in aquatic toxicity testing [23]. BT has a high water solubility, and is a very stable compound, and hence the BT stock solutions were prepared in water, not solvent. This helped to limit the overall solvent concentration. The rate of supply of the BT stocks was 0.1 ml/min. Tanks were equilibrated with the test chemicals for a period of 1 week prior to the introduction of the fish. The photoperiod was

maintained at 16:8 h light:dark throughout the study period. Temperature and dissolved oxygen were monitored daily.

*Experimental design*

Two experiments were run concurrently; one using adult male and the other using adult female fathead minnows. Since the aim of the study was to assess the potential for BT to inhibit estrogenic activity, E2 (100 ng/L) was added to the tank water in which the males were exposed, in order to stimulate an estrogenic response (in this case, vitellogenin (VTG) induction). This was not necessary for the female fish, since their endogenous levels of E2 were sufficient to detect high levels of VTG without the addition of external estrogens.

Both the males and the females were exposed to three different concentrations of BT: 10, 100 and 1000 µg/L. For females, the negative response was determined from fish exposed to water alone. For males, two controls were employed; a positive control consisting of exposure to 100 ng/l E2, for direct statistical comparison against the BT-treated groups, and a negative solvent control (67 µl/L DMF). The positive control was designed to induce a sub-maximal VTG response, according to previously obtained dose-response data for E2 in fathead minnows in this system [24]. The negative control was in place in order that if BT was observed to be anti-estrogenic, it could be judged whether this chemical was capable of completely inhibiting the effect of E2. A total of eight fish were deployed to each treatment tank, and the exposure was maintained for a period of two weeks. At the end of the study, fish were anaesthetised, and the length and weight of each fish was recorded, as well as

liver and gonad weights. Blood samples were collected from the caudal peduncle using heparinised capillary tubes, and were centrifuged at 4000 *g* for 5 m, following which plasma was removed and stored at -20˚C prior to analysis for VTG. Plasma VTG concentrations were determined using an Enzyme-Linked Immunosorbent Assay (ELISA) that had previously been validated for measurement of fathead minnow VTG [25].

Water samples were collected for chemical analysis at three points during the study, once prior to introduction of the fish (*t*=0), and after one week and two weeks of exposure (*t*=1 and *t*=2, respectively). Samples collected for BT analysis were stored at 4˚C (for a maximum of 24 h) prior to extraction, and those collected for E2 analysis were extracted directly into ethyl acetate, and subsequently stored at -20˚C until analysis by radioimmunoassay.

*Statistical analysis*

The data from the in vivo exposure were assessed for normality of distribution. Vitellogenin concentrations were log-transformed to normalise the data. The variances of the data sets were also confirmed to be homogeneous. Data were subsequently analysed by one-way analysis of variance (ANOVA).

***Chemical analyses***

*E2 analysis*

A volume of 1ml tank water was extracted with 1 ml ethyl acetate, which was subsequently analysed for E2 concentration using a radioimmunoassay as

described by Carragher [24]. The detection limit of this assay was 0.02 ng/ml; cross reactivity with other estrogens (estrone and estriol) was <0.5%, and with other steroids was <0.005% [26].

*BT analysis*

*Enrichment of BT.*

Benzotriazole was enriched from 100 ml of tank water through OASIS HLB cartridges (Waters; 60 mg, 3 ml). Solid-phase extraction was performed using a 12-port vacuum extraction manifold. The cartridges were sequentially conditioned with 2 x 3 ml of methanol and 2 x 3 ml of dd$H_2O$ water by applying a slight vacuum. Water samples were percolated through the cartridges at a flow rate of 5ml/min. The cartridges were dried under vacuum for 10 min and the analytes were eluted with 1.5 ml of dichloromethane containing 3% methanol. The eluates were evaporated to dryness under a gentle stream of nitrogen. Dry residues were redissolved in 0.5 – 1 ml of the liquid chromatography (LC) mobile phase (methanol: water 7:3 with 0.4% formic acid).

*Separation, Detection and Quantitation*

Underivatized extracts dissolved in the LC mobile phase were analyzed by LC-MS/MS (mass spectrometry/mass spectrometry) for BT. 5,6-Dimethyl-BT served as surrogate standard. An HP Series 1100 system from Agilent (Santa Clara, California, US), coupled with a triple quadrupole mass spectrometer (API 4000) from Applied Biosystems (Warrington Cheshire, UK) equipped with a vacuum solvent degassing unit, a binary high-pressure gradient pump, an automatic sample injector and a column thermostat was used. Separation was

accomplished with a 125 x 2.1 mm i.d. endcapped C8 column (Macherey-Nagel, Düren, Germany). Isocratic elution was used with a mixture of methanol/water/formic acid (70:30:0.4) as a mobile phase at a flow rate of 0.2 ml/min. Detection of the analytes was accomplished with electrospray ionization in positive mode and using multiple reaction monitoring (MRM). The following main ions $[M+H]^+$ and two or three fragment ions for MS determination were chosen: for BT from *m/z* 120 to 64.85 and 92.05, and for 5,6-dimethyl-BT from *m/z* 148 to 77.05, 90.95 and 92.85. Due to the highly specific detection, only a partial chromatographic separation was necessary. Thus, short analysis times of only 5 min could be applied. The quantification of BT was carried out by calculating the relative response factors based on the area of 5,6-dimethyl-BT. Six calibration standard solutions (10 to 500 ng absolute) were used to produce a calibration curve for BT relative to the internal standard. Recovery was 99±8%. The limit of detection calculated as three times the standard deviation of low level standard was 8 ng/l for BT, and the limit of quantitation (10 times the standard deviation) was 30 ng/l.

## Results and discussion

The activity of BT in the yeast estrogen screen and anti-estrogen screen can be seen in Figures 1a and 1b, respectively. There was no agonistic estrogenic activity exhibited by this chemical at the concentrations tested in this study. Conversely, there was clear evidence of anti-estrogenic activity, with BT possessing approximately one-hundredth the potency of the positive control, 4-OH-tamoxifen. Benzotriazole was subsequently assessed for its ability to inhibit

β-galactosidase activity (the key enzyme response in this assay), in the absence of the recombinant yeast organism. There was no evidence of inhibition of β-galactosidase by BT (data not shown), and it was therefore concluded that the anti-estrogenic activity observed in the yeast anti-estrogen screen was truly estrogen receptor-mediated.

There are few examples of industrial endocrine disrupting chemicals discovered to date which fall into the category of “anti-estrogen”; the majority have been found to possess either estrogen-agonistic or anti-androgenic properties. It was therefore considered that it would be interesting to determine whether BT could behave in an anti-estrogenic manner in vivo, in a commonly used ecotoxicology fish species, the fathead minnow, and if so, to examine the interaction of this chemical with other classes of endocrine disruptors. A study was designed to provide an answer to this question - is BT also anti-estrogenic in vivo? Both E2-stimulated male fish and adult female fish were used in this study. The former would provide a measure of whether BT could antagonise E2 activity via a receptor-mediated pathway. The rationale of using adult females as well as E2-stimulated males was that if no effect of BT was observed in the male fish, but a significant effect (a reduction in the plasma vitellogenin concentration) was observed in the female fish, the response in the females may be a result of antagonism via some other mechanism. For example, some azoles (including imidazoles and triazoles) are known to possess aromatase-inhibiting properties [27], and fadrazole is a member of the azole family which is used as a human pharmaceutical in breast-cancer treatment due to its ability to inhibit aromatase activity. Thus, there was the possibility that BT could have anti-estrogenic

properties not because it was an antagonist of the ER, but because it inhibited synthesis of estrogens by inhibiting aromatase, the enzyme that converts androgens to estrogens. Nevertheless, it is also apparent that the response observed in the yeast anti-estrogen screen was receptor-mediated, and so this was the expected mode of action.

Analysis of the BT concentrations during the in vivo exposure revealed that the levels were remarkably consistent between different tanks, as well as across all timepoints, with mean values falling between 80 and 86% of the nominal values in all tanks. The mean E2 concentrations in the tanks containing male fish were slightly lower than nominal concentrations, as is frequently observed in such studies. Nevertheless, the measured concentrations of 74, 70, 57 and 67 ng/L were sufficient to induce measurable, but submaximal, vitellogenin synthesis in all tanks. Despite the slightly lower concentration (57 ng/l) of E2 measured in one of the tanks (tank 5, which contained E2 plus 100 µg/L BT), it was estimated that the concentration of plasma vitellogenin inducible by the respective concentrations of E2 in the fish in all four E2-treated tanks would not be significantly different from one another (based on concentration-response data from Brian et al. [24]. Therefore, if any differences were observed, they would be attributable to the presence of BT and not to the slightly different concentrations of E2.

The gonadosomatic (GSI) indices of fish in each treatment group are shown in Figure 2. There were no significant differences observed due to any of the treatments. Although there was an apparent increase of GSI in the female fish exposed to the highest dose of BT (1000 µg/L), there were no statistically

significant differences between treatment groups. The groups of male fish exposed to E2 (with or without BT) all have slightly reduced GSIs compared to the negative control, which is almost certainly a result of exposure of the fish to E2 [28]; none of the BT treatments were significantly different from that of the (positive) E2-control. In summary, BT had no effect on the GSI of either sex of fish.

Plasma vitellogenin concentrations measured in male and female fathead minnows are shown in Figures 3a and 3b, respectively. There were no observable differences between the E2-control group, and the E2+BT-treated male fish. Likewise, there were no discernible differences between the control group and any of the groups of BT-exposed female fish. Thus, BT had no effect whatsoever on the plasma vitellogenin concentrations of either sex. These data imply that BT possesses no anti-estrogenic activity either receptor-mediated or via any other mechanistic pathway in fathead minnows at the concentrations tested here.

The log $K_{OW}$ of BT is 1.23 [18], indicating that this chemical is weakly lipophilic. It is therefore possible that, not only will BT be readily absorbed by fish from water bodies in which it is present, but that it may also accumulate to some degree in their tissues. Although BT itself has not yet been measured in aquatic organisms, two related chemicals (5-methyl-1H-benzotriazole and 4-methyl-1H-benzotriazole) have been detected in tissue extracts from fathead minnows placed downstream of an effluent outfall which receives ADAF-contaminated runoff [19]. It seems legitimate, therefore, to infer that BT is also capable of

crossing the water/organism interface in fathead minnows. To our knowledge, the bioconcentration factor (BCF) of BT has not yet been determined, and so the degree to which it accumulates in aquatic organisms is as yet unknown.

Although a positive control (in the form of either a known anti-estrogen or aromatase-inhibiting chemical) was not used in this study, it is unlikely that the lack of anti-estrogenic activity observed in our studies was a result of the lack of ability of our fish to respond to such chemicals. It has already been demonstrated that a suppressed vitellogenin response occurs in juvenile fathead minnows exposed to a pharmaceutical anti-estrogen [29]. Further, the aromatase inhibitor fadrazole has been shown to reduce vitellogenin concentrations in adult female fathead minnows [30, 31]. These data suggest that if BT does act via either a receptor-mediated anti-estrogenic pathway (as implied in the in vitro study conducted here), or via an anti-aromatase pathway similar to that of some of its related azole family members [27], effects would be observable using the fathead minnow vitellogenin assay undertaken here.

It is still possible that higher concentrations of BT would demonstrate some anti-estrogenic activity, but the concentrations used here (up to 1000 µg/L) far exceed those reported in surface waters (up to 3.69 µg/L in the River Glatt, Switzerland, [1]; 0.9 µg/L and 3.4 µg/L in Lake Tegel and the Landwehr Canal, respectively [8]). However, much higher concentrations of BT (up to 126 mg/L) have been measured in groundwater samples collected from a perched water monitoring well at an international airport [4]. It is also feasible that effects could be induced by longer-term exposures, which may give this moderately lipophilic

399 substance the potential to accumulate to an effective concentration in the tissues
400 of the exposed organism.
401
402 In summary, although clear anti-estrogenic activity of BT was
403 demonstrated in vitro, no evidence of anti-estrogenic activity was observed in the
404 in vivo assay undertaken for this study. Nonetheless, we consider that the lack of
405 chronic toxicity data currently available for this compound is unacceptable,
406 particularly given that it has been described as “toxic to aquatic organisms and
407 can cause long-term adversary effects in the aquatic environment” [12], is highly
408 persistent in the aquatic environment, and has been found to be ubiquitous in
409 Swiss river WWTP effluents and their receiving waters. More effort should be
410 applied in determining the long-term risk of this chemical to aquatic organisms.
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418
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420 **References**
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552 **Figure Legends**

553

554 **Figure 1**

555 Activity of benzotriazole in A) the recombinant yeast estrogen screen; B) the
556 recombinant yeast anti-estrogen screen. The 'control' sample shown in figure 1B
557 shows the activity of the assay with a submaximal concentration of estradiol
558 added to the yeast medium.

559

560 **Figure 2**

561 Gonadosomatic indices (GSI) of both male and female adult fathead minnows
562 exposed to benzotriazole (BT)for 14 d. Males were exposed concurrently to a
563 nominal concentration of 100 ng/l estradiol (E2). Results are presented as
564 means with standard error (*n*=8 in all cases).

565

566 **Figure 3**

567 Vitellogenin concentrations in A) male fathead minnows exposed to various
568 concentrations of benzotriazole (BT), plus a constant concentration of estradiol
569 (E2) for 14 d; B) female fathead minnows exposed to BT alone for 14 d. In every
570 treatment, *n*=8, and each dot represents the plasma vitellogenin concentration of
571 a single fish (some points overlay others, hence 8 dots are not apparent in each
572 treatment).