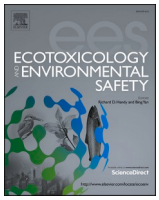
Ecotoxicology and Environmental Safety 307 (2025) 119411

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



In vivo measurement of xenobiotic detoxification in annelids: ECOD activity in a terrestrial, a freshwater, and an estuarine worm

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ARTICLE INFO

Keywords:
Enzymatic assay
ECOD
Cytochrome P450
Azole inhibition
Annelids

ABSTRACT

Cytochrome monooxygenases (CYPs) play a pivotal role in xenobiotic detoxification through chemical biotransformation. *In vitro* methods for measuring these CYPs commonly rely on samples obtained from homogenised tissues. Such preparations, however, often lead to enzyme degradation and auto-inhibition, rendering them unsuitable for some species. Methods to quantify CYP activity *in vivo* have been proposed as an alternative to the use of homogenised tissues. However, such methods remain limited, especially for soil- and sediment-dwelling species. This study aimed to adapt and validate an *in vivo* 7-ethoxycoumarin-O-deethyllase (ECOD) assay previously used for aquatic invertebrates to measure CYP-dependent enzyme activity in three annelid species: *Enchytraeus crypticus* (terrestrial oligochaete), *Capitella teleta* (estuarine sediment-dwelling polychaete), and *Tubifex tubifex* (freshwater sediment-dwelling oligochaete) exposed in water. The successful development and use of the assay demonstrated ECOD activity in *E. crypticus* and *C. teleta*, with activity in *C. teleta* up to 17 times higher than in *E. crypticus* per milligram wet weight. In contrast, no measurable ECOD activity was observed in *T. tubifex*, likely reflecting assay sensitivity limitations for this species. CYP inhibition studies with azole fungicides (propiconazole, imazalil) confirmed that the assay is able to detect concentration-dependent CYP inhibition in *E. crypticus*, highlighting the potential of the method for toxicological assessments of CYP activity and its inhibition or induction. To our knowledge, this study is the first to develop and apply an *in vivo* assay to measure CYP activity in a terrestrial and annelid species.

1. Introduction

Cytochrome monooxygenases (commonly abbreviated as CYPs or P450s) are enzymes that play a crucial role in the metabolism of endogenous and exogenous organic chemicals in organisms. CYPs are a complex group of enzymes, with a range of class members and families that have developed through various evolutionary pathways across diverse taxa (Reviewed in Nelson, 2013), including in plants (Schuler, 1996), bacteria (Greule et al., 2018), fungi (Črešnar and Petrič, 2011), invertebrates (Lu et al., 2021; Nelson, 2018; Snyder, 2000), and humans (Guengerich, 2015). CYPs have a range of functions in endogenous chemical biosynthesis. They produce fatty acids and sterols used in the formation of cell walls, membranes, cuticles, signalling molecules and defence compounds, and work in the metabolism of many compounds, turning contaminants into more excretable hydrophilic derivatives (Buhler and Wang-Buhler, 1998; De Montellano, 2013) and activating pro-mutagens into their mutagenic forms (Abu-Bakar et al., 2022). Research on this detoxification potential of CYPs is essential in various research areas, including pharmacology, environmental toxicology and crop protection science (De Montellano, 2013; Nelson, 2013).

Changes to CYP activity, whether through inhibition or induction, can significantly affect organism biology. CYP induction is widely recognised as an adaptive response to organic chemical exposure, enhancing xenobiotic metabolism, while CYP inhibition can increase the toxicity of certain chemicals by slowing their biotransformation (Lu et al., 2021) or, in the case of compounds that require oxidation to become active (e.g. organophosphate pesticides), can result in reduced toxicity as the more potent transformed metabolite (e.g. an organophosphate-oxon) is produced at a slower rate (e.g. Cedergreen et al., 2006; Rider and LeBlanc, 2005). Alterations to CYP activity can

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https://doi.org/10.1016/j.ecoenv.2025.119411
Received 11 July 2025; Received in revised form 10 November 2025; Accepted 11 November 2025
Available online 14 November 2025

also influence how organisms process combinations of chemicals, potentially exacerbating or mitigating the toxic effects of mixtures (Martin et al., 2021). Furthermore, increased CYP activity can indicate a transient response to ongoing pollutant exposure for use in biomonitoring (Swart et al., 2022) or be a permanent result of artificial selection of resistance traits (e.g. repeated pesticide use; (Nauen et al., 2022; Liu et al., 2015). Altogether, studying CYP activity can provide valuable insights into organism biology under pollution stress and detoxification mechanisms, helping to explain the toxicity of both single compounds and chemical mixtures on species.

In invertebrates, the most common method for quantifying CYP activity uses fluorometric assays that measure enzymatic cytochrome P450 conversion of a substrate into a fluorescent product, which can then be measured using a spectrofluorometer (Gagnaire et al., 2010; Tsyrlov and Lyakhavich, 1978). One such technique is the *in vitro* measurement of 7-ethoxycoumarin-O-deethylase (ECOD) activity, where 7-ethoxycoumarin, the substrate, is turned into 7-hydroxycoumarin, the product. The ECOD assay is part of a family of fluorometric assays that quantify CYP-dependent activity towards various substrates, including 7-ethoxy-, 7-methoxy-, or 7-pentoxyresorufin (EROD, MROD, and PROD assays). While the *in vitro* EROD assay has been associated with CYP1A content in fish (Burkina et al., 2018), the ECOD assay has a broader specificity, as found in human tissues, showing activity of multiple isoenzymes from the CYP1, CYP2, and CYP3 families (Waxman and Chang, 2006). Furthermore, it is noted for its low operational costs (Gottardi et al., 2016). These *in vitro* assays traditionally use homogenates either from specific tissue (e.g. organs) or the whole organism before measuring the CYP activity of the extracted microsomal fraction (Gottardi et al., 2016). Indeed, the *in vitro* ECOD assay has successfully revealed CYP ECOD activity for Lepidoptera (Chen et al., 2023; F. Li et al., 2023; Ngegba et al., 2023; Parra Morales et al., 2017), Diptera (Civolani et al., 2021; Frank et al., 1997; Gottardi et al., 2016; Lee and Scott, 1989, 1992), aphids (Wu et al., 2022), Coleoptera (Khan et al., 2021; Pedersen et al., 2019; Sun et al., 2021), termites (Haritos et al., 1994), molluscs (Bivalves: Dauberschmidt et al., 1997; gastropod: Lowe et al., 2006), nematodes (Kotze, 1999), and earthworms (Booth et al., 2002; Liimatainen and Hanninen, 1982). Despite successes, there have also been published cases where this approach fails to show CYP activity *in vitro*, such as in crustaceans (Gottardi et al., 2016), a chiton mollusc (Schlenk and Buhler, 1988) and a nematode, where Kotze (1999) found ECOD activity in L3-stage juveniles of the nematode *Haemonchus contortus*, but not in the adults.

A major critique of the *in vitro* ECOD assay is that it relies on tissue homogenisation. Gilbert and Wilkinson (1975) hypothesised that this homogenisation step can inhibit or deactivate CYP enzymes due to proteases or inhibitors in, specifically, eye tissue or the guts of some species. Gottardi et al. (2016) detected *in vivo* ECOD activity in *Daphnia magna,* but this activity was not found in a microsomal fraction of the same species, indicating potential degradation during sample preparation. Indeed, these authors further demonstrated that exposure to the homogenate inhibited ECOD activity in rat liver microsomes, while the fluorescence measurement remained unaffected. This inhibitory effect of homogenate on CYP activity has also been observed in terrestrial organisms, such as the beetle *Tenebrio molitor* (Pedersen et al., 2019) and the earthworm *Lumbricus rubellus* (Brown et al., 2004). In aphids, it was observed that the homogenate of *Acyrthosiphon pisum* and *Myzus persica* strongly inhibited CYP activity in pig liver microsomes, but the homogenate of *Rhopalosiphum padi*, a closely related species, did not (Wu et al., 2022). Together, these findings indicate that species-specific and life-stage-specific factors can strongly affect CYP activity after tissue homogenisation, making it difficult to conclude on the appropriateness of this approach for certain species.

The *in vivo* ECOD assay, an alternative to the *in vitro* method, is based on the addition of the chemical substrate directly to the liquid exposure medium in which an organism is kept. As the CYPs in the organism break down the substrate, the product is excreted into the surrounding water, where it can be directly measured. This *in vivo* ECOD assay approach has successfully been developed to measure CYP activity in caddis flies, may flies, an annelid, an isopod (Dalhoff et al., 2020), a flatworm (Li, 2016), gastropods (Dalhoff et al., 2020; Gagnaire et al., 2010), a midge (Cedergreen et al., 2021; Gottardi et al., 2016) and crustacean species (Ács et al., 2024; Farkas et al., 2022; Cedergreen et al., 2021; Dalhoff et al., 2020; Gottardi et al., 2016; David et al., 2012). To date, the *in vivo* ECOD assay has only been applied to freshwater and marine species, or the larval stages of hemimetabolous aquatic insects, following their incubation in a water medium. Indeed, *in vivo* assays are difficult to operationalise in soil due to the difficulty of extracting and measuring the enzymatic product (Pedersen et al., 2019).

Related to the difficulty of testing in soil, terrestrial annelid earthworms have not to date been assessed for CYP-dependent activity using an *in vivo* approach. Nevertheless, ECOD activity has been detected *in vitro* in the homogenised gut tissue of the larger earthworms *Aporrectodea caliginosa, Lumbricus rubellus* (Booth et al., 2002) and *Lumbricus terrestris* (Liimatainen and Hanninen, 1982). *Enchytraeus crypticus,* another smaller soil-dwelling oligochaete with recently acquired whole genome, has been posited as a soil model organism (Amorim et al., 2021) and has shown CYP-dependent activity in *in vitro* assays (i.e. 7-ethoxyresorufin-O-deethylase assay; Achazi et al., 1998). Bioassay studies in our laboratory have shown that this species can survive in a water medium for up to two weeks. This potential to live for an extended period in water could allow for *in vivo* ECOD measurement approaches as for aquatic and marine species. The sediment-dwelling estuarine polychaete, *Capitella teleta* (formerly *Capitella* sp. I), is commonly found in sites with high organic contamination (Blake et al., 2009; Pearson and Rosenberg, 1978). It has previously been investigated for detoxification potential, revealing that closely related subspecies of the *Capitella spp.* species complex can have large differences in CYP biotransformation rates (Selck et al., 2003). Whole genome annotation of *C. teleta* has revealed 96 functional CYPs, including CYP51A1, belonging to 9 out of the 11 metazoan CYP clans (Dejong and Wilson, 2014). *Tubifex tubifex*, a freshwater sediment-dwelling oligochaete, has also been shown to have *in vivo* ECOD activity, albeit $>$ 40 times lower than *Daphnia magna* (Dalhoff et al., 2020). The evidence for this CYP activity in these worms, combined with the known role of CYPs in detoxification and the tendency of annelids to inhabit contaminated environments, means there is an interest in understanding CYP functionality across species in this phylum.

In the present study, we aimed to adapt the protocol by Gottardi and Cedergreen (2019) to measure *in vivo* ECOD activity in the terrestrial oligochaete *E. crypticus* and the sediment-dwelling polychaete *C. teleta* and oligochaete *T. tubifex*. This method was used to assess CYP-dependent activity toward 7-ethoxycoumarin, as a proxy for xenobiotic detoxification. In a first experiment, we verified method sensitivity to detect CYP inhibition through the addition of the triazole fungicide propiconazole, a known CYP inhibitor in *Daphnia magna* and *Chironomus riparius* (Gottardi and Cedergreen, 2019) and a known synergist in *E. crypticus* (Bart et al., 2022). In a second experiment, we demonstrated the potential of the method in a full concentration-response relationship for CYP inhibition in *E. crypticus* by the imidazole fungicide imazalil. Finally, we discuss the strengths and limitations of the *in vivo* ECOD assay for terrestrial, freshwater, and estuarine annelid species, as well as its potential for measuring detoxification in experiments with higher ecological complexity.

2. Materials & methods

2.1. Chemicals

7-ethoxycoumarin (CAS: 31005–02–4, Catalogue number: E1379–25MG, purity: ≥99.45 %), 7-hydroxycoumarin (CAS: 93–35–6, purity: ≥98.0 %), propiconazole (CAS: 60207–90–1, purity: ≥98.0 %), imazalil (CAS: 35554–44–0, purity: ≥98.0 %), and acetonitrile (CAS:

75–05–8, purity: ≥99.8 %) were obtained from Sigma-Aldrich (Poole, UK). BCA Protein Assay Kit 1 L (catalogue number: 23225) and Oxoid™ phosphate-buffered saline (Dulbecco A)(catalogue number: BR0014G) were bought from Thermo Fisher Scientific (Loughborough, UK). Stock solutions of propiconazole, imazalil, 7-ethoxycoumarin and 7-hydroxycoumarin were prepared in acetonitrile. All stock solutions were stored in glass vials and were freshly prepared for every experiment.

2.2. Worm culturing

The *Enchytraeus crypticus* (soil-dwelling Oligochaete) (Westheide and Graefe, 1992) culture was sourced originally from the Vrije Universiteit Amsterdam (NL) and has been maintained in culture at our experimental facility for > 5 years. Cultures used to produce the organisms for testing were kept in plastic boxes on 2.5 % w/w agar medium made with artificial freshwater (Roembke and Knacker, 1989). These potworms were reared at 20 °C under complete darkness in a climate chamber and fed *ad libitum* on a batch-cooked porridge diet containing oatmeal (150 g), baker's yeast (50 g), milk powder (50 g), cod liver oil (50 mL), and milk (500 mL). This porridge was kept frozen in small plastic bags and defrosted before use.

Capitella teleta (sediment-dwelling Polychaete) (Blake, Grassle and Eckelbarger, 2009) were initially collected from a high organic matter sediment in Setauket Harbor, New York, USA and have been maintained at Roskilde University, Denmark, in clean culture for > 35 years (Ramskov et al., 2009). Worms were cultured in laboratory conditions in aquarium tanks (approx. 2 L), each containing 100 g (wet weight) of ≤ 125 μm purified sediment (following Grønlund et al., 2024) and 1500 mL of water. *C. teleta* were supplied with natural filtered seawater (<0.2 μm) with a salinity of 31–32 ppt, which was continuously aerated using a pump, silicon tube, and aeration stone and kept at a temperature of 20 (±1) °C.

Tubifex tubifex (sediment-dwelling Oligochaete) (Muller, 1774) were obtained from Bonnies Dyrecenter (Denmark) and were cultured under the same conditions as *C. teleta* but in artificial freshwater following OECD guideline 315 (OECD, 2008).

Sediment for rearing was collected from Munkholmbroen in Isefjord, Roskilde, Denmark (55° 40′25″N 11°48′44″E), in September 2022 by scraping the top few centimetres of the sediment surface. The organic matter content of the sediment was determined to be 20.8 % by the loss-on-ignition method. The sediment was wet sieved coarsely (≤ 125 μm) for rearing and finely (≤ 63 μm) for use as a food supplement, using deionised water as described by Grønlund et al. (2024). Both sieved sediments were then frozen until use. Before use in culture aquaria, sediment was thawed, rinsed with medium (salt- or freshwater) specific for the target organism, and homogenised with an immersion blender. The sediment was added to the aquaria (ca. 5 cm layer), overlayed with water medium, and worms were added. In addition to the coarse sediment, diet was supplemented weekly with ca. 15 g of fine (≤ 63 μm) wet sediment for *C. teleta* and with 0.5 g of artificial food for *T. tubifex*, which consisted of equal parts commercial fish food (Wardley*), baby cereal (Milupa), and spinach (Forbes et al., 1996), which was dried and ground before use.

2.3. Experimental design

This study consisted of two parts: optimisation of the method, followed by experimental testing of the approach. In the first part, we optimised the assay to measure the development of the ECOD activity over time for each of the three annelid species. This comparison allowed us to gauge the relative CYP activity profiles of the three species under normal physiological conditions. In the second part, a set of experiments was designed to optimise the assay to assess whether this ECOD assay was sensitive enough to detect the inhibition of CYP activity following exposure to azole fungicides, a class of pesticides known to impact the activity of CYPs in other taxa (Gottardi and Cedergreen, 2019). In the first of these experiments, all three species were exposed to propiconazole or the control treatment (solvent only) to verify the method's sensitivity to inhibition. In a second experiment, *E. crypticus* was further exposed to imazalil at various concentrations or a control treatment (solvent only) and analysed by concentration-response curve analysis. Both experiments included a pre-exposure phase of 18 h, during which the organisms were exposed only to the inhibitor or control (solvent only), and an exposure phase of 4 h, during which they were also exposed to 7-ethoxycoumarin, and *in vivo* ECOD activity was tested.

2.3.1. Method optimisation

The original method (Gottardi and Cedergreen, 2019) was optimised to measure the development of the ECOD activity over time for each of the three selected annelid species. We maintained the number of organisms per sample at four worms per well, but increased the experimental duration from 2.5 h to 4 h to achieve better resolution between control and inhibition treatments. Chemical concentrations were selected based on protocol consensus from previous literature (Cedergreen et al., 2021; Gagnaire et al., 2010; Gottardi and Cedergreen, 2019). No addition of cofactor (i.e. NADPH) was necessary to arrive at a significant difference from the background sample, as previously documented by Pedersen et al. (2019) using dissected guts of *Tenebrio molitor* and *Apis mellifera*. Following Gottardi and Cedergreen (2019), we minimised the amount of acetonitrile solvent to 0.06 % v/v. This is extremely low, and optimisation focused further on minimising chemical weighing errors and avoiding contamination. Precise weighing of chemicals is crucial to obtaining accurate concentrations. Hence, we used a point balance with sub mg precision, utilising *antistatic* weighing funnels (TWD Scientific, ASWF1S). Furthermore, 7-ethoxycoumarin was dissolved straight from the vial containing 25 mg to minimise measuring errors. After method development in the lab, we started to observe contamination in our setup, indicated by extremely high fluorescence values in the experiment. We traced this contamination back to the use of glassware from a previous experiment, despite washing and autoclaving (i.e. these steps might not be sufficient to fully remove 7-hydroxycoumarin from the glass). We therefore implemented an additional cleaning step by acid washing (1 % nitric acid) glassware overnight and thorough RO water rinsing before use in an experiment.

2.4. E. crypticus experiment

The pre-exposure phase for *E. crypticus* was 18 h, over which time we expected significant internal exposure of the worms to the azole fungicide (as in Rösch et al., 2016). This exposure time further allows a direct comparison with the 10 species tested using this approach by Dalhoff et al. (2020). Individual adult *E. crypticus* with developed clitella were taken from the lab culture and put into 48-well plates (Starlab CytoOne non-treated plate) containing artificial freshwater (Roembke and Knacker, 1989) CaCl2 294 mg L^{-1}, MgSO4 123 mg L^{-1}, NaHCO3 65 mg L^{-1}, KCl 5.8 mg L^{-1}). The individual potworms were then exposed to a range of concentrations of propiconazole (0, 6.25, 12.5, 25, 50 mg L^{-1}; Fig. 1A) or imazalil (0, 4.5, 9, 13.5, 18 mg L^{-1}; Fig. 2) dissolved in acetonitrile as the solvent carrier (exposure volume: 500 μL). This solvent was also added to controls to ensure consistent solvent exposure across treatments (solvent conc: 0.06 % v/v)(n.b. acetonitrile was selected as a solvent as it has previously been shown to have the least effect, out of a range of solvents, on CYP activity of various CYPs in rat liver microsomes, Li et al., 2010).

For the exposure, *E. crypticus* were transferred using a small hook to 24-well plates (Starlab CytoOne non-treated plate), with 4 worms per well and 16 replicates per treatment. Each well contained 1.9 mL of artificial freshwater. During the 4-hour exposure, worms were kept at the pre-exposure phase concentrations of propiconazole, imazalil or control conditions. The experiment started by adding 100 μL of 7-ethoxycoumarin to each well (final concentration: 0.02 mM). Aliquots (100 μL) were taken immediately and at 30-minute intervals for 4 h (9

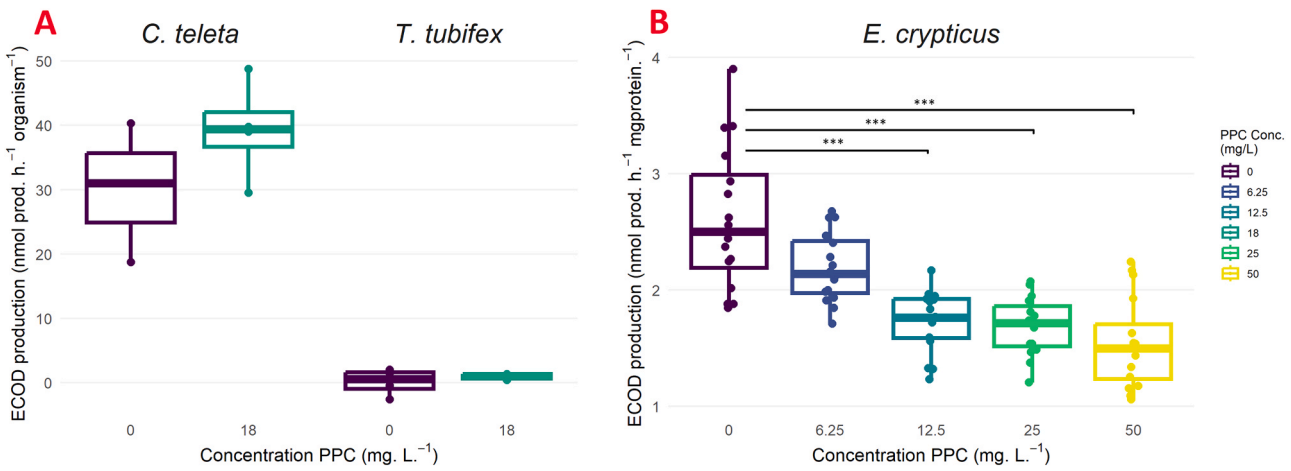

Fig. 1. *In vivo* ECOD activity during four-hour exposure to various concentrations of propiconazole (PPC) in A) *C. teleta* and *T. tubifex*, and B) *E. crypticus*. Boxplots represent median and quartile values of the data and are colour-coded from purple to yellow, corresponding to PPC concentrations of 0–50 mg L^{-1}.

sampling times), transferred to a microwell plate (96 well, opaque base; Thermo Scientific Sterilin Black Microtiter plate), and measured for fluorescence (excitation: 380 nm, emission: 480 nm) using a microplate reader (Agilent Cytation 5) operated at 20 °C. These measurements were calibrated using a dilution series of 7-hydroxycoumarin (0–2 pM).

2.4.1. Protein quantification

To allow ECOD activity of *E. crypticus* to scale to protein levels, protein content was determined for *E. crypticus* using the microplate procedure of the Pierce BCA protein assay kit (ThermoFisher Scientific). Worms were loaded per sample into screw-top Eppendorf tubes and homogenised with a single tungsten bead in 1 mL of PBS (Oxoid; pH 7.3 ± 0.2) for 30 s at 30 Hz using a Qiagen Tissuelyser II. Absorbance was read simultaneously for samples and BCA kit protein standard (working range: 20–2000 μg mL^{-1}) at 562 nm using a microplate reader (Agilent Cytation 5), and sample protein concentrations were calculated from the protein standard calibration curve.

2.5. C. teleta & T. tubifex experiment

The pre-exposure for *C. teleta* and *T. tubifex* was conducted for 18 h to correspond to the *E. crypticus* experiment. Initially, adult worms were sieved (500 μm) from the laboratory culture, weighed and allocated to a treatment group to ensure consistent weight distribution across samples. Worms were placed into pre-exposure vials with four worms per replicate and four replicates per treatment. Each replicate vial lacked sediment, but contained a pre-aerated medium, saltwater for *C. teleta* and artificial freshwater for *T. tubifex* (described for both species under 2.2 Worm Culturing) that was spiked with either 18 mg L^{-1} propiconazole or a control treatment (solvent only) (Fig. 1A). Both treatments contained 0.06 % acetonitrile solvent used as a carrier.

For ECOD activity measurements (i.e. the exposure phase), the four worms per replicate of *T. tubifex* or *C. teleta* were taken from the pre-exposure and placed together into a well of a multi-well plate containing 2 mL of the same spiked culture medium as in the pre-exposure phase. The exposure experiment started by adding 2 mL of this medium, which was spiked with 7-ethoxycoumarin (final concentration: 0.02 mM). During the assay, aliquots of 100 μL were taken over 4 h; sampled every 15 min for 60 min, and thereafter every 60 min until the end of the exposure (i.e. 7 sampling times). These aliquots were transferred to a microwell plate and stored at −20 °C until measurement by fluorescence (excitation: 380 nm, emission: 480 nm) using a microplate reader (Molecular Devices SpectraMax i3x) operated at 20 °C. These measurements were calibrated using a dilution series of 7-hydroxycoumarin (0–8 pM).

2.6. Data analysis

All statistical analyses were conducted in RStudio (RStudio-v2024.04.2 +764; R-v4.4.0) and visualised in GGplot2 (v3.5.1). Blank values from the fluorescence calibration curve were subtracted from all experimental fluorescence values, and divided by the calibration slope, transforming relative fluorescence units into pmol 7-hydroxycoumarin contained per well. Values were then standardised for volume reduction and divided by protein content (*E. crypticus*) or the number of organisms (*E. crypticus, C. teleta,* and *T. tubifex*). Subsequently, the 7-hydroxycoumarin formation rate was calculated as the slope of a fitted linear regression (pmol/h/mg protein or pmol/h/organism). For *E. crypticus* and *C. teleta,* this formation rate has also been recalculated to pmol/h/mg wet weight using the average weight of adult worms (2.49 ± 0.34 mg and 6.16 ± 2.08 mg, respectively) to facilitate direct comparison between these two species. Product formation rate was compared between controls and treatments using a one-way ANOVA, with significance at $P < 0.05$. For the *E. crypticus* inhibition experiment with imazalil, product formation rate of all samples was fitted with a 3-parameter log-logistic model as a concentration-response curve (Fig. 2B) using the drc package in RStudio (v.3.0–1).

3. Results

3.1. In vivo ECOD activity in E. crypticus, C. teleta and T. tubifex

Measurements for *E. crypticus, C. teleta* and *T. tubifex* indicated quantifiable ECOD activity for *E. crypticus* and *C. teleta*, but not for *T. tubifex*, across the 4 h measurement period (Fig. S1). Linear regressions fitted to the time series of 7-hydroxycoumarin fluorescence data, indicated a ca. 17–43 times higher ECOD activity for *C. teleta* at 29.45 (±SE 4.14) pmol h^{-1} organism^{-1} (R^2=0.74), or 4.78 (±SE 0.67) pmol h^{-1} mg wet weight^{-1}, than for *E. crypticus* at 0.69 (±SE 0.05) pmol h^{-1} organism^{-1} (R^2=0.59) or 5.10 (±SE 0.33) pmol h^{-1} mg protein^{-1} (R^2=0.64), or 0.28 (±SE 0.02) pmol h^{-1} mg wet weight^{-1}, whereas the activity for *T. tubifex* was at near negligible values at 0.126 (±SE 0.724) pmol h^{-1} organism^{-1} (R^2=0.044) (Table 1).

3.2. Inhibition studies in E. crypticus, C. teleta and T. tubifex with propiconazole

Method sensitivity to CYP inhibition was verified by co-exposing all three species to the known inhibitor propiconazole for 4 h (Fig. 1). For *C. teleta* ($F(1,5)= 1.758$, $p = 0.24$) and *T. tubifex* ($F(1,6)= 0.62$, $p = 0.46$), no significant difference was found in *in vivo* ECOD activity

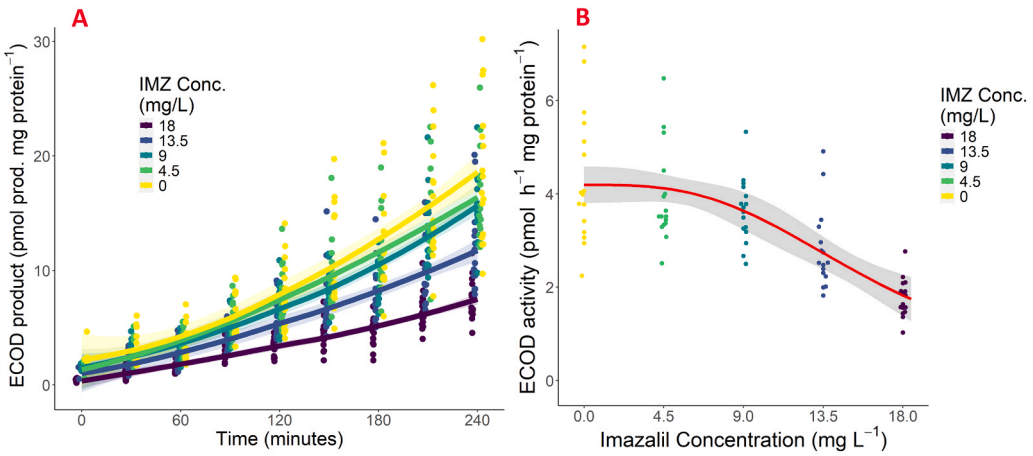

Fig. 2. *In vivo* ECOD activity of *E. crypticus* while exposed to differing concentrations of imazalil A) over time and B) at 4 h exposure. A) Lines represent a LOESS regression (span = 1) coloured and fitted to their respective concentration; 0 (yellow) to 18 mg L^{-1} (purple). Every timepoint treatment includes 16 replicates with 4 worms in each sample. B) 3-parameter log-logistic model (red line) is fitted to the data. Ribbon is 95 % confidence interval.

Table 1
Summary of studies on ECOD activity measured in annelids. Data shows ECOD activity for multiple species - gathered and normalised using various methods.

Study	Species	Compartment	Method	ECOD activity (pmol HC h^{-1} […] ±SE)	Extended Unit
This study	*E. crypticus*	Terrestrial	*in vivo*	0.69 ± 0.05	organism^{-1}
				0.28 ± 0.02[a]	$mg^{-1}_{wet\ weight}$
				5.10 ± 0.33	$mg^{-1}_{protein}$
	C. teleta	Estuarine	*in vivo*	29.45 ± 4.14	organism^{-1}
				4.79 ± 0.67[a]	$mg^{-1}_{wet\ weight}$
	T. tubifex	Aquatic	*in vivo*	0.126 ± 0.724	organism^{-1}
Booth et al. (2002)[a]	*A. caliginosa*	Terrestrial	*in vitro*	6.0 ± 1.8[b]	$mg^{-1}_{protein}$
	L. rubellus			12.6 ± 3.0[b]	
Liimatainen and Hanninen (1982)	*L. terrestris*	Terrestrial	*in vitro*	1.7 ± NA	$mg^{-1}_{protein}$
Dalhoff et al. (2020)	*T. tubifex*	Aquatic	*in vivo*	0.070 ± 0.021 SD	$mg^{-1}_{wet\ weight}$
	A. aquaticus			0.029 ± 0.006 SD	
	C. riparius			0.018 ± 0.003 SD	
	C. villosa			0.945 ± 0.350 SD	
	D. magna			2.847 ± 0.530 SD	
	E. danica			0.020 ± 0.000 SD	
	G. pulex			0.005 ± 0.003 SD	
	H. pellucidula			0.190 ± 0.003 SD	
	H. sulphurea			0.035 ± 0.005 SD	
	R. balthica			0.155 ± 0.020 SD	

[a] data estimated from average adult worm weight gathered in other experiments.
[b] data collected from figure and recalculated from [pmol min^{-1} $mg^{-1}_{protein}$].

between the control and an exposure to 18 mg L^{-1} propiconazole (Fig. 1A); with group means of 30.02 (±SE 6.24) and 39.30 (±SE 3.93) for *C. teleta*, and 0.126 (±SE 1.04) and 0.964 (±SE 0.241) for *T. tubifex*, respectively. In contrast, for *E. crypticus* ($F(4,75) = 17.8$, $p < 0.001$), *in vivo* ECOD activity indicated significant concentration-dependent differences from control for ECOD measurements made in worms exposed to propiconazole concentrations of 12.5 mg L^{-1} and above (Fig. 1B); with group means for control and treatments of 6.25, 12.5, 25 and 50 mg L^{-1} of 2.61 (±SE 0.154), 2.19 (±SE 0.075), 1.73 (±SE 0.066), 1.76 (±SE 0.095), and 1.54 (±SE 0.097), respectively. These contrasting results between species indicate that propiconazole has a lower potential to reduce ECOD activity in *C. teleta* than in *E. crypticus*.

3.3. Inhibition studies in E. crypticus with imazalil

Because *E. crypticus* showed CYP inhibition during exposure to the triazole fungicide propiconazole, we further assessed the method in *E. crypticus* to determine whether this inhibition was also observed for the representative imidazole imazalil in a time-series concentration-response curve analysis. We first visualised the calibrated data over the full time course of the experiment and fitted a concentration-response curve to the calculated ECOD activity rates to study inhibition due to imazalil at the end of the exposure period (Fig. 2A). The concentration-response curve illustrates a monotonic response, with greater ECOD inhibition at higher imazalil concentrations starting from a threshold of 13.5 mg L^{-1} (Fig. 2B). The absolute EC50 for ECOD inhibition by imazalil was estimated at 16.54 mg L^{-1}.

4. Discussion

ECOD activity measurements quantify the CYP450-dependent metabolisation of 7-ethoxycoumarin. This assay can serve as a proxy for the xenobiotic metabolism of chemicals and can be conducted at individual level (*in vivo*), tissues (*ex vivo/in vitro*) or homogenates (*in vitro*). However, the homogenisation process for the *in vitro* ECOD assay can lead to the (auto)inhibition of enzymatic activity for many invertebrates (Gilbert and Wilkinson, 1975; Gottardi et al., 2016). *In vivo* measurement of ECOD activity offers a solution to this problem by avoiding the homogenisation step to limit inhibition potential. In this study, we developed and tested an *in vivo* ECOD assay for measuring CYP-dependent xenobiotic detoxification in three annelid species: *E. crypticus, C. teleta*, and *T. tubifex*; reporting interspecies variability in

ECOD activity, and differences in species sensitivity to CYP-inhibiting chemicals.

4.1. In vivo ECOD activity

Assay results from the current study and the literature show that it is possible to measure the rate of ECOD activity in terrestrial, freshwater, and estuarine annelids using an adaptation of the *in vivo* methods previously used for aquatic species (Gottardi and Cedergreen, 2019). To our knowledge, this is the first time that an *in vivo* ECOD assay has been used to measure CYP activity in a polychaete and a terrestrial annelid species. Our assay results showed a clear interspecies variability in ECOD activity. Thus, measurements of *C. teleta* and *E. crypticus* demonstrated basal or induced activity of the CYP enzymes capable of degrading the substrate, whereas this was not observed for *T. tubifex*. The absence of activity in *T. tubifex* in our results suggests either inherently low ECOD activity in this species or otherwise species- or assay-specific limitations to detection of ECOD activity (e.g. differences in substrate uptake, binding affinity, presence of ECOD-relevant CYPs). This contrasts with results by Dalhoff et al. (2020) who reported *in vivo* activity for *T. tubifex* of 0.070 (±SD 0.021) pmol h^{-1} mg wet $weight^{-1}$. Measured ECOD activity in *C. teleta* was up to 17 times higher than in *E. crypticus* on a per mg wet weight basis, and up to 43 times higher on a per organism basis. This higher ECOD activity reflects potentially greater biotransformation rates for *C. teleta,* which has been known to thrive in sites high in organic pollution (Blake et al., 2009; Pearson and Rosenberg, 1978), likely due to the range and activities of its functional CYPs (Dejong and Wilson, 2014).

Our *in vivo* results for *E. crypticus* and *C. teleta* are in the range reported by Dalhoff et al. (2020) for 10 different aquatic species, indicating that our assay is comparable to previous findings. Despite our lack of significant enzyme activity in *T. tubifex*, Dalhoff et al. (2020) reported this species to be the 5th highest-ranking species out of 10, with medium to low *in vivo* ECOD activity. Our observed *C. teleta* activity is almost twice that reported for *Daphnia magna*, their highest-ranking species, while *E. crypticus'* activity is a factor of 10 below that. Indeed, *E. crypticus* activity is comparable to *Radix balthica,* a freshwater snail, and larvae of *Hydropsyche pellucidula*, a caddisfly, and at about a factor of 3 below the activity observed in *Chaetopteryx villosa*, another caddisfly (Dalhoff et al., 2020).

Comparing our *in vivo* results with previous *in vitro* findings of ECOD activity or other CYP-dependent enzyme assays (e.g. EROD, PROD, MROD) in Annelida is challenging for multiple reasons. First, *in vivo* results will generally be lower than those gathered using intact, undegraded homogenates obtained from *in vitro* approaches, for the *in vivo* approach only measures the 7-hydroxycoumarin released into the test medium and so does not measure all that is produced by CYP activity. Second, because different metrics have been used to normalise ECOD activity measurements. Some researchers have quantified their CYP activity per milligram of protein, while others have used per milligram wet weight or per organism. However, this lack of standardisation points to a larger problem in the wider research area; with which there is a general lack of protocol transparency, underreporting of data, and lack of FAIR access to data. This is particularly evident in the study of pesticide resistance, where CYP activity of a resistant population is often only presented as a percentage of the control population without providing a calibrated baseline activity. We encourage further implementation of the FAIR principles in this research area and the reporting of CYP activity measurements in multiple formats, including standardisation against a weight standard (i.e. mg protein, mg wet weight), which would allow comparisons between species as we have done in Table 1.

4.2. Inhibition studies

The *in vivo* ECOD assay demonstrated sensitivity to CYP inhibition by representative triazole and imidazole class azole fungicides in *E. crypticus* (Fig. 1B & Fig. 2), confirming the inhibitory effects of these chemicals on CYP enzymes as measured by the ECOD assay. The concentration-response relationship observed for imazalil supported the assay's capacity to detect concentration-dependent inhibition in *E. crypticus*. Interestingly, exposure to either azole fungicide resulted in a reduced variance of enzyme activity compared to the control treatment (Fig. 1B & Fig. 2). This reduction in variability seems consistent with the buffering concept of canalisation, which is a tendency of stress, in this case chemical stress, to constrain phenotypic variability of a population (read more Hallgrimsson et al., 2019; Waddington, 1942). In all, our finding aligns with prior studies on azole fungicides that inhibit CYP enzymes in aquatic invertebrates (Gottardi and Cedergreen, 2019) and offers a mechanistic explanation for previously observed synergistic interactions between azoles and other pesticides in *E. crypticus* (Bart et al., 2022).

In contrast to *E. crypticus, C. teleta* did not show significant inhibition of ECOD activity following azole (propiconazole) exposure (Fig. 1A). This can be explained in multiple ways. The presence of 96 functional CYPs in this species (Dejong and Wilson, 2014), from 9 of the 11 metazoan CYP groups, could confer a high detoxification potential to many different chemicals simultaneously, as well as provide functional redundancy in detoxification of compounds, both singly and in mixtures. Hence, the high CYP activity observed in *C. teleta* indicates rapid detoxification of propiconazole, possibly explaining our inability to detect inhibitory effects at this concentration. However, *C. teleta* is also known to contain CYP51A1 (Dejong and Wilson, 2014), which is the CYP target of azole fungicides (Peyton et al., 2015), but it is also known to bind propiconazole tightly, as shown for the CYP51s found in *Candida albicans* and humans (Warrilow et al., 2013). The presence of this specific CYP in *C. teleta* could lead to strong off-target binding of propiconazole, potentially resulting in reduced binding at the target site (e.g. either the CYPs relevant for the ECOD assay, or the toxic target). Such off-target stoichiometric binding has previously been linked to a reduced sensitivity to neonicotinoids in earthworm species (Short et al., 2021). We suggest that future studies should focus on the direct effects of azole exposure, such as inhibition of steroid biosynthesis and endocrine disruption, as well as the indirect effects of azole exposure, including synergistic interactions with toxic compounds (e.g. pesticides).

4.3. Strengths and limitations of the in vivo ECOD assay

The *in vivo* ECOD assay offers several advantages over traditional *in vitro* approaches. As noted earlier, the lack of tissue homogenisation avoids the potential for enzyme inhibition caused by the release of proteases from tissue, something that has proved problematic in some studies of CYP activity in invertebrates (Gilbert and Wilkinson, 1975; Gottardi et al., 2016). The non-destructive nature of the assay also allows for more ecologically relevant assessments by preserving the species' natural and biochemical context, and means it can even be performed in parallel with other assays (e.g. biochemical/enzymatic assays, gene expression). Indeed, with proper controls in place, the *in vivo* CYP approach can simultaneously study multiple ecological compartments. For example, it would be possible to study sediment-dwelling organisms in sediment and water medium simultaneously, while also controlling for microbiota effects.

While there are clear benefits to the assay, there are also some noteworthy limitations. The absence of significant ECOD activity in *T. tubifex* suggests that this assay may not be sensitive enough to detect ECOD activity in organisms with a naturally low level of response, at least using the relatively small numbers of organisms, and therefore biomass, used here. This suggests the need to use species that can be cultured at a larger scale, like *E. crypticus,* allowing higher throughput experiments with greater numbers of organisms. Alternatively, this absence of activity could indicate that the ECOD assay is not a good proxy for CYP activity in *T. tubifex*. It is known that the detoxification

battery of any organism comprises multiple different CYPs and CYP isozymes, leading to metabolisation pathways that might not be detected by the ECOD assay (Feng et al., 2018). This would be the case if the organisms lack the CYPs capable of metabolising a certain substrate or lack a CYP upregulation response after exposure. Further refinement of the sensitivity of the ECOD assay can build upon the recent work of Ács et al. (2024) in the aquatic crustacean *D. magna*. Their optimisation resulted in accurate and repeatable results for individual organisms with well-defined limits of detection for this species. Additionally, there is an opportunity to further develop the method's versatility by incorporating additional substrates, such as EROD, PROD and MROD. Especially EROD shows potential as the most sensitive among those substrates tested (Melo de Almeida et al., 2022). Using multiple substrates in this *in vivo* test would improve the validity of this approach as a proxy for xenobiotic detoxification.

Despite our success in testing the *in vivo* ECOD activity of *E. crypticus* in water, the reliance on a water medium still excludes many soil-dwelling species from *in vivo* assessment of xenobiotic detoxification. Our observation that the majority of enchytraeids survive for over two weeks in artificial freshwater suggests that this medium is not particularly stressful. However, it is important to note that in these medium conditions, this model organism has no access to food. Altogether, the successful application of the *in vivo* ECOD activity assay in *E. crypticus* allows high-throughput and cost-effective screening of (potentially CYP-mediated) synergistic chemical interactions in a terrestrial, soil-dwelling annelid. Such screening will prioritise chemical combinations requiring further testing in more environmentally relevant (and more costly) soil exposure systems. To expand the applicability of the assay, we recommend exploring the possibility of expanding the range of species tested to include both marine (e.g. molluscs, gastropods) and terrestrial species (e.g. annelids) when feasible.

5. Concluding remarks

The demonstrated utility of the *in vivo* ECOD assay in detecting CYP activity and its inhibition underscores the potential of this method to quantify aspects of xenobiotic detoxification in environmental species. We were able to use our *in vivo* protocol to measure CYP-dependent ECOD activity across soil and sediment-dwelling annelids. This method has the potential to compare CYP activity within and between species to support studies on inherent chemical sensitivity and resistance development.

Biodiversity decline in cropland habitats (Mancini et al., 2023) highlights the need to assess the consequences of detoxification disruption resulting from pesticide exposure in terrestrial species. Azole fungicides are widely used in agriculture and have been found to exhibit synergistic interactions in many cases where co-exposure to other pesticides occurs in non-target organisms (Bart et al., 2022; Cedergreen, 2014; Martin et al., 2021). By quantifying the inhibitory effects of azoles on CYP activity, our assay provides valuable tools for studying the sublethal effects of azoles and their potential disruption of xenobiotic detoxification in critical terrestrial, marine, and aquatic taxa (Fonte et al., 2023).

Funding

This Ph.D. position is part of the CHRONIC project "Chronic exposure scenarios driving environmental risk of Chemicals", which is an Innovative Training Network (ITN) funded by the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie Actions (MSCA). Grant no. 956009.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used Grammarly in order to remove typos and improve readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

I want to thank Emily Eagles and Alex Robinson for their support in the laboratory, particularly in worm culturing. Further thanks to my CHRONIC colleagues and supervisors for the regular discussion of results during project meetings. We thank the reviewers of the manuscript for their constructive comments and suggestions throughout the peer review process.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.119411.

Data availability

Data will be made available on request.

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