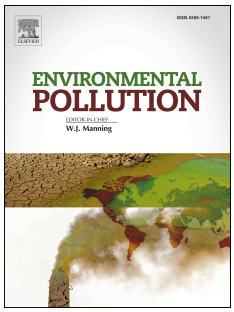

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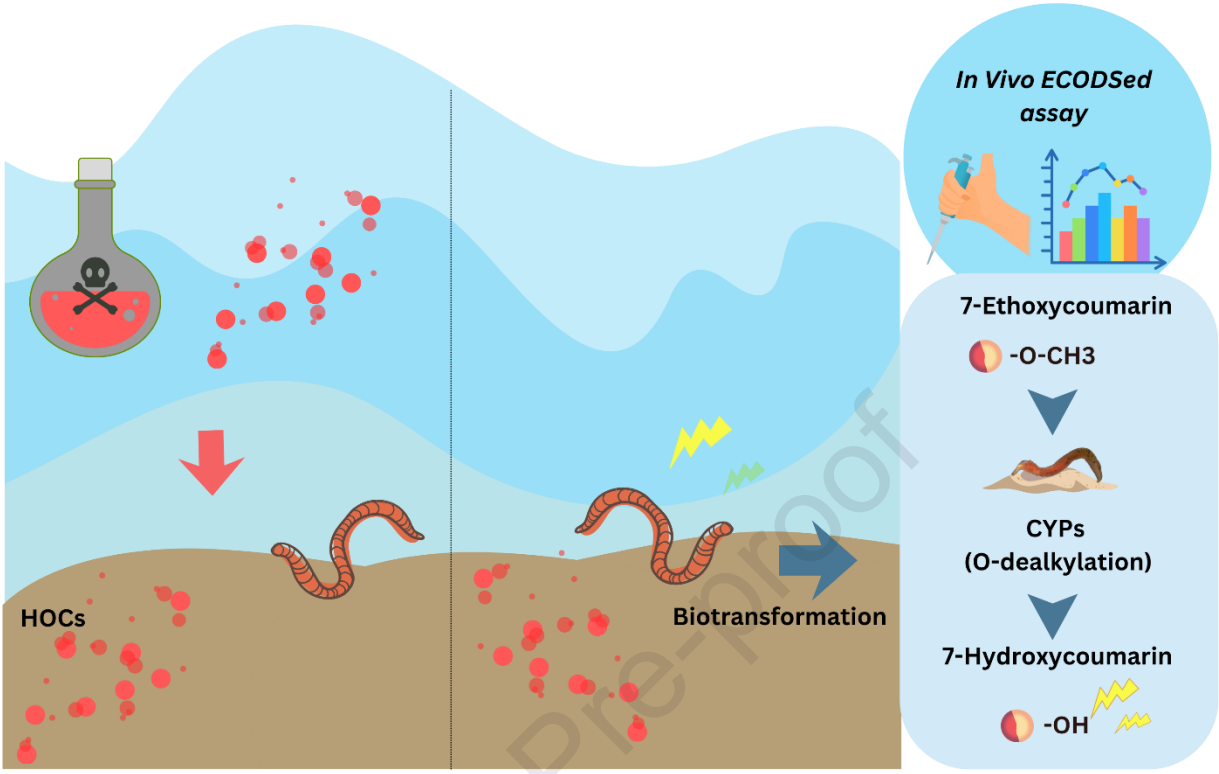

The *in vivo* ECOD$_{Sed}$ assay: an optimised method for measuring cytochrome P450 activity in sediment-dwelling organisms.

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Keywords: ECOD *in vivo* activity, enzymatic activity, sediment, aquatic sediment-dwelling invertebrates, *Capitella teleta*, HOCs

Abstract

Cytochrome P450 (CYP450) enzymes are central to xenobiotic biotransformation, yet traditional *in vivo* water-based ethoxycoumarin-O-deethylase (ECOD) assays are not an efficient method for deposit-feeding species. We developed and validated $ECOD_{Sed}$, a sediment-integrated *in vivo* assay, by incorporating sediment through both pre-exposure and assay phases. $ECOD_{Sed}$ reflects environmentally relevant exposure pathways for species exposed to sediment-associated contaminants during ingestion. We evaluated three assay formats representing different pre-exposure scenarios prior to assessing ECOD *in vivo*, namely $ECOD_{Sed14}$ (14-day pre-exposure), $ECOD_{Sed7}$ (7-day pre-exposure), and Quick $ECOD_{Sed}$ (no pre-exposure). We found that CYP inducers, such as polychlorinated biphenyls (PCBs) and the polycyclic aromatic hydrocarbon fluoranthene (FLUO), had the strongest induction of ECOD activity after 14 days in $ECOD_{Sed14}$ (~55%, PCB), and after 7 days in $ECOD_{Sed7}$ (~129%; FLUO). In both $ECOD_{Sed7}$ and $ECOD_{Sed14}$, the antidepressant sertraline (SER; CYP inhibitor) produced persistently lower activity (~30% on average) than controls. In the Quick assay, sertraline significantly inhibited activity (~50%), while fluoranthene showed no deviation from controls. Overall, the hierarchy of ECOD activity followed: fluoranthene > PCBs > control > sertraline. Our results demonstrate that $ECOD_{Sed}$ reliably captures chemical-specific metabolic dynamics across multiple exposure durations *in vivo*.

Together, the three $ECOD_{Sed}$ assays establish a tiered framework that allows users to match assay duration and complexity. The Quick assay offers rapid screening of chemical modulators, species responses, substrate suitability; $ECOD_{Sed7}$ captures early phase induction dynamics; $ECOD_{Sed14}$ reveals sustained metabolic responses under long term exposure. $ECOD_{Sed}$ advances CYP450 mediated activity assessment in sediment dwelling organisms, enabling rapid evaluations and deeper mechanistic insight into contaminant effects.

1. Introduction

Benthic organisms are exposed to sediment-associated hydrophobic organic chemicals (HOCs) through ingestion of contaminated particles and organic matter, as well as to dissolved HOCs via porewater and overlying water (Lamoureux and Brownawell, 1999; Dai et al., 2012). As hydrophobicity increases, HOCs preferentially partition into sediments, making sediment and dietary uptake dominant exposure routes for sediment-dwelling organisms (Selck et al., 2003a). For compounds with log $K_{OW} \geq 5.5$, sediment ingestion is

considered the primary uptake pathway for deposit feeders (Fowler et al., 1978; Klump et al., 1987; Selck et al., 2003b).

Upon xenobiotic exposure, organisms initiate detoxification processes, with Phase I biotransformation, largely mediated by cytochrome P450 (CYP450) enzymes, playing a central role in converting hydrophobic contaminants into hydrophilic, thus more excretable metabolites (Livingstone, 1998; Zhao et al., 2021). Changes in CYP450 activity are widely used as indicators of chemical exposure (Gottardi et al., 2016; Ács et al., 2024), although most existing assessments rely on *in vitro* assays measuring ethoxyresorufin-O-deethylase activity (EROD) or ethoxycoumarin-O-deethylase (ECOD) activity in pelagic species. *In vivo* extracellular enzymatic assays (EEAs) exploit the use of selective xenobiotic substrates, such as 7-ethoxycoumarin or ethoxyresorufin, which are readily taken up by organisms and subsequently metabolized by Phase I cytochrome P450 enzymes. These substrates undergo CYP-mediated oxidative reactions to form fluorescent metabolites (e.g. 7-hydroxycoumarin or hydroxyresorufin), whose fluorescence intensity is directly proportional to their concentration.

Temporal changes in fluorescence therefore provide a quantitative measure of the rate of CYP-dependent biotransformation, integrating the activity of the set of CYP isoforms capable of converting the parent substrate into its corresponding metabolite. Importantly, these assays selectively reflect Phase I oxidative metabolism, as the formation of the fluorescent metabolites is strictly catalyzed by CYP monooxygenases. Phase II conjugation products (e.g. glucuronides or sulfates) are not detected, because subsequent conjugation reactions do not generate fluorescent signals and, thus, fall outside the analytical scope of these assays (Binelli et al., 2006; Gagnaire et al., 2010; Le Bihanic et al., 2013). Furthermore, because the substrate itself can induce or activate CYP enzymes, an increase in biotransformation activity may also be observed in control organisms exposed only to 7-ethoxycoumarin, independent of contaminant treatment, providing a baseline Phase I CYP activity (Waxman and Chang, 2006).

Despite their ecological relevance, CYP-mediated metabolism in aquatic invertebrates, especially sediment-dwellers, remains poorly characterized. Current *in vivo* studies are limited to a few species and primarily address waterborne exposures (Noort et al., 2025), leaving metabolism under sediment-based exposure conditions largely unexplored. Advances in *in vivo* ECOD and EROD methods for various aquatic taxa (including gastropods, fish embryos, *Daphnia magna*, chironomids, and planarians) allow real-time

assessment of target CYP activity (Gagnaire et al., 2010; Le Bihanic et al., 2013; Gottardi et al., 2016; Li, 2016). For example, Gottardi et al. (2016) developed an *in vivo* ECOD assay based on the CYP-mediated transformation of 7-ethoxycoumarin into the fluorescent metabolite 7-hydroxycoumarin. This compound is metabolized by multiple CYP isoforms, mainly within the CYP1, CYP2, and CYP3 families, which are key groups in xenobiotic biotransformation (Nebert and Russell, 2002; Waxman and Chang, 2006; Doelle et al., 2009; Rendic and Guengerich, 2015; Loerracher and Braunbeck, 2021). However, existing *in vivo* ECOD assays apply exclusively to waterborne exposures and cannot assess dietary uptake of sediment-associated HOCs in sediment-dwelling organisms. To address this gap, we developed a novel *in vivo* sediment-based CYP assay, $ECOD_{Sed}$, designed to measure real-time *in vivo* ECOD activity as a proxy for CYP-dependent Phase I biotransformation during sediment ingestion. It should be noted that ECOD activity reflects the activity of CYP isoforms capable of metabolising 7-ethoxycoumarin and should therefore be interpreted as a proxy for CYP-dependent Phase I biotransformation rather than a measure of total CYP450 activity (Gottardi et al., 2016; Noort et al., 2025).

We performed $ECOD_{Sed}$ on the opportunistic estuarine polychaete *Capitella teleta*, which thrives in organically enriched and contaminated sediments (e.g., from wastewater effluents, aquaculture waste, and oil pollution) (Blake et al., 2009; Forbes et al., 2001; Selck et al., 2003a, 2003b). *C. teleta* is capable of metabolizing PAHs; however, the specific biotransformation pathways are not yet fully understood (Forbes et al., 2001; Selck et al., 2003a, 2003b). While recent work by Hochstein et al. (2019) identified gut microbiota taxa potentially associated with PAH degradation, experimental evidence indicates that fluoranthene transformation primarily requires the presence of the host itself (Jang et al., 2020). The presence of multiple CYP genes in *C. teleta* genome (Li et al., 2004; Dejong and Wilson, 2014) further supports its suitability as a model for sediment-associated xenobiotic metabolism assessment.

To validate $ECOD_{Sed}$, *C. teleta* was exposed to a hydrophilic CYP inhibitor (i.e., propiconazole) and three representative HOCs: sertraline (weak CYP-inhibitor), fluoranthene (highly biotransformable and CYP-inducing), and PCBs (poorly biotransformable yet CYP-inducing). Through this approach, we aimed to establish an ecologically realistic tool for quantifying ECOD activity in sediment-dwelling organisms and to advance understanding of how sediment-associated contaminants influence benthic metabolic responses and tolerance mechanisms.

2. Materials and methods

2.1 Chemicals and ECOD Solutions

Propiconazole (CAS: 60207901; ≥98% purity) was purchased from Sigma Aldrich and dissolved in acetonitrile (Merck; CAS: 75058; 99.9% purity) to prepare the stock solution. Three PCB congeners (PCB 52 (CAS: 35693993), PCB 153 (CAS: 35065271), and PCB 209 (CAS: 2051243)) were selected as representative legacy pollutants. PCB 52 and PCB 153 (Th. Geyer Skandinavien, Denmark) were dissolved in acetone (Merck 1.00014; CAS: 67641; 99.9% purity), whereas PCB 209 (Sigma Aldrich) was dissolved in chloroform (Merck; CAS: 67663; 99.99% purity). Fluoranthene (CAS: 206440), sourced from Th. Geyer Skandinavien and Sigma Aldrich, and the stock solution was prepared in acetone. Sertraline hydrochloride (CAS: 79559-97-0; >98% purity; Sigma-Aldrich) was dissolved in methanol (CAS: 67-56-1; ≥99.9% purity) for stock preparation.

For the $ECOD_{Sed}$ assay, the ECOD stock solution was freshly prepared by dissolving 5 mg of 7-ethoxycoumarin (EC; CAS: 31005024; Sigma Aldrich) in 1 mL of acetone or ethanol (Merck; CAS: 64175). This stock was diluted with filtered seawater (≤0.2 μm; salinity 31 ± 1 ppt; pH 7.88), hereafter referred to as "seawater", to yield an ECOD working concentration of 3.8 mg/L (ECOD solution), following Gottardi and Cedergreen 2019). The final solvent content in the ECOD solution was kept below 0.06% v/v (0.038% v/v), in accordance with Noort et al. (2025).

To generate the calibration curve for quantifying ECOD activity, serial dilutions of 7-hydroxycoumarin (also called umbelliferone, HC; CAS: 93356; Sigma Aldrich) were prepared in ethanol and diluted with seawater. These standards were used to convert fluorescence measurements into picomoles of metabolite produced (see Supplementary Material (S.M.), S1 for more details).

2.2 Sediment collection and spiking

Sediment collection and spiking is detailed in S2 in S.M. and in Grønlund et al (2023). Briefly, sediment was collected from the Munkholmbro area (Denmark) and sieved to <125 μm (worm cultures) and <63 μm (experimental use). Sediment was frozen (-20 °C) until use, was then thawed and homogenized using an immersion blender.

Sediment spiking was performed by first transferring wet sediment into glass beakers assigned to each treatment, whereafter the wet sediment was spiked by adding either propiconazole, sertraline (SER), fluoranthene (FLUO), or PCB mixture (PCB), at nominal

concentrations of 18 μg/g dw (dry weight), 100 μg/g dw respectively, and 50 μg/g dw for both fluoranthene and ∑PCB congeners. The PCB mixture consisted of PCB 52, PCB 153 and PCB 209. Each congener was added at a nominal concentration of 17 μg/g dw sediment. A total of 1.2 mL acetone and 1 mL chloroform were added to 400 g wet sediment (80 g dw equivalent) during spiking (corresponding to approximately 0.53% (v/w) of wet sediment). Fluoranthene-spiked sediment received 2 mL acetone per 200 g wet sediment batch (40 g dw equivalent), while sertraline-spiked sediment received 4 mL methanol (corresponding to approximately 1% and 2% (v/w), respectively). Corresponding solvent controls received identical solvent volumes (i.e., not added test chemicals).

All sediment samples, including solvent controls, were placed on a shaking table in the dark for at least 12 hours to evaporate solvents and ensure thorough mixing of contaminants within the sediment.

2.3 Model organism

C. teleta was originally sourced from Long Island, New York (USA) in 1984, and was since then kept in continuous culture at the Department of Science and Environment, Roskilde University, Denmark. Cultures were maintained in 2-liter aquaria containing 1-liter of natural, seawater. Each aquarium was layered with approximately 100 g of natural sediment (<125 μm). A spoonful of natural sediment (<63 μm) was added biweekly as a food source. Aeration was provided via air tubes connected to pumps, and all aquaria were kept in climate-controlled rooms at 21 °C.

2.4 Optimisation strategy

Developing a robust *in vivo* assay to quantify ECOD activity in sediment-dwelling organisms requires careful optimisation of both exposure conditions and assay design. Because *C. teleta* interact with HOCs through multiple pathways, like waterborne uptake and ingestion of contaminated sediment, we adopted a stepwise optimisation strategy to evaluate how these different exposure routes influence measurable ECOD activity. First, we established whether the ECOD assay could detect CYP-mediated metabolism under controlled water-only conditions (as in Gottardi et al. (2016)), and subsequently we adapted the method to sediment-based exposure that more closely reflects the species′ natural ecology. By systematically refining pre-exposure duration, exposure medium, and contaminant selection, we aimed to develop an assay capable of capturing real time-dependent CYP450 responses to HOCs in sediment systems.

2.4.1 Overall experimental design

2.4.1.1. Rationale

$ECOD_{water}$

In $ECOD_{water,}$ we tested two distinct pre-exposure setups stemmed from both biological and methodological considerations. Preliminary trials showed that *C. teleta* placed in water without sediment exhibited signs of stress responses that could compromise metabolic processes and reduce ECOD assay reliability (Hand and Hardewig, 1996; Sokolova et al., 2012). Exposure routes also differ between media: in water-only setups, contaminants are primarily taken up over the outer surface epithelium, whereas in sediment exposures, uptake occurs mainly from the gut lumen following ingestion of contaminated sediment particles. Additionally, the duration of exposure was further adjusted to reflect these differences: while 18 hours are considered sufficient for waterborne exposure, sediment exposure requires a longer duration (24h) to allow worms to acclimate, burrow, and initiate feeding, which are critical steps for ingesting and metabolizing sediment-associated contaminants (Tsutsumi et al., 2005; Dorgan et al., 2016). Testing both water and sediment pre-exposure approaches, we assessed the efficiency of the $ECOD_{water}$ assay under varying pre-exposure conditions.

$ECOD_{Sed}$

Following $ECOD_{water}$, we developed the first version of the $ECOD_{Sed}$ assay to assess cytochrome P450 activity in *C. teleta* during sediment exposure to HOCs. Unlike the $ECOD_{water}$, this method incorporates a sediment layer in the experimental wells to mimic the natural exposure route of sediment-dwelling organisms through feeding. Including sediment during both the pre-exposure and assay phases was intended to provide a more realistic representation of *in vivo* ECOD activity over time for sediment-dwelling organisms.

Additionally, we tested two different pre-exposure durations: 14- and 7-days pre-exposure to sediment-associated contaminants, referred to as $ECOD_{Sed14}$ and $ECOD_{Sed7}$, respectively.

$ECOD_{Sed14}$ included a control (no chemical), a CYP-inducer (PCBs) and a CYP-inhibitor (SER), whereas the $ECOD_{Sed7}$ assay incorporated fluoranthene as an additional CYP-inducer. This choice was based on the findings from Selck et al. (2003a, 2003b), who demonstrated that *C. teleta* is capable of biotransforming fluoranthene and that its biotransformation peak occurs after 4 and 7 days of exposure (Selck et al., 2003a, 2003b). Therefore, fluoranthene was included as a positive control to assess the time-dependent ECOD activity in *C. teleta*.

Faecal Pellets

In parallel with the $ECOD_{Sed7}$, we established an identical sediment setup to determine whether faecal material could interfere with the quantification of 7-hydroxycoumarin. *C. teleta* faecal pellets (FPs) host distinct microbial communities shaped by both the worms' gut microbiome and the surrounding environment (Hochstein et al., 2019). We therefore hypothesized that microbial activity associated with FPs produced during the assay could contribute to the biotransformation of 7-hydroxycoumarin, potentially affecting fluorescence-based ECOD measurements.

Because gut clearance of worms after pre-exposure would require 8-12 hours, during which contaminant depuration could occur compromising exposure integrity, we conducted a separate FPs-only experiment to isolate any ECOD-like microbial activity (see S.M., S5 for more details). Based on published egestion rates (13 mg dw FPs /worm in 7 days; Gomes et al. (2014)) and average FPs gut content per worm (~ 30.2 FPs; Forbes and Lopez (1987)), we estimated that four worms would produce ~ 1.54 mg/dw FPs in 5h (*in vivo* assay duration). For the FPs assay, we standardised the material to 30 FPs per replicate (average: 3.15 mg/dw) to ensure sufficient fluorescence signal.

Quick $ECOD_{Sed}$

This experiment aimed to evaluate the responsiveness of *C. teleta* to sediment-associated HOCs without a pre-exposure phase, using a rapid version of the $ECOD_{Sed}$ assay, referred to as the Quick $ECOD_{Sed}$. This approach allows for the detection of early-phase cytochrome P450 activity and can also provide insight into how quickly the species respond to a given contaminant through either baseline activity, induction or inhibition of CYP enzymes.

The Quick $ECOD_{Sed}$ assay is particularly useful when information on the species' metabolic capacity or the contaminant's biotransformation potential is lacking. It also serves to assess the suitability of the substrate for the species and to identify the time window at which ECOD activity peaks in acute exposures. This version of the assay lasted 5 hours (300 minutes) and does not require a pre-exposure period, making it a practical tool for rapid screening of metabolic responses.

2.4.1.2. Experimental during pre-exposure

$ECOD_{water}$

C. teleta were sieved (<500 μm) from laboratory cultures and transferred to individual wells (6-well plate containing oxygenated seawater) for manual removal of residual sediment and tubes. Worms were then grouped in groups of four worms, placed in fresh oxygenated seawater, and left overnight for gut clearance. After gut clearance, two pre-exposure setups were established: one for water- and one for sediment exposure. In the water-only pre-exposure, worms were exposed to propiconazole in seawater for 18 hours following Gottardi et al. (2016), with controls kept in uncontaminated seawater. In the sediment-based pre-exposure, worms were exposed for 24 hours to sediment spiked with propiconazole; corresponding controls received sediment spiked only with solvent (see S.M., S3 for details). All treatments were prepared in triplicate (n=3 vials per treatment). For both setups, pre-exposure was conducted in 20 mL glass vials filled with 20 mL of pre-oxygenated seawater or around 5 g/ww (approximately equivalent to 1 g/dw) sediment and 15 mL of pre-oxygenated seawater and kept in darkness at 21°C with continuous aeration via silicone tubes and needles.

$ECOD_{Sed}$

C. teleta collection from stock cultures, grouping, and gut clearance were performed as described for $ECOD_{water}$. Pre-exposure vials for $ECOD_{Sed}$ were prepared by adding 7–8 g wet sediment (~1.5 g dry weight) to 20 mL glass vials and filling them with 15 mL oxygenated seawater. Sediment was spiked with the chemicals used respectively in the $ECOD_{Sed14}$ and $ECOD_{Sed7}$ setups (see Table 1), with each treatment prepared in triplicates. After allowing the sediment to settle overnight, half of the overlying water was replaced with oxygen rich newly prepared seawater.

Pre-exposure began when the worms were added (four individuals per vial; n=3 replicate vials per treatment). Pre-exposure experimental conditions matched those used in

$ECOD_{water}$, with one modification: deionized water was added every second day if water had evaporated to maintain stable salinity. This adjustment was necessary because the pre-exposure periods extended to 7 days for $ECOD_{Sed7}$ and 14 days for $ECOD_{Sed14}$, during which evaporation could otherwise alter salinity.

Faecal pellets

A secondary pre-exposure, identical to the $ECOD_{Sed7}$ setup, was initiated one day prior to experimental start to allow worms to undergo an 8 h gut-clearance period in clean seawater after the exposure. FPs produced during this clearance period were collected directly from the water phase to avoid sediment contamination. All environmental and chemical pre-exposure conditions matched those of $ECOD_{Sed7}$.

2.4.1.3. Experimental during *in vivo* ECOD assay

$ECOD_{water}$ assay

Following pre-exposure, all groups were incubated in 5 ml of ECOD solution (see section 2.1) spiked with propiconazole for 3 hours as described in Noort et al. (2025). In parallel control groups were incubated in ECOD solution only. The formation of the fluorescent metabolite 7-hydroxycoumarin was measured as an indicator of CYP450 Phase I dependent activity: every 30 minutes a subsample of 100 µL were collected in triplicates from each well and loaded in a 96 black well plate (Nunc™ 96-Well Optical-Bottom Microplate, black, TC surface, Thermo Scientific™) for fluorescence analysis (excitation: 380 nm; emission: 480 nm, performed with fluorescence reader Molecular Devices, SpectraMax i3x) (see S.M., S3 for more details).

$ECOD_{Sed14}$ assay

After 14 days of pre-exposure to either control conditions or HOCs (PCBs and SER;), worms were sieved (<125 µm sieve) from the pre-exposure vials and each group was placed into individual wells of a 6-well plate, pre-filled with 5 mL of ECOD solution and a bottom layer of ~0.5 g wet sediment spiked with the same contaminant as used during pre-exposure. Control wells and blanks were included in triplicates (see S.M., S4 for more details).

The $ECOD_{Sed14}$ assay was initiated (t_0) by placing each group of worms into their designated wells. Aside from the inclusion of a bottom sediment layer, aliquots intervals and total duration of the assay (4 hours instead of the 3 hours of the $ECOD_{water}$), all procedures followed the $ECOD_{water}$ protocol (see S.M., S3 for assay details). Briefly, aliquots were

sampled from each well in triplicates at t_0 and every 15 minutes for the first 2 hours, and every 30 minutes for the following 2 hours, and fluorescence was measured to assess *in vivo* Phase I dependent CYP450 activity in response to exposure conditions: solvent-spiked sediment, PCBs-spiked sediment, SER-spiked sediment, and blanks for background fluorescence.

ECOD$_{Sed7}$ assay

Following 7 days of pre-exposure to either control conditions or HOCs (PCBs, FLUO, and SER) worms were sieved (<125 μm sieve) from the respective vials, grouped (n=4 worms per group, in triplicates), and transferred into pre-prepared 6-well plates with 8 ml ECOD solution, the rest was handled as described in ECOD$_{Sed14}$. Control and blank wells with and without sediment were also included. The ECOD$_{Sed7}$ assay was initiated upon placement of worms into each well (t_0). At t_0 and thereafter every 30 min, triplicate aliquots were collected from each well as in ECOD$_{Sed14}$ (see S.M., S5 for more details).

Faecal pellets assay

Collected FPs were transferred with Pasteur pipettes into 6-well plates, with 30 FPs per well (n=3 replicate wells per treatment). Each well received 8 mL of ECOD solution, and the FPs assay was run in parallel with ECOD$_{Sed7}$. Sampling intervals and fluorescence measurements followed the ECOD$_{Sed7}$ protocol. Fluorescence generated in FPs-only wells was used to quantify background 7-hydroxycoumarin formation attributable to microbial activity, enabling correction of ECOD$_{Sed7}$ data.

Quick ECOD$_{Sed}$ assay

Worms were selected directly from stock cultures and, after overnight gut clearance, exposed to either control or HOC-contaminated sediment (fluoranthene and sertraline; same concentrations as in ECOD$_{Sed7}$). The *in vivo* Quick ECOD$_{Sed}$ assay followed the same protocol as ECOD$_{Sed14}$, with the modification that triplicate aliquots were sampled from each well at t_0 and every 30 minutes thereafter until the end of the assay.

This setup allowed us to assess *in vivo* biotransformation capacity under short-time exposure conditions and to determine how quick contaminant metabolism, via Phase I CYP450 activity, is initiated in *C. teleta*. The information can be used to establish the best measurement time window for e.g., ECOD$_{Sed14}$.

Assay name	Pre-exposure type	Pre-exposure duration	Assay type	Chemicals	Study
ECODwater	water	18 hours	water	ctrl; propiconazole	Noort et al. (2025)
ECODwater	sediment	24 hours	water	ctrl; propiconazole	Noort et al. (2025)
ECODSed14	sediment	14 days	sediment	ctrl; PCBs; SER	this study
ECODSed7	sediment	7 days	sediment	ctrl; PCBs; FLUO; SER	this study
Quick ECODSed	0	0	sediment	ctrl; FLUO; SER	this study

Table 1. Overview of the different experimental setups implemented during the optimization process. Ctrl = control; PCBs = polychlorinated biphenyls; SER = sertraline; FLUO = fluoranthene.

2.5 Data Treatment and Statistical analysis

Data were analysed using linear mixed-effects models (LMMs) to account for repeated measurements over time and the hierarchical structure of the experimental design.

For each dataset, the response variable (enzyme activity related to metabolite concentration) was analysed as a function of treatment (i.e., control, PCBs, FLUO, SER), time, and interaction. Time was treated as a continuous predictor and expressed in minutes; for numerical stability and interpretability, time was rescaled and mean-centered prior to modelling. Rescaling affects the units of slope estimates but does not influence statistical inference. Treatment was included as a categorical fixed effect. Replicate was included as a random intercept to account for non-independence of repeated measurements within the same experimental unit. Models were fitted using restricted maximum likelihood (REML). Degrees of freedom and p-values for fixed effects were estimated using Satterthwaite's approximation. Type III analyses of variance were used to assess the significance of main effects and treatment × time interactions. Fixed-effect significance was assessed using t statistics and corresponding p values based on the Kenward–Roger approximation for degrees of freedom.

Treatment-specific temporal trends were quantified by estimating rates of change over time (slopes). Differences in slopes between treatments were assessed using pairwise contrasts

with Holm adjustment for multiple comparisons. These slope comparisons were used as the primary basis for inference on treatment-dependent kinetics, as they directly assess differences in trajectories over time. Time was centered prior to analysis to facilitate interpretation of model intercepts and main effects at the mean experimental time point and to reduce collinearity among fixed-effect terms. Estimated marginal means and model-based predictions were generated and visualized together with 95% confidence intervals. Model assumptions (normality and homoscedasticity of residuals) were assessed visually by inspection of residual and Q–Q plots and were considered acceptable. Results are reported as model estimates ± 95% confidence intervals, and statistical significance was assessed at $\alpha = 0.05$ unless otherwise stated.

3. Results

3.1 $ECOD_{water}$ was not suitable for measuring ECOD activity in *Capitella teleta*.

Initial attempts to apply the *in vivo* ECOD assay to *C. teleta* under water-only conditions did not show reliable ECOD activity signals. Both the assay readout and the water-only pre-exposure setup were ineffective for detecting modulation of ECOD activity in *C. teleta* (Noort et al., 2025). This motivated the development of a protocol integrating sediment in the assay ($ECOD_{Sed}$).

3.2 $ECOD_{Sed14}$

The results from $ECOD_{Sed14}$ showed that ECOD activity increased over time in all treatments (Fig. 1a), but both the magnitude and the rate of increase differed significantly among treatments (linear mixed-effects model, Treatment × Time interaction, table S1 in S.M.). At the mean experimental time point (~90 min), PCB-exposed organisms displayed significantly higher ECOD activity than CTRL (estimated difference = +1.85 $pmol \cdot min^{-1} \cdot org^{-1}$, p = 0.001, table S1 in S.M.), whereas sertraline showed a non-significant tendency toward lower activity compared to CTRL (estimated difference = −1.02 $pmol \cdot min^{-1} \cdot org^{-1}$, p = 0.070, table S1 in S.M.). ECOD activity increased significantly over time in CTRL (slope = +1.01 $pmol \cdot min^{-1} \cdot org^{-1} \cdot h^{-1}$, p < 0.001, table S2 in S.M., see also time effect in table S1 in S.M.), and this increase was significantly steeper under PCB exposure (slope = +2.06 $pmol \cdot min^{-1} \cdot org^{-1} \cdot h^{-1}$, table S2 in S.M), corresponding to a ~104% higher induction rate relative to CTRL (CTRL vs PCBs slope contrast, p = 0.049, table S3 in S.M). In contrast, the time-dependent increase under sertraline exposure was weak and not statistically

significant (slope = +0.45 pmol·min^{-1}·org^{-1}·h^{-1}, table S2 in S.M), corresponding to an approximately 55% lower induction rate relative to CTRL.

No significant differences in ECOD activity among treatments were detected at early time points (0–60 min; Fig. 1a). From 75 min onwards, however, PCB exposure resulted in significantly higher ECOD activity compared to sertraline and CTRL (e.g. +2.16 pmol·min^{-1}·org^{-1} at 75 min, p = 0.027, table S5 in S.M.). Differences between PCBs and CTRL were statistically significant from 90 min onward (−1.65 pmol·min^{-1}·org^{-1} for CTRL − PCBs at 90 min, p = 0.033, table S5 in S.M.) and increased progressively with time. At the same time points, PCBs consistently exceeded sertraline (e.g. +2.56 pmol·min^{-1}·org^{-1} at 90 min, p = 0.010; +3.37 at 120 min, p = 0.001; +4.18 at 150 min, p < 0.001, table S5 in S.M.). Differences between CTRL and sertraline emerged later and were smaller in magnitude, reaching statistical significance at 150–210 min (e.g. +1.48 pmol·min^{-1}·org^{-1} at 150 min, p = 0.041; +2.04 at 210 min, p = 0.046, table S5 in S.M.), but were not consistently significant across all late time points.

At the end of the exposure (240 min), ECOD activity followed the hierarchy PCBs > CTRL > SER. Mean predicted activity under PCB exposure (75.9 pmol·min^{-1}·org^{-1}) was approximately 6% higher than CTRL (71.6 pmol·min^{-1}·org^{-1}; p = 0.001) and ~10% higher than sertraline (69.3 pmol·min^{-1}·org^{-1}; p < 0.0001), whereas sertraline remained ~3% lower than CTRL. Overall, these results indicate a pronounced, time-dependent induction of ECOD activity by PCBs, characterized by both elevated activity levels and a substantially higher induction rate, while sertraline did not induce ECOD and instead showed a tendency toward suppressed activity over time.

3.3 ECOD$_{Sed7}$

ECOD$_{Sed7}$ results show that ECOD activity increased with time in all treatments, but treatments differed in how rapidly and to what extent that increase occurred; as a result, activity levels were similar on average but diverged substantially over the course of the exposure (Fig. 2a, table S6 in S.M.).

Under CTRL conditions, ECOD activity increased significantly over time (slope = +0.46 pmol·time^{-1}·org^{-1}·h^{-1}, p < 0.001, tables S6 – S7 in S.M.), as expected. The time-dependent increase was moderately steeper under PCB exposure (slope = +0.68 pmol·time^{-1}·org^{-1}·h^{-1}, table S7 in S.M.), corresponding to an approximately 47% higher rate of ECOD activity relative to CTRL, although this difference was not statistically significant after Holm correction (CTRL vs PCBs slope contrast, p = 0.26, table S8 in S.M.). In contrast, sertraline

exposure was associated with a markedly attenuated increase in ECOD activity over time (slope = +0.26 pmol·time^{-1}·org^{-1}·h^{-1}, table S7 in S.M.), corresponding to an approximately 44% lower rate relative to CTRL, indicating suppression of time-dependent CYP activation. The strongest increase in ECOD activity over time was observed under fluoranthene exposure, which displayed a significantly steeper slope (slope = +0.97 pmol·time^{-1}·org^{-1}·h^{-1}, table S7 in S.M.), corresponding to a ~110% higher rate relative to CTRL (p = 0.002) and a ~275% higher rate relative to sertraline (p < 0.0001, table S8 in S.M.).

No significant differences in ECOD activity were detected among treatments at early time points (30–60 min; Fig. 2a), which is consistent with similar baseline ECOD activity across exposures (table S10 in S.M.). From intermediate time points onward, fluoranthene exposure resulted in progressively higher ECOD activity relative to the other treatments, reflecting the strong Treatment × Time interaction (table S6 in S.M.). Pairwise contrasts showed that FLUO consistently exceeded SER and CTRL at later sampling times (e.g. 210–240 min), with the magnitude of these differences increasing toward the end of the exposure (table S9 in S.M.). PCB-exposed organisms generally exhibited higher ECOD activity than sertraline at late time points, although these differences were smaller and less consistent than those observed under fluoranthene exposure (table S10 in S.M.).

At the end of the exposure (240 min), ECOD activity followed the hierarchy FLUO > PCBs > CTRL > sertraline. Mean predicted ECOD activity under fluoranthene exposure was approximately 65–70% higher than CTRL, whereas PCB exposure resulted in a more moderate increase of ~20–25%. In contrast, sertraline consistently exhibited the lowest ECOD activity, remaining approximately 15–20% lower than CTRL and 30–35% lower than fluoranthene (end-point percentages, refer to total ECOD activity at 240 min). Overall, these results indicate a pronounced, time-dependent induction of ECOD activity by fluoranthene, a moderate activation under PCB exposure, and a clear suppression of ECOD activity in the presence of sertraline.

3.4 Quick ECOD$_{Sed}$

ECOD activity , assessed via Quick ECOD$_{Sed}$, increased significantly over time in all treatments (Fig. 3a); however, both the magnitude and the temporal dynamics of this increase differed significantly among exposures (linear mixed-effects model, significant Treatment × Time interaction; $F_{2,75}$ = 13.04, p < 0.0001, table S11 in S.M.). At the mean experimental time point (~165 min), ECOD activity did not differ significantly between

CTRL and fluoranthene exposures, whereas sertraline exposed organisms showed significantly lower ECOD activity than CTRL (estimated difference = −8.24 pmol·min^{-1}·org^{-1}, p = 0.027; table S11 in S.M.), indicating early suppression of CYP mediated activity.

Under CTRL conditions, ECOD activity increased strongly over time (slope = +6.17 pmol·h^{-1}·org^{-1}, $p < 0.001$, tables S11 – S12 in S.M.), reflecting robust time-dependent CYP activation as expected. A similarly strong but slightly less steep increase was observed under fluoranthene exposure (slope = +5.33 pmol·h^{-1}·org^{-1}, table S12 in S.M.), corresponding to an approximately 14% lower rate relative to CTRL, although this difference was not statistically significant (CTRL vs FLUO slope contrast, $p = 0.23$, table S13 in S.M.). In contrast, sertraline exposure resulted in a markedly attenuated time-dependent response (slope = +2.72 pmol·h^{-1}·org^{-1}, table S12 in S.M.), corresponding to a ~56% lower rate compared to CTRL ($p < 0.001$) and a ~49% lower rate compared to fluoranthene ($p < 0.001$, table S13 in S.M.), indicating strong suppression of ECOD activity.

No pronounced differences in ECOD activity among treatments were evident at the earliest time points (Fig. 3a, table S14 in S.M.); however, divergence among exposure conditions became increasingly apparent as the assay progressed, consistent with the significant Treatment × Time interaction (table S11 in S.M.). FLUO exposed organisms exhibited consistently higher ECOD activity than sertraline at later sampling times, with significant differences emerging from ~210 min onward (table S14 in S.M.). CTRL and fluoranthene treatments exhibited broadly comparable ECOD activity across most time points.

At the end of the exposure (300 min), ECOD activity followed the hierarchy CTRL ≈ FLUO > SER. Relative to CTRL, fluoranthene-exposed organisms displayed comparable terminal ECOD activity, whereas sertraline-exposed organisms remained substantially lower throughout the assay. The strongly reduced slope and persistently lower ECOD activity observed under sertraline exposure are consistent with sustained inhibition of CYP-mediated biotransformation in the Quick ECOD assay. Overall, these results demonstrate rapid, time-dependent Phase I CYP activation in CTRL and fluoranthene treatments, contrasted by pronounced suppression of ECOD activity in the presence of sertraline.

3.5 Comparison of ECOD activity across ECOD$_{Sed}$ assays

After 14 days of pre-exposure, ECOD activity decreased to intermediate levels: PCBs-exposed worms averaged ~2.4–2.6 pmol h^{-1} organism^{-1}, controls around ~1.5–1.7 pmol h^{-1} organism^{-1}, and sertraline-exposed worms ~1.1–1.4 pmol h^{-1} organism^{-1} (Fig. 1B). This

may indicate persistent, but attenuated, Phase I activity after longer contact with sediment-bound contaminants.

The 7-day pre-exposure assay displayed the lowest rates overall, with fluoranthene-exposed worms reaching ~1.1–1.3 pmol h^{-1} $organism^{-1}$, PCBs-exposed worms ~0.7–0.9 pmol h^{-1} $organism^{-1}$, controls ~0.4–0.6 pmol h^{-1} $organism^{-1}$, and sertraline-exposed worms ~0.3–0.4 pmol h^{-1} $organism^{-1}$ (Fig. 2B).

In the Quick $ECOD_{Sed}$ assay, activity levels ranged from approximately 3 pmol h^{-1} $organism^{-1}$ in sertraline-exposed worms to ~5 pmol h^{-1} $organism^{-1}$ in fluoranthene-exposed worms, with controls reaching ~6 pmol h^{-1} $organism^{-1}$ (Fig. 3B). These values are substantially higher than those observed in either assay with pre-exposure.

3.6 Faecal pellets and microbial contribution to biotransformation

Fluorescence readings of FPs in this study revealed negligible metabolite formation, with no significant differences among treatments, confirming that microbial transformation of 7-hydroxycoumarin was minimal under our experimental conditions (see S.M., figure S2 for more details).

4. Discussion

4.1 The implementation of sediment in the *in vivo* ECOD assay was a game-changing element for detecting modulation of ECOD activity in *Capitella teleta*.

Early attempts to apply the *in vivo* ECOD assay to *C. teleta* under water-only conditions failed to generate consistent ECOD activity signals (see Noort et al. (2025)). Likely explanations may relate both to biological and methodological considerations: *C. teleta* shows stress symptoms when kept without sediment (i.e., in water-only), which may impair metabolic function (Hand and Hardewig, 1996; Sokolova et al., 2012), making the $ECOD_{water}$ assay less reliable. Further, *C. teleta* is a deposit-feeder capable of ingesting large amounts of sediment within short timeframes (Forbes et al., 2001; Selck et al., 2003a), which may enhance the concentration of contaminants passing through the gut and thus increasing the bioaccumulation of sediment-associated contaminants, particularly hydrophobic

compounds. In contrast, water-only exposure limits uptake to transdermal absorption, thus omitting uptake of the gut epithelium which is likely the most efficient route of exposure for HOCs in this species. Additionally, we observed an increased secretion of mucus in control and more accentuated in HOC exposed *C. teleta* during 12-hour water-only exposure. In addition to being a stress response (Iori et al., 2014; Stabili, 2019; Huan et al., 2023) the mucus layer may also serve as sponge for HOC accumulation hence reducing contaminant concentrations in the water and thus the amount of HOC available for absorption over the outer worm surface, eventually rendering propiconazole unavailable.

The use of sediment during both the pre-exposure and assay phases in $ECOD_{Sed14}$ and $ECOD_{Sed7}$ substantially improved the detection of ECOD activity response in *C. teleta*.

Unlike the $ECOD_{water}$ approach, the sediment-based assays produced results that were consistent with the theoretical expectations of xenobiotic-induced CYP modulation: overall, fluoranthene and PCBs exposure increased ECOD activity, whereas sertraline tended to suppress or maintain activity at levels lower than or comparable to the control. These patterns align with well-established mechanistic pathways, as both PAHs and PCB congeners are known activators of the aryl hydrocarbon receptor (AhR), through which they can upregulate transcription and enzymatic activity of multiple xenobiotic metabolizing CYP isoforms, with a magnitude that depends on the contaminant's chemical structure (Safe, 1994; Honkakoski and Negishi, 2000; Casarett, Louis J., 2019). Whereas sertraline is a mild inhibitor of several CYP isoforms (e.g., CYP2D6, CYP1A2, CYP2C9/10) (Masubuchi and Kawaguchi, 2013; Huddart et al., 2020).

4.2 Temporal shifts in CYP modulation: comparing $ECOD_{Sed14}$ and $ECOD_{Sed7}$

In $ECOD_{Sed14}$, PCBs exposure led to the strongest significant induction, with ECOD induction rate (slope) approximately two-fold higher than controls and nearly twice the induction observed after 7 days of exposure (47% compared to controls). However, the apparent increase from day 7 to day 14 requires cautious interpretation, as additional factors may be influencing the observed pattern. From a toxicokinetic perspective, CYP-mediated metabolism is subject to saturation kinetics, meaning that under constant exposure a steady state would be expected once induction stabilizes and substrate turnover approaches enzyme capacity (Lin et al., 2001). Moreover, many PCB congeners can competitively inhibit or irreversibly bind CYP enzymes (Safe, 1994; Müller et al., 2025), so even strong induction does not necessarily translate into a monotonically increasing ECOD signal. Physiologically based toxicokinetic (PBTK) models of PCBs metabolism similarly account for limits on CYP

capacity at high doses, indicating that ECOD activity cannot rise indefinitely and may instead plateau or fluctuate over time (Sasso et al., 2012). In addition, prolonged CYP induction can shift metabolism toward Phase II pathways, such as glucuronidation or sulfation, which reduce the measurable 7-hydroxycoumarin signal even when Phase I activity remains active (Ács et al., 2024). Together, these mechanisms may provide explanation for the non-linear ECOD patterns in the $ECOD_{Sed}$ assay formats. However, further studies are required to fully understand the underlying mechanisms.

The comparison between PCBs and fluoranthene in the 7-day assay further illustrates the importance of contaminant-specific dynamics in CYP induction. Fluoranthene elicited the strongest response, with ECOD activity at the end of the exposure approximately 65–70% higher than controls, reflecting both a steep time-dependent increase and elevated terminal activity. In contrast, PCB exposure resulted in a more moderate elevation of ECOD activity at the final time point, in the order of ~20–25% relative to controls and was less consistently significant across time points. These differences likely reflect the distinct affinities of PAHs and PCB congeners for the aryl hydrocarbon receptor (AhR), as well as differences in their metabolic rates and capacities to induce specific CYP isoforms (Li et al., 2004; Koenig et al., 2012). At the same time, sertraline-exposed worms consistently displayed reduced ECOD activity relative to controls. In the 7-day assay, terminal ECOD activity under sertraline exposure remained approximately 15–20% lower than CTRL, while in the 14-day exposure sertraline resulted in a smaller but persistent reduction of ~3% relative to CTRL at the final time point. Taken together, these results support the interpretation that sertraline acts as a weak but persistent inhibitor or suppressor of CYP-mediated biotransformation in sediment-dwelling polychaetes, with inhibitory effects becoming less pronounced but still detectable at longer exposure durations (Huddart et al., 2020). The different responses observed between the two assays and across different chemicals likely stem from the diverse array of CYP isoforms expressed by *C. teleta*. Marine invertebrates possess broad CYPomes with numerous isoforms exhibiting overlapping or partially redundant substrate specificities (Rewitz et al., 2006; Waxman and Chang, 2006).

C. teleta possesses a highly diverse CYPome comprising 96 predicted functional CYP genes distributed across multiple CYP families and representing 9 of the 11 known metazoan CYP clans (Dejong and Wilson, 2014), indicating substantial functional redundancy and a strong capacity for compensatory metabolic responses. This diversity suggests that prolonged exposure to potent inducers or inhibitors may selectively affect some isoforms while others compensate, potentially explaining the modest inhibition observed with sertraline during long-term exposure.

These dynamics become even more complex when considering biological variation within the population. Ontogenetic and reproductive stage differences can markedly influence CYP expression and metabolic capacity, as documented in multiple marine invertebrates (Langston, 2020; Lewis et al., 2024). Since *C. teleta* size does not correlate with life stage (Seaver, 2016), individuals at different stages of sexual maturation or with varying metabolic demands may allocate resources differently, including gametogenesis. Such biological heterogeneity can therefore amplify variability in ECOD responses, particularly across different pre-exposure durations where physiological states continue to diverge over time.

4.3 The Quick ECOD$_{Sed}$ assay provides a rapid screening tool for assessing CYP450 activity involved in Phase I biotransformation in sediment-dwelling organisms.

Quick ECOD$_{Sed}$'s primary value lies in 1) determining substrate suitability for the target species, 2) evaluating metabolic responsiveness to specific contaminants and their concentrations, and in 3) identifying the time window in which peaks of induction or inhibition occur.

In our study, the 5-hour Quick ECOD$_{Sed}$ assay was insufficient for fluoranthene to elicit a full metabolic response in *C. teleta*, consistent with previous findings that induction of PAH biotransformation in this species requires several days of exposure (Forbes et al., 2001; Selck et al., 2003a). This delayed response likely reflects the time needed for transcriptional induction and subsequent synthesis of CYP enzymes following PAH exposure (Stoddard et al., 2021). In contrast, sertraline produced a markedly faster effect.

In the Quick ECOD$_{Sed}$ assay, sertraline reduced the overall time-dependent ECOD induction rate by approximately 56% relative to the control, as determined from slope estimates calculated across the full 300-minute assay period, despite showing no significant inhibition after 7- or 14-day pre-exposure. This apparent discrepancy may be explained by differences in mode of action and toxicokinetics. Unlike PAHs, which require metabolic activation and enzyme induction over time (Forbes et al., 2001; Selck et al., 2003a), the fast response to sertraline in the Quick ECOD$_{Sed}$, aligns with the design of many pharmaceuticals, which are intended to be readily bioavailable to quickly exert their mechanisms of action over short timescales (Masubuchi and Kawaguchi, 2013; Huddart et al., 2020). Therefore, the rapid decline in ECOD activity observed during the Quick ECOD$_{Sed}$ is likely attributable to sertraline´s pharmacokinetic profile, particularly its relatively short half-life (~26 hours) and rapid hepatic metabolism. Moreover, desmethylsertraline, sertraline's primary metabolite,

which has a considerably longer half-life (~56-120 hours), is generated by several CYP isoforms and displays similar but substantially weaker inhibitor potency (Huddart et al., 2020). This pharmacokinetic profile suggests that the strong early inhibition may reflect transient exposure to parent sertraline, whereas the subsequent loss of effect is consistent with the predominance of desmethylsertraline, whose weaker inhibitory activity is insufficient to maintain CYP suppression. Although the pharmacokinetic behaviour of sertraline has been most extensively characterised in vertebrates, these findings provide a possible mechanistic framework for interpreting the observed response in *C. teleta*. However, the CYP isoforms involved and their interaction with sertraline remain unknown in annelids and therefore this explanation should be regarded as a hypothesis requiring further investigation.

The Quick ECOD$_{Sed}$ approach, thus, revealed an acute inhibitory effect by sertraline that would likely remain undetected in longer pre-exposure assays, highlighting its utility for early-phase screening and for contaminants with fast-acting pharmacodynamics.

Overall, the Quick ECOD$_{Sed}$ assay represents a further advancement, offering a rapid (5 hour) method to screen CYP activity interactions before performing pre-exposure. This streamlined version of the ECOD$_{Sed}$ assay is particularly valuable for screening contaminants with poor information on CYP interactions or species with uncharacterized CYP profiles, as it enables efficient evaluation of metabolic responsiveness in a wider range of CYPs compared to EROD. Additionally, it facilitates the determination of substrate suitability for CYP measurement and helps identify optimal time windows for CYP induction or inhibition. Due to its speed and operational simplicity, the Quick ECOD$_{Sed}$ assay is especially well-suited for early-phase screening in laboratory settings where time or resources may be limited.

4.4 Comparison of ECOD activity across ECOD$_{Sed}$ assays

When comparing ECOD activity rates among the three ECOD$_{Sed}$ formats, a clear quantitative pattern emerges across all treatments. Although absolute values differ across assays due to experimental design and scaling, a consistent pattern of relative CYP activation was observed: quick ECOD$_{Sed}$, which is performed without any sediment pre-exposure, consistently showed the highest ECOD activity, followed by ECOD$_{Sed14}$, and finally ECOD$_{Sed7}$. These differences likely reflect the absence of prolonged pre-exposure in the

Quick assay, which captures an acute metabolic response before potential feedback regulation or Phase II conjugation reduces detectable Phase I activity (Ács et al., 2024), since the ECOD method can only detect Phase I metabolites. At the same time, the variability of CYP response observed in both the controls (exposed to 7-ethoxycoumarin) and the contaminant-exposed groups likely reflects shifts in developmental and reproductive stages explained in section 4.2, which can drive substantial changes in baseline CYP expression patterns and overall metabolic capacity.

4.5 Faecal pellets and microbial contribution to biotransformation

Microbial communities in sediments play an important role in contaminant degradation, but their efficiency varies widely depending on chemical properties, environmental conditions, and the presence of macrofauna (Ellegaard-Petersen et al., 2010; Dai et al., 2012). *C. teleta* faecal pellets (FPs) host distinct microbial assemblages shaped by both gut microbiota and surrounding sediment (Hochstein et al., 2019), raising concerns that microbial activity could interfere with $ECOD_{Sed}$ fluorescence measurements by transforming 7-hydroxycoumarin, the assay's target metabolite. Our findings align with previous studies showing that while sediment microbes contribute to contaminant breakdown, their role is minor compared to worm-mediated metabolism (Ellegaard-Petersen et al., 2010; Dai et al., 2012). For example, *C. teleta* accelerates removal of sediment-associated acetyl cedrene by up to 99% within 14 days, compared to only 13–31% in systems without worms (Dai et al., 2012). Similarly, fluoranthene degradation does not occur in the absence of the worm hosting bacteria (Jang et al., 2020), reinforcing that CYP enzymes within *C. teleta* are the primary drivers of biotransformation.

5. Concluding remarks

$ECOD_{Sed}$ and Quick $ECOD_{Sed}$ provide a promising framework for assessing CYP-dependent Phase I biotransformation in sediment-dwelling organisms. Their flexibility makes the assays suitable for contaminant screening, mechanistic studies, and as a supporting tool for future risk-assessment applications following further validation, with or without pre-exposure and at different stages of toxicity testing. Incorporating sediment exposure greatly enhances ecological relevance, reflecting the natural conditions experienced by deposit feeders. The strong divergence between sediment-based and water-only results demonstrate that water-only assays are inadequate for species such as *C. teleta*, emphasizing

683 the need to consider organismal ecology and exposure pathways when developing
684 biotransformation assays. Our findings further show that $ECOD_{Sed}$ predominantly reflects
685 worm-derived enzymatic activity rather than microbial interference, supporting its
686 reliability for measuring *in vivo* CYP450 responses.

687 Sediment-based ECOD assays capture natural exposure routes and reveal dynamic CYP
688 responses shaped by exposure kinetics, induction limits, and physiological state. These
689 insights are essential for interpreting ECOD data and for designing assays that accurately
690 represent the metabolic capacity of sediment-dwelling organisms. Finally, the influence of
691 assay duration and exposure history on Phase I CYP profiles highlights the complementary
692 roles of Quick $ECOD_{Sed}$ for acute screening and $ECOD_{Sed7/14}$ for longer-term assessments.

693

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

706 During the preparation of this work, the author(s) used Copilot-Microsoft to improve the
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710

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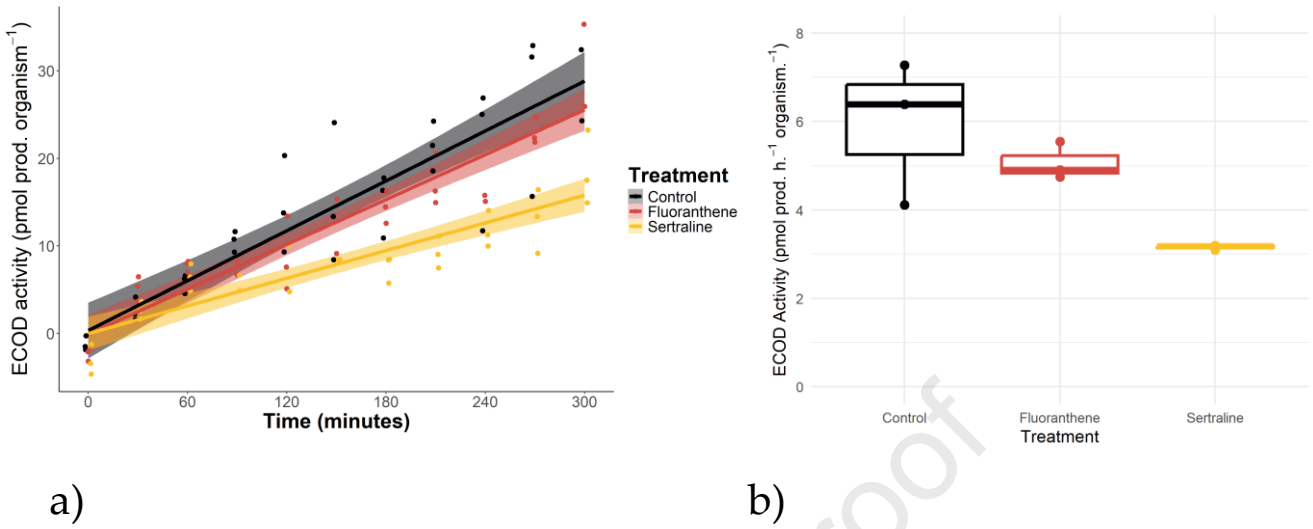
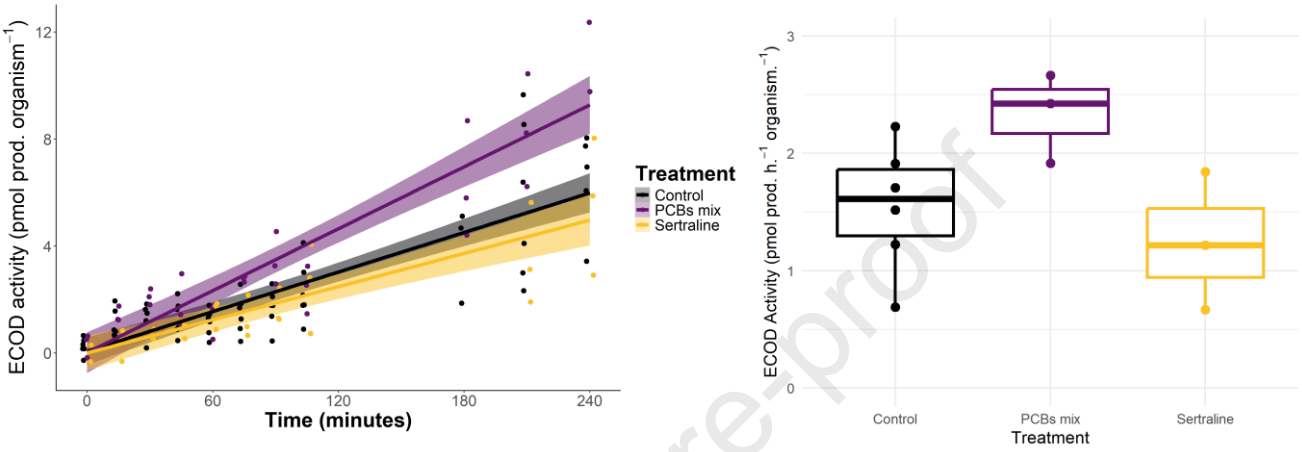


a) b)

Figure 3. Quick $ECOD_{Sed}$. *In vivo* ECOD responses in *C. teleta* during direct exposure to fluoranthene, sertraline, or control conditions in the Quick ECODSed assay. a) Time-resolved accumulation of metabolite (7-hydroxycoumarin) expressed as pmol·org^{-1}. b) ECOD activity expressed as the rate of metabolite formation (pmol·org^{-1}·h^{-1}), calculated as the slope of the accumulation curves for each treatment.



a) b)

Figure 1. $ECOD_{Sed14}$. *In vivo* ECOD responses in *C. teleta* following 14-day sediment pre-exposure to PCBs, sertraline, or control conditions. **a)** Time-resolved accumulation of metabolite (7-hydroxycoumarin) expressed as pmol·org^{-1}. **b)** ECOD activity expressed as the **rate of metabolite formation** (pmol·org^{-1}·h^{-1}), calculated as the slope of the accumulation curves for each treatment.

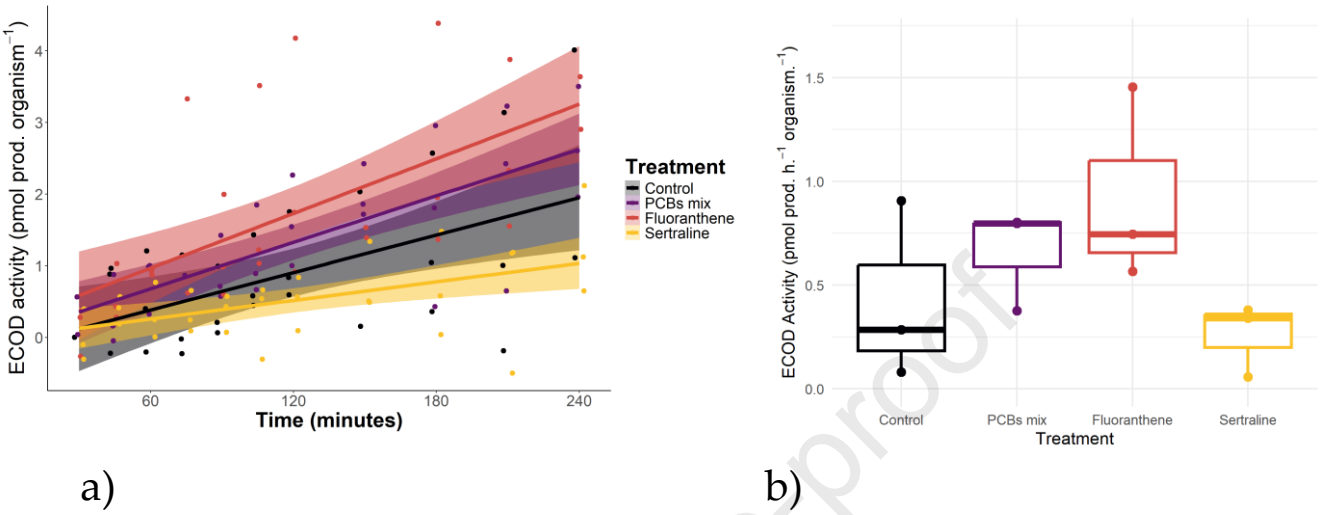


Figure 2. $ECOD_{Sed7}$. *In vivo* ECOD responses in *C. teleta* following 7-day sediment pre-exposure to PCBs, fluoranthene, sertraline, or control conditions. a) Time-resolved accumulation of metabolite (7-hydroxycoumarin) expressed as pmol·org^{-1}. b) ECOD activity expressed as the rate of metabolite formation (pmol·org^{-1}·h^{-1}), calculated as the slope of the accumulation curves for each treatment.

Highlights

- Water-based ECOD failed to reveal clear CYP modulation in *Capitella teleta*.
- $ECOD_{Sed}$ reliably detected ECOD activity in sediment-dwelling invertebrates.
- 3 $ECOD_{Sed}$ assays to capture either rapid, early, or long-term metabolic responses.
- Hierarchy of CYP responses: fluoranthene > PCBs > control > sertraline.
- $ECOD_{Sed}$ offers a flexible, tiered framework for toxicological assessment.

Ethics declaration

Animal subject

Ethics committee approval was not required.

Declaration of interests

☐ 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.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Elettra D'Amico reports financial support was provided by Centre European des silicones (CEH). Martina Santobuono reports financial support was provided by European Union. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.