CYP35: Xenobiotically induced gene expression in the nematode *Caenorhabditis elegans*

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Abstract:

Although over 80 cytochrome P450 (CYP) encoding genes have been identified in the genome of the nematode *Caenorhabditis elegans* very little is known about their involvement in biotransformation. This paper demonstrates a concentration dependent relationship of *C. elegans* CYP35A1, A2, A5, and C1 gene expression in response to four organic xenobiotics, namely atrazine, PCB52, fluoranthene, and lansoprazole. The toxicity of these xenobiotics was determined using a reproduction assay. CYP-specific messenger RNA expression was analyzed by semi-quantitative RT-PCR resulting in a strongly increasing, concentration-dependent induction well below the EC_{50} for reproduction. For PCB52, approximately 0.5 % of the EC_{50} induces a two-fold increase of CYP35 gene expression. Using a double mutant and multiple RNAi of CYP35A/C it was possible to diminish the reproduction decline caused by PCB52 and fluoranthene.

Key words: *Caenorhabditis elegans*, Cytochrome P450, CYP35, Organic xenobiotics, Gene expression, Reproduction assay, RNAi

Introduction

The nematode *Caenorhabditis elegans* is perhaps the only multi-cellular animal having the experimental convenience of a single-celled organism [1]. The worm's short life cycle, developmental invariance, and the completion of its genome sequence are greatly advantageous. During the last decades, its culture conditions, anatomy, genetic properties, and developmental staging have been better defined than for any other animal. Nevertheless, the knowledge about one of the most numerous and diverse enzyme superfamilies, the cytochromes P450, and their functional importance in the nematode, is rather limited. In excess of 80 cytochrome P450 genes (CYP) have been identified to be present in the genome of *C. elegans* (http://drnelson.utmem.edu/CytochromeP450.html), and with the exception of CYP22 [2,3] almost nothing is known about them.

CYP genes are found in all eukaryotes and most bacteria and Archaea. They code for NADPH dependent monooxygenases involved in the metabolism of various endogenous and exogenous compounds. The cytochrome P450 enzymes have, in particular, been implicated in the bioactivation or detoxification of many hydrophobic drugs and xenobiotics. The CYP gene expression can be modulated by these substances [4–8], and the potential of xenobiotically induced gene expression has been established both in toxicological and ecotoxicological studies [9,10].

A previous study [11] investigated the potential of 18 different xenobiotics to induce *C. elegans* CYPs. It could be shown that in particular CYP35s are strongly inducible by polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB) and certain drugs. Our ongoing interest is to further characterize the CYP specific genetic response and thereby not only provide new insights regarding the nematode's reaction to man-made chemical stress, but also exploit CYP as a biomarker for common environmental stressors. *C. elegans* has

proven useful in classical *in vivo* toxicity testing (lethality, reproduction, growth or developmental toxicity) [12–14] as well as in more recently developed molecular biological approaches like differential display [15,16], and DNA microarray analyses [17,18]; or systems with transgenic animals [19–22]. This latter system is based on heat shock promoter elements inducible by various kinds of natural and man-made stress. A CYP promoter driven reporter construct, in contrast, should have an increased substance class specific response potential.

This paper reports concentration dependent relationships of CYP35A1, A2, A5, and C1 gene expression in response to four different xenobiotics. The selected compounds comprise representative substances of ecotoxicological relevant groups: a PAH (fluoranthene), a polychlorinated biphenyl (PCB52), an herbicide (atrazine), and the ulcer preventive pharmaceutical lansoprazole.

In parallel to the effect monitoring on the molecular level, the toxicity of the four substances was analyzed using a reproduction assay in liquid medium. In addition, the consequences of CYP35A/C1 gene knockdown were determined by gene disruption and RNA interference (RNAi) in the presence and absence of xenobiotics. The authors postulate that the specific knockdown of all CYP35A forms and C1 results in a significant change in sensitivity towards the xenobiotics tested which in turn may act as a P450 substrate in the biotransformation process.

Materials and methods

Strains and cultivation

Throughout this study either *C. elegans* wild type strain Bristol N2 was used or a homozygous double mutant XA6700, which was generated by crossing *cyp-35A2* (gk317) and *cyp-35A4* (ok1393). Synchronous worms were generated by rinsing worms from NGM plates

and subsequently filtered through a 10 µm membrane filter (SM 16510/11, Sartorius, Germany) which retains all but first-stage juveniles. With the exception of the RNAi experiments, worms were cultivated in S basal liquid medium [23] at 25°C with *Escherichia coli* OP50 as a food source. The following exposure protocol was used per replicate for all experiments: 10 L1 larvae, 250 µg (fresh weight) *E. coli* OP50 and the xenobiotic, dissolved in 0.3 µl DMSO were added to 100 µl S basal liquid medium in a small glass vessel,

Xenobiotics

The effects of the following xenobiotics were analyzed: Atrazine [2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazin] (Riedel de Haën, Germany); fluoranthene [Benzo[j,k]fluorine]; lansoprazole [$C_{16}H_{14}F_3N_3O_2S$] and PCB52 [2,2',5,5'-tetrachlorbiphenyl]. If not stated otherwise the chemicals were obtained from Sigma-Aldrich, Germany. Due to their hydrophobic nature, the selected compounds had to be dissolved in an organic solvent. After a detailed selection procedure including different concentrations of ethanol, methanol, and DMSO, a final concentration of 0.3 % (v/v) DMSO in the medium was found to have no effect on reproduction of the nematodes and CYP35 mRNA expression.

Reproduction test

First-stage juveniles were initially transferred by micropipette to the test glass vessels containing 100 µl of liquid medium. The samples were placed in an incubator at 25°C for 4 days (96 ± 2 h). For each xenobiotic two controls, with or without 0.3 % DMSO, respectively and a dilution series of five to six concentrations was used. Twelve replicates were used for each test concentration. At the end of the exposure period the number of living adult individuals was noted and the offspring, regardless of the developmental stage, was determined. Finally offspring per worm was calculated from the number of second-generation juveniles per test

vessel. After the 96-h exposure period, the number of offspring was calculated from the number of second-generation juveniles at all stages. The reproduction rate in the control without solvent was 152.95 ± 15.69 (SD) and 142.05 ± 14.61 (SD) in the control with 0.3 % DMSO (in both cases n=60). The effect concentrations (EC_{50}) were calculated by probit analysis.

RNA Preparation and semi-quantitative RT-PCR

Nematodes were harvested as young adults (after 48 h) for the preparation of RNA . The RNA isolation followed standard procedures using Trizol reagent (Invitrogen), however modified to include a homogenization step with 0.5 mm glass beads to maximize cell breakage. Subsequently, RNA was purified using an RNeasy kit followed by a DNase digestion kit (QIAGEN). The reverse transcription was performed using M-MLV reverse transcriptase (PROMEGA) at 42°C for 90 min. Thereafter a PCR amplification of the *act-1* cDNA was performed using the HotStar Taq DNA polymerase system from QIAGEN with the primers act1-1 (5' GAGGCCCAATCCAAGAGA 3') and act1-2 (5' TGTTGGAAGGTGGAGAGG 3'). To quantify amplified DNA, the PCR samples were separated in an agarose gel and imaged using an AlphaImager 2200 (Alpha Innotech Corp., USA). The appropriate cDNA dilutions were empirically determined to ensure that the PCR amplification of *act-1* cDNA, as well as of each CYP35 cDNA, did not reach saturation. In each case calibration curves were constructed with known amounts of a commercial DNA mass standard. In the next step, the cDNA amounts used for the CYP35 PCRs were determined, calculating the *act-1* cDNA amount as internal standard (for CYP35 gene specific primers see [11]). To minimize potential error due to cDNA variability, the data was presented as a relative ratio between the specifically amplified CYP cDNA and *act-1* as internal reference. Each experiment was performed in triplicate.

RNAi by feeding

The RNAi feeding assay was performed on NGM agar plates. For each of the five CYP35A genes and for CYP35C1 an individual bacterial feeding strain was generated. We used the L4440 feeding vector [24] as a basis for cloning of restriction fragments of previously isolated CYP35A1-5/C1 cDNAs. In reference to the coding sequence the resulting inserts correspond with +21 to +899 (35A1), +1 to +748 (35A2), +1 to +1183 (35A3), +1 to +798 (35A4), +21 to +1100 (35A5), and +514 to +816 (35C1), respectively. The resulting plasmids, and an empty control vector, were transformed separately into the *E. coli* feeding strain HT115(DE3). The cultivation and induction of the bacteria followed in principal the instructions of Hull and Timmons [25]. Briefly, the plasmid carrying HT115(DE3) strains were cultivated in the presence of 50 µg/ml Ampicilin and 12.5 µg/ml Tetracycline until the freshly inoculated culture reached OD_{600}= 0.4–0.6. IPTG was added to a final concentration of 0.4 mM, and the culture incubated for an additional four hours at 37°C. The cells were harvested by centrifugation, if multiple RNAi feeding strains were utilized equal amounts of cell suspension were harvested. The bacteria were resuspended in resh LB medium, spiked with additional antibiotics (50 µg/ml Ampicilin, 12.5 µg/ml Tetracycline) and IPTG (to a final concentration of 0.8 mM). Finally, the cells were added to NGM agar plates, containing the same concentration of antibiotics and IPTG as described below. If a xenobiotic treatment was performed, fluoranthene (0.5 mg/L), PCB52 (10 mg/L), lansoprazole (10 mg/L) or atrazine (50 mg/L) were added to both the bacterial suspension and to the agar. In each case an individual first-stage juvenile of N2 wild type or XA6700 was transferred to each plate. Commencing with the egg laying period, each parental worm was transferred to new, freshly prepared RNAi feeding plates at regular intervals to facilitate the counting the entire F1 generation. Each experiment was performed twelve times.

To ensure that dsRNA feeding was successful, 10 adult worms from each RNAi condition were assessed by CYP35A specific RT-PCRs (for details see paragraph before). To obtain an overview regarding potential cross reactivities, these control experiments were performed with 5 mg/L PCB52 induced N2 wild type. To exclude the possibility that traces of the original feeding construct were amplified in the RT-PCR different reverse primers (Tab. 1) were used, binding in each case outside of the derived RNAi construct in the 3' non coding region.

Data analysis

For the reproduction assay (n=12), the relative reproduction [%] was calculated relative to the control (treated with 0.3 % DMSO). For the RNAi test (n=12), the total amount of F1 offspring was determined. All data were analyzed using the SPSS® computer software. If the data were normally distributed, a one way ANOVA was run to test significant differences between treatments followed by the Bonferroni T test to identify treatments that were significantly different from the control. If there was a lack of homogeneity of normality, the non-parametric Kruskal-Wallis one way analysis of variance on ranks was used followed by the Dunnett's test. The effect concentrations were determined using the probit analysis according to Finney [26]. For the RT-PCR tests (n=3), the concentration response plots were fitted to the respective data using Sigma Plot 2001 (SPSS®), for statistical comparison the standard deviation is presented.

Results

Dose dependent induction of CYP gene expression

In the semi-quantitative RT-PCR assay, all four xenobiotics caused a strong and reproducible induction of CYP35A1, A2, A5, and C1 gene expression. Fig. 1 presents representative DNA agarose gels showing the relative gene expression of each individual CYP form in relation to *act-1*, an invariant housekeeping gene in response to the respective xenobiotics. It is obvious that the increasing concentrations of the chemicals did not affect the actin gene expression, whereas the expression of all CYP35 genes increased in a concentration dependent manner.

The semi-quantitative evaluation of the data is presented in Fig. 2. This figure shows the increase of CYP35 specific gene expression (n=3), normalized to the *act-1* expression as internal control. A two-fold increase in gene expression was defined as the cut-off, highlighted in the graph by dashed lines. The concentration of each xenobiotic substance is shown on a molar basis (µmol/L), facilitating the comparison between the RT-PCR results and the toxic potency determined by the reproduction assay. PCB52 displayed the most significant CYP35 induction (Fig. 2B), where (with the exception of CYP35A5) 0.342 µmol/L (0.1 mg/L) resulted in excess of a two-fold increase. In the case of the PAH fluoranthene, 1.236 µmol/L (0.25 mg/L) was found to be sufficient to induce a two-fold increase of CYP35A1 and A5 gene expression (Fig. 2A). 1.353 µmol/L (0.5 mg/L) of the ulcer preventing drug lansoprazole also caused a two-fold increase of CYP35A1 and A2 (Fig. 2C). Atrazine (Fig. 2D) was found to be a less potent CYP35A/C inducer than the other chemicals, as the cut-off level for CYP35A1 and C1 induction was reached at concentrations equal or higher than 23.18 µmol/L (5 mg/L). Based on the combined data, but excluding the fluoranthene results, there is evidence that CYP35A5 gene induction is the least sensitive and CYP35A2 the most response CYP tested.

Reproduction assay

Fig. 3A–D visualizes the concentration effect relationship for fluoranthene, PCB52, lansoprazole, and atrazine as relative data (% reproduction) in single box plots. The associated control values were set to 100%. All xenobiotics negatively affect the reproduction in a strong concentration dependent manner with increasing concentrations causing decreasing reproduction. Only with lansoprazole, a hormetic effect was observed; low concentrations increased reproduction of *C. elegans*. While fluoranthene was the most toxic compound used in the study, the worms were insensitive to the herbicide atrazine. Based on the EC_{50}, the following order of toxicity was observed: Fluoranthene > PCB52 > lansoprazole > atrazine.

Efficacy of CYP35A RNAi

Based on the knowledge that CYP35A forms were found to be strongly inducible by PAK and PCB, it was considered important to analyze the effect of their gene expression knockdown during a continued xenobiotic exposure. RNAi refers to the introduction of homologous double stranded RNA (dsRNA) to specifically target a gene's mRNA, resulting in null or hypomorphic phenotypes. Generally, RNAi feeding assays are performed by using one bacterial strain harboring one plasmid at a time. To obtain multiple gene knockdowns, however, a feeding approach with multiple dsRNA producing strains is most useful. For predicting the efficacy of the selected CYP35A dsRNA feeding constructs as well as to control side-effects among each other, specific RT-PCRs were performed. N2 wild type worms were fed with each of the individual CYP35A1-5 constructs as well as with a mixture of all five feeding strains. As negative control a feeding strain harboring an empty L4440 vector was used. Furthermore, 1 mg/L PCB52 was added into the agar providing strong CYP35A gene induction. After a 48 h incubation period, 10 nematodes were analyzed by CYP35A1-5 and *act-1* specific RT-PCRs. It became obvious that each individual feeding construct showed a high specificity to its targeted gene, indicated by the absence of a RT-PCR product (Fig. 4). This

was valid also in the case where all five feeding strains were used in a mixed bacterial feeding cocktail, resulting in a complete loss of detectable CYP35A1-5 specific mRNA (Fig. 4, last lane). A strong cross-reactivity was found between CYP35A3 and A4 (Fig. 4, rows 3 and 4, lanes 3 and 4).Similarly, the A3 feeding construct knocked down CYP35A4 expression, the A4 construct the expression of CYP35A3 and the A2 feeding construct disabled CYP35A1 expression (Fig. 4, rows 1, lanes 2). Only CYP35A2 and A5 gene expression could be knocked down exclusively by its specific dsRNA construct. Because of its significantly lower homology CYP35C1 was not included in this cross-reactivity test.

CYP35A gene knockdown phenotype

Although multiple CYP35A1-5 RNAi succeeded in the intended gene silencing of the entire 35A subfamily, the inclusion of available *cyp-35* knock-out strain is desirable. The alleles *cyp-35A2* (gk317) and *cyp-35A4* (ok1393) both do not show any deviant phenotype, even in the presence of xenobiotics at concentrations used in this study (data not shown). The same results were obtained with the derived homozygous double mutant XA6700, generated by crossing *cyp-35A2* (gk317) and *cyp-35A4* (ok1393). Besides the unchanged phenotype, the reproduction rate remained constant in comparison to the wild type, even following the exposure to fluoranthene, PCB52, lansoprazole, or atrazine (Fig. 5). Cytochrome P450s are well known for their redundancy, so that more than one isoenzyme is capable of metabolizing a single agent. The intention to knockdown all CYP35A/C forms was achieved by feeding XA6700 CYP35A1, A3, A5 and C1 dsRNA. Again, the CYP35A/C depleted worms resembled wild type in terms of morphology and reproduction rate under control condition. However in the presence of any of the four xenobiotics, reproduction increased in comparison to N2 wild type (Fig. 5).

Discussion

CYP35 gene expression and xenobiotics

A systematic gene expression screen of *C. elegans* cytochrome P450 genes revealed that almost all CYP35 forms are moderately or strongly inducible by different xenobiotics [11]. This report focused on CYP35A1, A2, A5, and C1, all of which were basally expressed and displayed a strong inducibility. It is important to add that the missing CYP35A forms 3 and 4 are just as well inducible as the other four selected forms [11]; however, their basal expression is often hardly detectable. From the four xenobiotics used in the recent investigation, PCB52, fluoranthene, and lansoprazole were found to be able to induce CYP35 gene expression significantly in a concentration dependent relationship. In case of atrazine, an approximately 100-fold increase in concentration was necessary to cause a moderate induction.

All four xenobiotics are known inducers of human CYP forms [27] as well as *C. elegans* CYPs [11]. Lansoprazole, like other benzimidazole derivates, is both a potent inducer of human CYP forms (1A1, 1A2 and 1B1) [28, 29] and a suitable P450 substrate (3A4, 2C18, 2C19) [30, 31]. Fluoranthene is a rather weak inducer of CYP1A1 and 1B1 [32, 33]. For atrazine, heterologously expressed human CYP1A1 and A2 were described as the major P450 species metabolizing this substance [34]. Moreover, this s-triazine was found to be able to increase 7-ethoxyresorufin O-deethylase (EROD), 7-methoxyresorufin O-demethylase (MROD), and 7-pentoxyresorufin O-depentylase (PROD) activities significantly [35], all closely related to P450 1A forms.

The regulation of mammalian CYP1A is well understood. It is based on the aryl hydrocarbon receptor complex (AHR-ARNT) binding to the xenobiotic response element (XRE) in the CYP1A promoter region [for review, see 36]. Even though CYP35A/C transcription can be activated by inducers of the CYP1A subfamily, there is no significant homology of the

derived amino acid sequences. The highest homologies exist to CYP family 2C forms. It is important to add that in *C. elegans*, homologous proteins to the regulators AHR and ARNT could be identified, designated as *ahr-1* and *aha-1*, respectively [37]. In addition, XRE like elements could be found in the promoter regions of almost all CYP35 genes. In *ahr-1* minus worms (ju145), however, the strong CYP35A/C gene expression induced by PCB52, lansoprazole, and fluoranthene was not affected at all (data not shown). Powell-Coffman's laboratory recently demonstrated [38, 39] that AHR-1 is localized in various nervous cells of *C. elegans* and not in the intestine like P450 35A forms [11]. The obvious function of *ahr-1* is the regulation of GABAergic motor neuron fate specification [38, 39]. To facilitate this, AHR-1 requires AHA-1, but not Hsp90, indispensable, however, in the mediation of the PAH ligand-activated transcription. Moreover, *C. elegans* AHR-1 was not able to bind a dioxin analogue [37] and, thus, probably has a different substrate specificity than mammalian aryl hydrocarbon receptors. Hence, we assume that PAH induced CYP35A/C gene expression is independent of AHR-1. This is also confirmed by the finding that CYP35A/C gene expression is also inducible by PCB52. PCB52 (2,2',5,5'-tetrachlorbiphenyl) is an *ortho*-substituted, non-coplanar PCB unable to induce mammalian CYP1A [40]. All non-dioxin like PCB congeners are believed to be unable to bind to AHR in *C. elegans*. On the other hand, these PCB congeners are able to bind CYP2 and 3 forms. PCB52 binds preferentially to CYP2B and to a lesser extent to CYP3A enzymes [41]. Combining all results, the xenobiotically induced CYP35A/C gene expression pathway seems to be different to any known mammalian CYP inducing pathway. Further effort will be necessary to unravel the molecular genetic background of this observation.

Xenobiotics and toxicity

Besides the CYP inducing properties of the xenobiotic substances used, it was also important to characterize their toxic potential. The effect of xenobiotics on the reproduction of test organisms is a broadly accepted test parameter and was found to be a much more sensitive indicator of toxicity than lethality in *C. elegans* [13]. The observed mean reproduction rate in control medium of about 150 offspring per worm was significantly higher than the reproduction rate of 18.9 ± 8.0 (SD) reported by Traunspurger et al. [42]. This observed difference is likely due to the culturing conditions used. Whilst Traunspurger et al. used test vessels placed on a shaking desk we favored a less disruptive method by reducing the volume to 0.1 ml thereby ensuring a sufficient supply of oxygen without the need of shaking.

This modified *C. elegans* reproduction test produced a robust concentration dependent relationship to the xenobiotic substances. To date nearly all *C. elegans* ecotoxicological studies investigated the toxic effects of heavy metals, such as cadmium and lead. Although a comparison with available toxic endpoint values of other invertebrates (Tab. 2) reveals that *C. elegans* is less sensitive to organic compounds than, for example, daphnids or oligochaetes, the observed order of toxicity, based on the EC_{50} values (fluoranthene > PCB52 > > atrazine) could be confirmed. PAHs and PCBs have been classified as narcotics. In addition to death, exposure to narcotic chemicals also may affect sublethal processes including growth, reproduction, and developmental time.

Toxicity and CYP35 gene expression

It is interesting, particularly for ecotoxicological purposes, to compare effect levels of acute toxicity and induction thresholds of CYP35A/C gene expression, reached under subacute conditions. In case of PCB52, a two-fold increase of CYP35A1, A2, and C1 gene expression was reached at 0.52 %, of the corresponding EC_{50} for reproduction. In the case of lansoprazole, 2.2 % of the EC_{50} induced a two-fold expression increase of CYP35A1 and A2.

Fluoranthene and atrazine showed a low inducing potential, where 25 % of the EC_{50} values caused a two-fold expression increase of several CYP35 genes. The main advantage of the gene expression test compared to the reproduction test is the increased sensitivity. The parallel determination of a variety of stress inducible genes (e.g. by using DNA microarrays) will considerably enlarge the implementation of this approach. Depending on the selected marker genes, this approach has the potential to identify substance class specific effects.

In this context, it is of fundamental importance to reveal potential relationships between the observed toxicity and the induced gene expression. Specific gene knockdown is a proven approach to study the function of the encoded protein in more details. Cytochromes P450 are noted for forming families of isoenzymes whose members are often able to act as a substitute for several other members. It was therefore the intention of this work to knockdown all six CYP35A/C forms in parallel. This was successfully achieved by a multiple RNA interference (RNAi) assay, and by using the homozygous *cyp-35A2/cyp-35A4* double mutant (XA6700). However, cross-reactivity, whereby multiple genes may be simultaneously targeted by a single dsRNA fragment, can potentially jeopardize correct interpretation of gene function. Individual CYP35A1-5 RNAi by feeding experiments with induced N2 wild type demonstrated that cross-reactivity occurred in particular between CYP35A3 and A4, and, to a lesser extent, between CYP35A1 and A2. These results accentuate the need for testing the specificity of each dsRNA fragment prior to a full phenotypic analysis, in particular if it is intended to produce a simultaneous knockdown of an entire subfamily of genes.

The consequences of complete CYP35A/C knockdown are rather low. There is not any striking abnormal phenotype, even in the presence of xenobiotic substances. When the brood size was taken into account, however, a surprising trend became visible. The knockdown of CYP35A/C rescued, to some extent, the decrease in reproduction caused by PCB52 and fluoranthene. Some PAHs and PCBs have been shown to cause cellular transformation

only after metabolic activation by cytochromes P450 and epoxide hydrolases producing highly reactive electrophiles [43]. In rat liver microsomes, PCB52 is metabolized to the 3-hydroxy- and 3,4-dihydroxy-forms, which were further oxidized to form a reactive intermediate that produce quinonoid-derived protein adducts in the liver [44]. Moreover, a very reactive PCB52 derived arene oxide intermediate was detected both in rat and rhesus monkey liver microsomes [45]. In case of the very related 2,5,2',5'-tetrachlorbiphenyl congener, Koga et al. [46] suggested that in hamster liver different P450 isoforms are involved in the 3- and 4-hydroxylation. The major metabolic activation pathway of fluoranthene resulting in the production of mutagenic species involves the formation of the 2,3-diol [47] and the subsequent oxidation to the 2,3-diol-1,10b-epoxide [48]. Another minor activation pathway with mutagenic endpoints may involve the formation of 7,8-dihydroxyfluoranthene [48]. Shimada et al. [32] showed that human CYP1A1 is able to activate fluoranthene-2,3-diol. Althgough it is unknown if *C. elegans* is able to activate PAH and/or PCB, preliminary experiments indicate that the worm is able to metabolize benzo[a]pyrene to at least two metabolites in a cytochrome P450-dependent manner (personal communication by M. Amichot, INRA, Antibes, France). Recent evidence was provided that biotransformation of fluoranthene may have produced a more toxic metabolite, analyzed by Schuler et al. [49] in the invertebrate *Chirnonomus tentans*.

Metabolites acting as specific toxins has been known to occur in vertebrates and is well-established for PAH compounds such as benzo[a]pyrene, but there are only few studies with invertebrates, such as insect larvae (*Chironomus*) or nematodes (this study). In fact the subtlety of metabolic channeling in which reactive intermediates transiently appear is decisive for their biological effect and depends mainly on proper coupling of all involved biotransforming enzymes. Our results indicate that single or several P450 35A/C forms are involved in the activation of fluoranthene and/or PCB52. It is suggested that by knocking down

CYP35A/C gene expression the generation of more toxic metabolites is prevented, which results in an increase in reproductive capacity.

A whole-animal biomonitor and environmental realism

Xenobiotically induced gene expression and other new approaches are becoming more and more prevalent in biomonitoring, ecotoxicology, and stress ecology. However, they are still not widely accepted, which tends to retard their implementation. One reason for this is that these systems are not able to completely simulate the highly integrated functions of a living organism [50]. The other reason is that usually only drastic, but ecologically almost meaningless effects, like increased mortality and, less frequently, reduced reproduction, are accepted as endpoints in validated and standardized biotests. Today, reliable, sensitive, and specific test systems are needed, in particular for risk assessment of low-level mixtures of xenobiotics in the environment which affect both wildlife and human health on a subcellular level. Furthermore, the chemical stress by man-made compounds must be differentiated from common natural stresses the organisms are exposed to. The natural stress may be caused by environmental matrices, such as humic substances. This applies particularly to a soil inhabitant, such as *C. elegans*. That means that all defense systems that respond to xenobiotic chemicals, must have evolved by natural stressors, and the response to xenobiotic exposure is only an additional, but severe, chemical stress. In this respect, it has recently been shown that humic substances have the potential to act as 'natural' xenobiotics [51] causing a modulation of offspring number [52, 53].

Using sensitive and reproducible detection methods, such as RT-PCR, it is possible to establish significant pollution and natural stress induced changes of specific gene expression and to generate accurate new assays. As shown, one main intention of this paper was to reveal a new link between toxicity and upstream induced gene expression. Future studies will show

whether or not these approaches will be able to distinguish between natural and man-made chemical stresses.

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Table 1

C. elegans CYP35A gene specific primer

Name	Sequence 5‘-3‘
35A1-2	ACATGTGAGCGATAAGATGA
35A2-2	AAAAACATTTTATTCGAAGA
35A3-2	TTTATTTAAACATTCACTAC
35A4-2	ATTCGTTGCATTAAGATTC
35A5-2	TTTATTTACTAAAATCTCTGAAC

Table 2

Comparative toxic endpoint values for three xenobiotics exposed to invertebrates.

SUBSTANCE	SPECIES	TOXIC END-POINT	TOXIC DOSE [mg/L]	SOURCE
Atrazine	*Lumbriculus variegatus*	LC_{50}	37.10[a]	[b]
	Daphnia pulex	EC_{50}	5.00	[32]
	Daphnia magna	LC_{50}	21.50[a]	[b]
	Helobdella stagnalis	LC_{50}	9.00	[32]
	C. elegans	EC_{50}	**86.64**	[c]
Fluoranthene	*Lumbriculus variegatus*	LC_{50}	> 0.178	[33]
	Daphnia magna	LC_{50}	0.117	[33]
	C. elegans	EC_{50}	**0.90**	[c]
PCB52	*Enchytraeus crypticus*	LOEC(70%)	2.00 [mg/kg soil]	[d]
	C. elegans	EC_{50}	**15.61**	[c]

All shown EC_{50}/LOEC values correspond to the reproduction. *L. variegates* and *E. cyrpticus* – Oligochaete worms; *T. tubifex* – Tubificid worm; *Daphnia spec.* – Water flea; *H. stagnalis* Freshwater leech

[a] Average species LC_{50} value [[b]] calculated for each chemical/species combination by adding the LC_{50} values for each study and dividing by the number of studies.

[b] http://www.pesticideinfo.org (resource of the Pesticide Action Network North America)

[c] This study

[d] http://anubis.uba.de/etox//bin/default.asp (ETOX database, a resource of the German Federal Office for Environment – UBA)

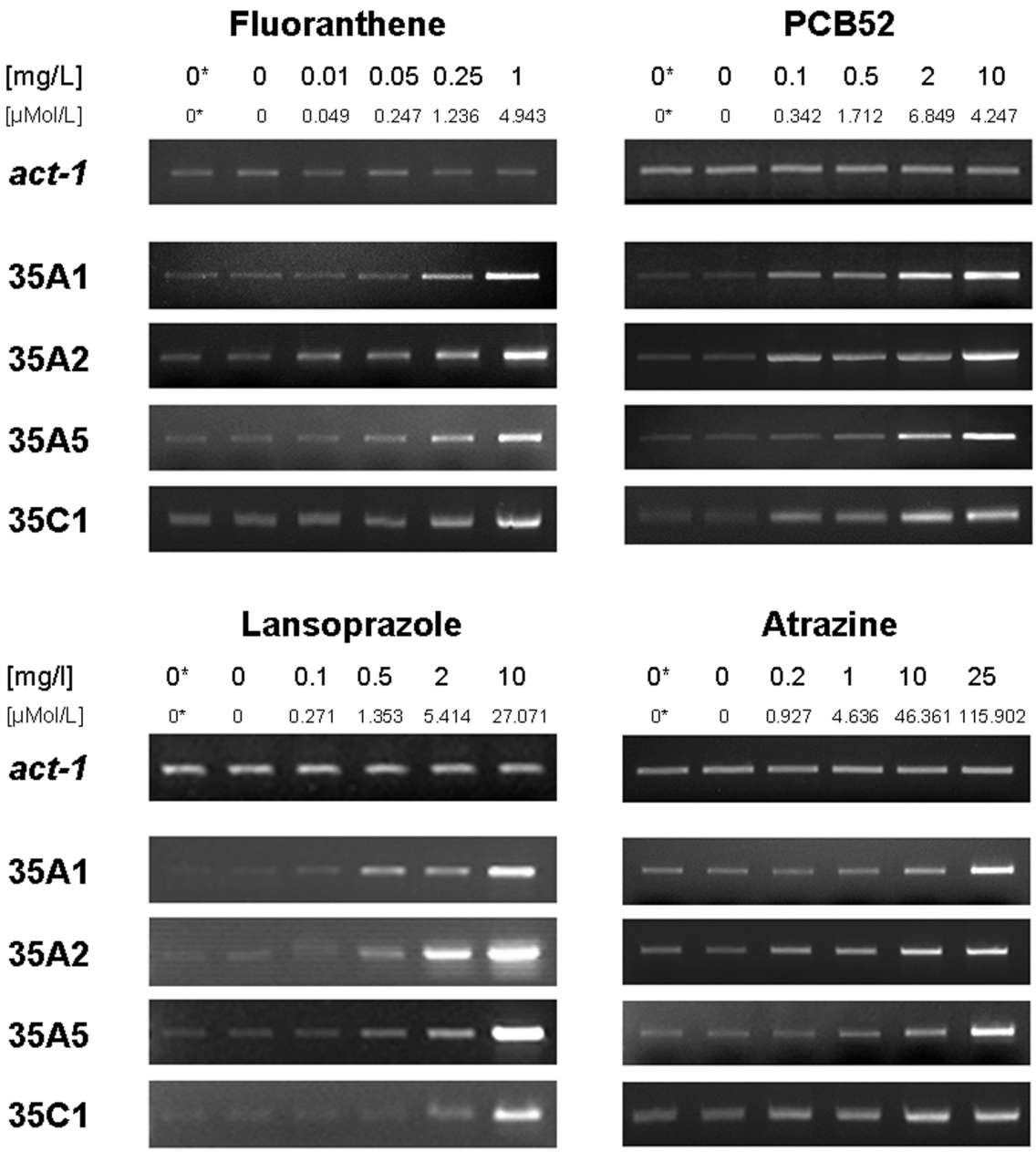

Fig. 1. CYP mRNA levels in control and xenobiotically induced worms determined by RT-PCR. Pictures are a representative image from three replicated analyses. *control without 0.3 % DMSO in the medium.

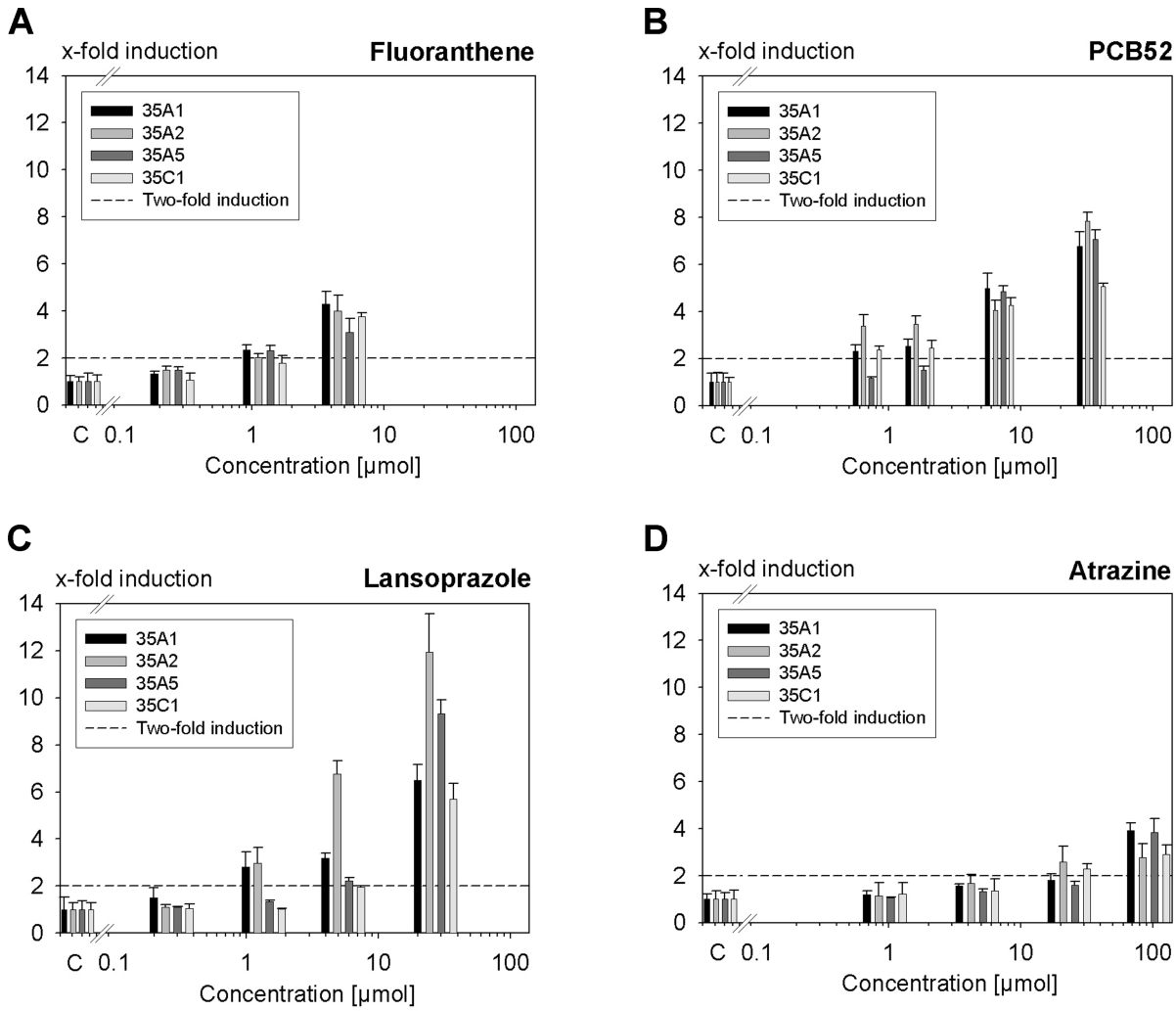

Fig. 2. Induction of CYP35A/C gene expression in response to (**A**) fluoranthene, (**B**) PCB52, (**C**) lansoprazole, and (**D**) atrazine. Shown are relative data as ×-fold increase of CYP35 gene expression normalized to *act-1* expression. The means for three trails are semi-log plotted, error bars denote SD (n=3). The mean of the control (C), with 0.3 % DMSO in the medium, corresponds to 1. The dotted line marks a two-fold increase of CYP35 specific gene expression.

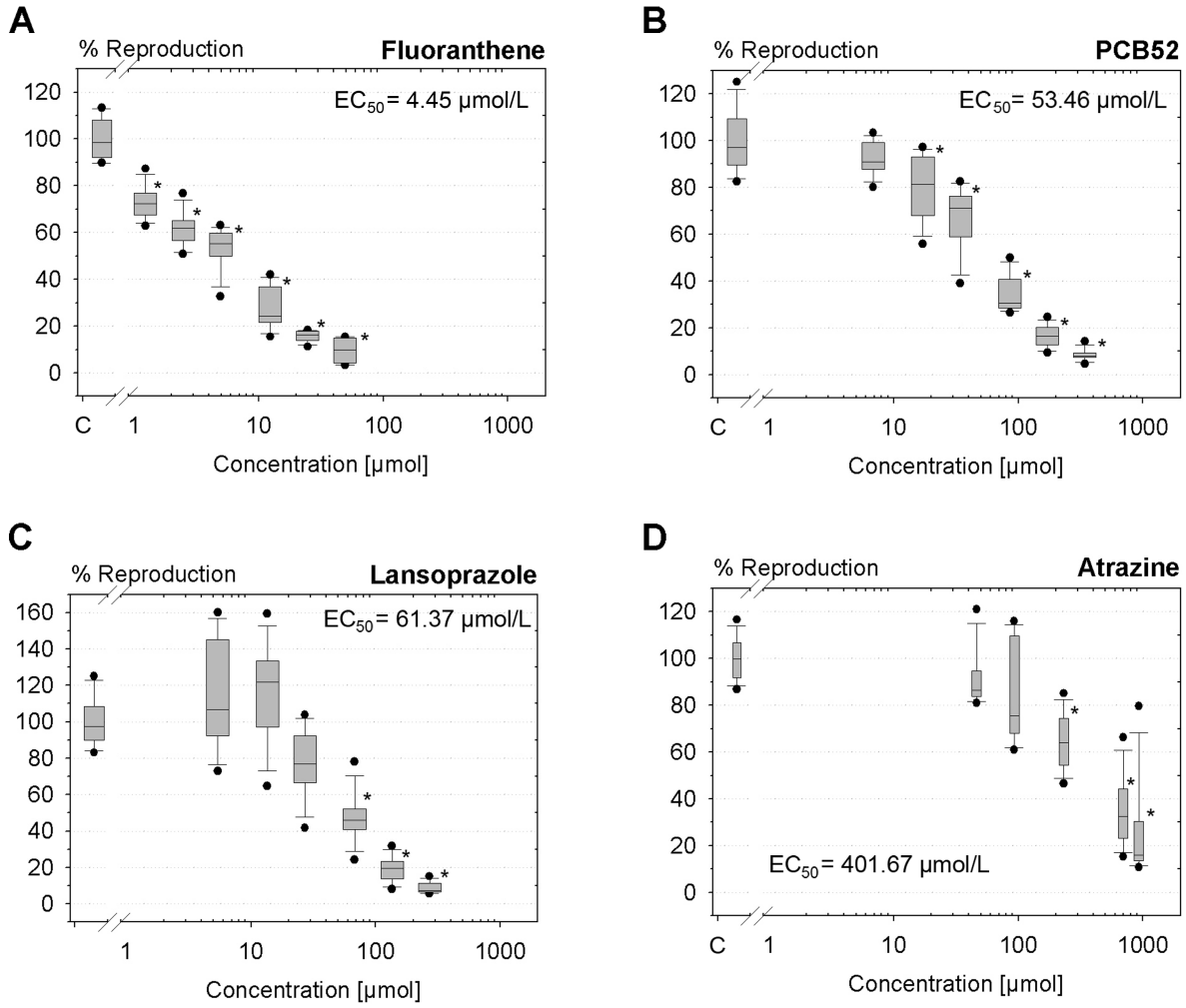

Fig. 3. Xenobiotically induced decrease in reproductive capacity. The F1 offspring was counted after a 96 h exposure in liquid medium (n=12); shown are relative data in percent. The mean of the control (C) with 0.3 % DMSO in the medium corresponds to 100 % reproduction. The data are semi-log plotted and each outlier is indicated: (**A**) fluoranthene, (**B**) PCB52, (**C**) lansoprazole, and (**D**) atrazine. The effect concentrations were determined according to Finney's probit analysis [26]. $^{*}p<0.05$.

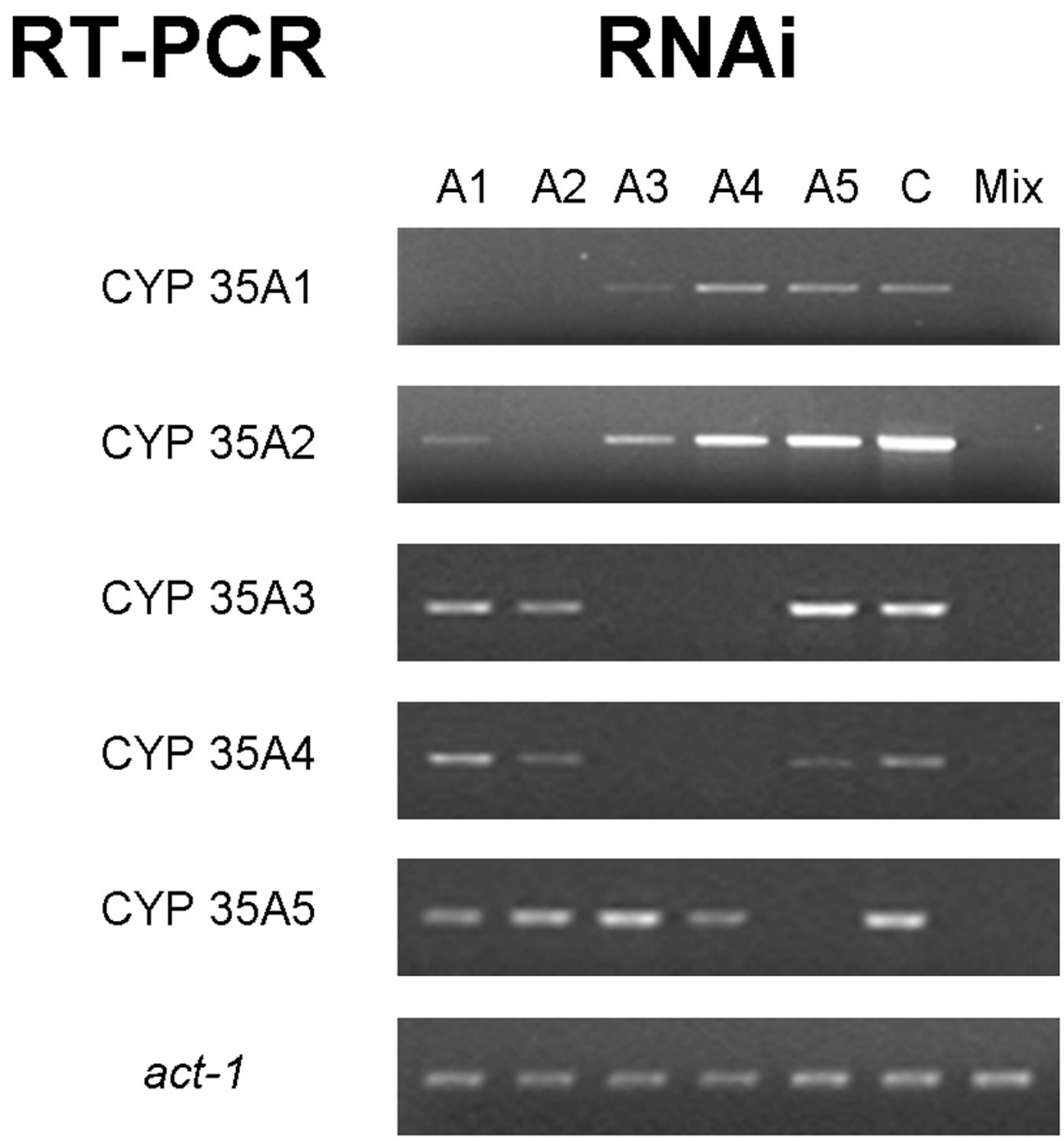

Fig.4. Cross reactivity of CYP35A1-5 RNAi by feeding assays. Pictures show typical images of a ten-worm RT-PCR assay loaded on an agarose gel. N2 wild type worms were cultivated for 48 h on single feeding plates (lanes A1-A5), control plates (lane C), and multiple feeding plates (lane Mix), respectively. In each case all five CYP35A specific RT-PCR (rows 1-5) and an *act-1* specific RT-PCR (row 6) was performed.

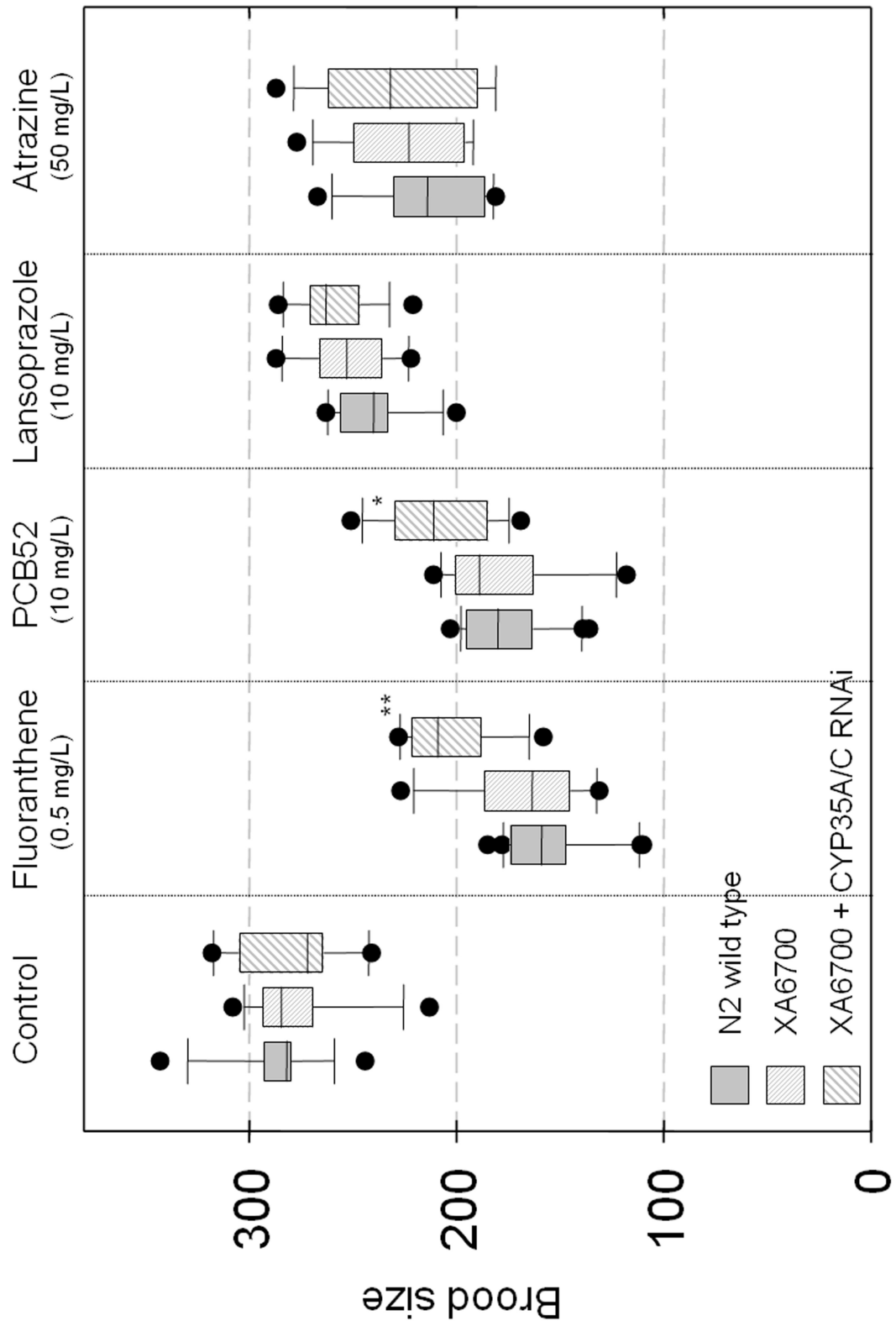

Fig. 5. CYP35A/C gene knockdown diminishes xenobiotically affected reproduction decline. L1 larvae of N2 wild type, XA6700 alone and combined with CYP35A/C RNAi by feeding, respectively, were cultivated on agar plates in the absence or presence of four different xenobiotics (n=12). The amount of complete F1 offspring are presented in box plots showing each outlier. $^{*}p<0.05$; $^{**}p<0.01$.