Ecotoxicology (2016) 25:419–430
DOI 10.1007/s10646-015-1600-z

Measuring cytochrome P450 activity in aquatic invertebrates: a critical evaluation of in vitro and in vivo methods

Michele Gottardi[1] · **Andreas Kretschmann**[1,2] · **Nina Cedergreen**[1]

Accepted: 9 December 2015 / Published online: 19 December 2015

Abstract The first step in xenobiotic detoxification in aquatic invertebrates is mainly governed by the cytochrome P450 mixed function oxidase system. The ability to measure cytochrome P450 activity provides an important tool to understand macroinvertebrates' responses to chemical stressors. However, measurements of P450 activity in small aquatic invertebrates have had variable success and a well characterized assay is not yet available. The general lack of success has been scarcely investigated and it is therefore the focus of the present work. In particular, the suitability of the substrate selected for the assay, the sensitivity of the assay and the possible inhibition/attenuation of enzymatic activity caused by endogenous substances were investigated. 7-ethoxycoumarin-O-dealkylation activity of *Daphnia magna*, *Chironomus riparius* larvae and *Hyalella azteca* was assessed in vivo and in vitro and possible inhibition of enzymatic activity by macroinvertebrates homogenate was investigated. Activities of *D. magna* and *C. riparius* larvae measured in vivo were 1.37 ± 0.08 and 2.2 ± 0.2 pmol h^{-1} organism^{-1}, respectively, while activity of *H. azteca* could not be detected. In vitro activity could be measured in *C. riparius* larvae only (500–1000 pmol h^{-1} mg microsomal protein^{-1}). The optimization of the in vitro assay has been especially long and resource consuming and particularly for *D. magna*, substances that inhibited cytochrome P450 activity seemed to be released during tissue homogenization preventing activity measurements in vitro. We therefore recommend testing the P450 inhibition potential of homogenate preparations prior to any investigation of P450 activity in vitro in macroinvertebrates.

Keywords Metabolic enzymes · Xenobiotic detoxification · *Chironomus riparius* · *Daphnia magna* · *Hyalella azteca*

Abbreviations

ACN	Acetonitrile
BSA	Bovine serum albumin
DTT	DL-dithiothreitol
ECOD	7-ethoxycoumarin-O-dealkylation
EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate
EROD	7-ethoxyresorufin-O-dealkylation
$K_2HPO_4 \cdot 3H_2O$	Potassium phosphate dibasic trihydrate
KH_2PO_4	Potassium dihydrogen phosphate
MFO	Mixed function oxidase
$MgCl_2 \cdot 6H_2O$	Magnesium chloride hexahydrate
NADPH	ß-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate
PMSF	Phenylmethanesulfonyl fluoride

Electronic supplementary material The online version of this article (doi:10.1007/s10646-015-1600-z) contains supplementary material, which is available to authorized users.

✉ Michele Gottardi
migo@plen.ku.dk

[1] Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark

[2] Present Address: Department of Pharmacy, Analytical Biosciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark

Introduction

Cytochrome P450 mixed function oxidase (MFO) systems play the major role in oxidation of drugs and other foreign compounds in humans (Guengerich 2005; Timbrell 2008) and in a vast variety of species such as bacteria (Kelly and Kelly 2013), plants (Gonzalez-Mendoza 2007), aquatic invertebrates (James and Boyle 1998; Rewitz et al. 2006; Snyder 2000), fish (Uno et al. 2012) and insects (Feyereisen 1999). Cytochrome P450 contribution to xenobiotic detoxification has driven the attention of environmental toxicologists for at least the past two decades (Feyereisen 1999; James and Boyle 1998; Rewitz et al. 2006; Snyder 2000; Uno et al. 2012). The activity of P450 enzymes (Binelli et al. 2006; Zelnickova et al. 2013) and their induction (Tian et al. 2014; Vanderweiden et al. 1993) have been used as biomarkers of xenobiotic exposure, while overexpression of P450 genes has been attributed to pesticide resistance in several insects (Daborn et al. 2001; Komagata et al. 2010; Yang et al. 2006). In addition, P450 activation or inhibition seems to play a key role for the mode of action of a range of synergizing chemicals (Cedergreen 2014). P450 induction can lead to a faster activation of chemicals that need oxidation to reach their most active form (Belden and Lydy 2000), while P450 inhibition can increase the toxicity of compounds that are otherwise rapidly detoxified via P450 (Cedergreen et al. 2006; Chalvet-Monfray et al. 1996; Johnson et al. 2013; Pilling et al. 1995). The knowledge of P450 activity in different species exposed to various types of stress, its regulation over time and through the life stages and the resulting effects on the organisms' fitness and susceptibility to chemicals is therefore paramount for our understanding of chemical effects and interactions in ecologically important species.

The majority of aquatic risk assessment is currently based on tests that employ a small suit of macroinvertebrates for which standardized protocols have been developed, for example *Daphnia magna*, *Chironomus riparius* larva and *Hyalella azteca* (EPA 2000; OECD 2004, 2010, 2011, 2012). As a result, these species also form the basis of a large range of ecological studies generally concerned with chemical stress. The ability to measure cytochrome P450 activity in these species and potentially in any species of macroinvertebrates, both collected in nature or cultured in the laboratory, would provide an important tool for our understanding of the organism responses to chemical stress as well as synergistic chemical interactions.

A well characterized and robust method for cytochrome P450 activity measurements in small aquatic invertebrates is to the best of our knowledge not yet available. Measurements of P450 activity have been carried out with variable success (Baldwin and Leblanc 1994; Binelli et al. 2006; Sturm and Hansen 1999). The lack of success may be due to several complications: (1) Substrates commonly used for human P450 might be unsuitable for the invertebrates due to differences in P450 isoforms (James and Boyle 1998; Snyder 2000). (2) Aquatic invertebrates, and particularly crustaceans, have very low P450 activities as compared to aquatic vertebrates (Livingstone 1998). Moreover, due to their small size, the analyses can generally be performed only on the whole organisms and not on organs such as the liver, hepatopancreas or gut system, which typically contain concentrated amounts of P450. Therefore, P450 activity in whole-body extracts from small invertebrates may not be high enough to be detected with current spectrophotometric assays. (3) Finally, since the target organs can usually not be dissected from small invertebrates, though exceptions exist (Anderson and Zhu 2004), endogenous substances released during homogenization of the whole organism might inhibit and/or alter the measurable enzymatic activity (Baldwin and Leblanc 1994).

The most common way to measure cytochrome P450 enzymatic activities is by using a range of fluorometric assays. These methods are based on the transformation of substrate to a fluorescent product catalyzed by cytochrome P450 and its quantification with a spectrofluorometer (Aitio 1978; Koenig et al. 2012). In vitro fluorometric assays have been developed extensively for humans (Ong et al. 2013), but have also been employed for other mammals (Glockner and Muller 1995; Routti et al. 2008), fish (Bach and Snegaroff 1989; Koenig et al. 2012; Uno et al. 2012), molluscs (Binelli et al. 2006), insects (Fisher et al. 2003; Mayer et al. 1977), nematodes (Kotze 2000) and large crustaceans (James 1984; Koenig et al. 2012). In the majority of these assays, P450 activity has been measured on the microsomal fraction of target organs which presumably contain high P450 activity. In addition to in vitro assays, in vivo assays, where a fluorescent product is formed during exposure of the living test organisms to a specific substrate, are also available but much less commonly used. These assays have been employed for molluscs (Gagnaire et al. 2010), zebrafish larvae (David et al. 2012; Jones et al. 2010; Noury et al. 2006) and *D. magna* (David et al. 2012).

Among the different substrates applied in the existing literature, 7-ethoxycoumarin (7-Ethoxy-2H-chromen-2-one) and 7-ethoxyresorufin (7-Ethoxy-3H-phenoxazin-3-one) are standard artificial substrates that have been used for the fluorometric determination of cytochrome P450 dependent 7-ethoxycoumarin-O-dealkylation (ECOD) and 7-ethoxyresorufin-O-dealkylation (EROD) activities, respectively. In particular, ECOD activities have been measured in vitro and in vivo in species as different as mammals (Moon et al. 1998; Waxman and Chang 2006), fish (Bach and Snegaroff 1989; Jones et al. 2010), molluscs

(Gagnaire et al. 2010), nematodes (Kotze 2000) and insects (Fisher et al. 2003; Sturm and Hansen 1999). Although the specificity of 7-ethoxycoumarin toward macroinvertebrate CYPs is largely unknown and other substrates might be more suitable for macroinvertebrates, its relatively broad P450 isoenzyme specificity in human (Waxman and Chang 2006), higher reported in vitro activities in fish liver microsomes (Bach and Snegaroff 1989), higher in vivo activities in zebrafish larvae (Jones et al. 2010) and fresh water snails (Gagnaire et al. 2010) compared to 7-ethoxyresorufin as well as its low cost and high water solubility, made 7-ethoxycoumarin our first substrate of choice for the present study.

This work investigates the applicability of in vitro and in vivo ECOD assays for small aquatic invertebrates. The focus was set on the three major challenges mentioned above: (1) Substrate suitability (2) Method sensitivity and (3) possible auto-inhibition of cytochrome P450 activity during preparation of microsomes for in vitro tests. The suitability of the substrate 7-ethoxycoumarin for measurement of cytochrome P450 activity in small aquatic invertebrates was tested under in vivo and in vitro conditions with the standard organisms *D. magna*, *C. riparius* larvae and *H. azteca*. Moreover, the ability of the ECOD assay to detect cytochrome P450 inhibition after exposure to xenobiotics was tested in vivo with the azole fungicide prochloraz. In order to measure low enzymatic activities of small aquatic invertebrates in vitro, existing methods were optimized with focus on the fluorometric assay sensitivity as well as microsomes preparation procedures. Since very low or absent in vitro P450 activities were reported previously for *D. magna* (Baldwin and Leblanc 1994; Sturm and Hansen 1999) and a large discrepancy was found between the in vitro and in vivo activities of *D. magna* in the present study, a fast and cheap method was designed to quantify possible auto-inhibition of P450 activity during homogenization of the whole organisms. Finally, the strengths and limitations of the investigated in vitro and in vivo ECOD assays are discussed.

Materials and methods

Chemicals

Potassium dihydrogen phosphate (KH_2PO_4, CAS: 7778-770, purity: ≥99.0 %), magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$, CAS: 7791-18-6, purity: ≥99.0 %) and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, CAS: 6381-92-6, purity: 99.0 %) were bought from VWR—Bie and Berntsen. Potassium phosphate dibasic trihydrate ($K_2HPO_4{\cdot}3H_2O$, CAS: 16788-57-1, purity: ≥99.0 %) was obtained from Merck (Germany), acetonitrile (ACN, CAS: 75-05-8, purity: ≥99.9 %) from Rathburn (U.K.), 7-hydroxycoumarin (CAS: 93-35-6 purity: 99.5 %), 7-ethoxycoumarin (CAS: 31005-02-4, purity: 99.7 %), bovine serum albumin (BSA, CAS: 9048-46-8, purity: ≥98 %), Bradford reagent (Product nr: M9066), ß-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH, CAS: 2646-71-1, purity: ≥98 %) and 2-methoxyethanol (CAS: 109-86-4, purity: ≥99.9 %), from Sigma-Aldrich (U.S.), DL-dithiothreitol (DTT, CAS: 03-12-3483, purity: ≥99.5 %) from Sigma-Aldrich (Canada), glycerol (CAS: 56-81-5, purity: ≥99 %), phenylmethanesulfonyl fluoride (PMSF, CAS: 329-98-6, purity: ≥99.0 %) and prochloraz (CAS: 67747-09-5, purity: 98.6 %) from Sigma-Aldrich (Germany).

Rat liver microsomes (Product nr: M9066, pooled from male rat) were obtained from Sigma-Aldrich (U.S.).

Phosphate buffer (0.30 M) was prepared with KH_2PO_4 (final conc. in buffer: 0.10 M) and $K_2HPO_4{\cdot}3H_2O$ (final conc. in buffer: 0.20 M) in water from a MilliQ system. Stock solution of 7-ethoxycoumarin (25 mM) was made in 30 % v/v ACN in MilliQ water and 7-hydroxycoumarin (1.36 mM) in 30 % v/v ACN in phosphate buffer (0.13 M, pH 7.5) and glycerol (10 % v/v). The stock solutions of BSA (5 $mg_{protein}$ mL^{-1}) and NADPH (2 mM) were prepared in phosphate buffer (0.13 M, pH 7.5), of PMSF in 2-methoxyethanol (100 %) and of DTT (50 mM), EDTA (10 mM) and $MgCl_2{\cdot}6H_2O$ (50 mM) in MilliQ water.

Organisms

D. magna originally obtained from the "Technical University of Denmark, Denmark" were cultured based on the "OECD guideline for Testing of Chemicals" (OECD 2012). During culturing, the organisms were fed with approx. 9×10^5 cells $organism^{-1}$ day^{-1} of the algae *Pseudokirchneriella subcapitata* and kept in glass beakers (1 L) containing 0.800 L M7 daphnia medium (OECD 2012) per 12 individuals at 20 ± 2 °C with a 16:8 h light: dark photoperiod. Neonates were discarded three times a week and M7 medium renewed once a week.

C. riparius larvae originally obtained from the "Swedish University of Agricultural Sciences, Sweden" were cultured based on the "OECD guideline for Testing of Chemicals" (OECD 2011). During culturing, the organisms were kept in 5 L crystalline dishes containing constantly aerated M7 medium (ca. 8 cm depth) and quartz sand (ca. 1 cm depth) at 20 ± 2 °C with 16:8 h light: dark photoperiods. Evaporated water from the medium was replaced with demineralized water to maintain the ion balance; moreover, 1 L of water was changed once a week to remove excess nutrients. The larvae were fed with 0.150 g of Tubifex® Biotal Basic Vegetable flakes per crystalline dish, three times a week.

H. azteca originally obtained from "Roskilde University, Denmark" were cultured based on the "USEPA Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates" (EPA 2000). During culturing, the organisms were kept in an aquarium (56 L) containing M7 medium at 25 ± 1 °C with 16:8 h light: dark photoperiods and fed with approx. 0.75 g of commercially available rabbit food pellets (Centrum Dyrehandel, Ballerup, Denmark) every 2 days. Evaporated water from the aquarium was regularly replaced with M7 medium. Twice a month, 5 L of medium present in the aquarium was replenished with fresh M7 medium.

In vivo cytochrome P450 activity assay

The in vivo method was adapted from Gagnaire et al. (2010) (for details see Section 1 in Supporting Information). Five days old *D. magna*, four instar *C. riparius* larvae or randomly selected adult *H. azteca* were used. The organisms (10 *D. magna*, 3 *C. riparius* larvae or 10 *H. azteca*, $n = 3$ biological replicates) were incubated with 7-ethoxycoumarin dissolved in M7 medium (0.006 mM, tot. vol. 2 mL) for 3 h at room temperature (22–25 °C). Blanks were made with the incubation medium containing no organisms to check for background fluorescence of the substrate (Aitio 1978). In order to verify whether product formation was cytochrome P450 dependent, the organisms were continuously exposed to the azole fungicide prochloraz (500 μg L^{-1} corresponding to 1.3 μM) before (18 h) and during incubation. In previous studies performed in our lab, prochloraz was found to be the strongest inhibitor of in vitro ECOD activity of rat liver microsomes as compared to other azole fungicides (tebuconazole, epoxiconazole, propiconazole) and the cytochrome P450 inhibitor piperonyl butoxide (see Fig. S1 in Supporting Information). During incubation, 100 μL aliquots were removed from the incubation medium every 30 min and transferred directly on a transparent microwell plate (Greiner Bio-one flat bottom 96-well plate, VWR). The samples were stored at −20 °C until fluorescence measurement. 7-hydroxycoumarin formation (excitation: 380 nm, emission: 480 nm) was measured with a Multi-mode microplate reader (SpectraMax M5 Microplate Reader, Molecular Devices, U.S.) at room temperature (22–25 °C).

Preparation of microsomes

The procedure for microsomes preparation was based on four methods reported in literature (Baldwin and Leblanc 1994; Fisher et al. 2003; Kretschmann et al. 2011; Sturm and Hansen 1999) and optimized (for details see Section 3 in Supporting Information). In short, 5 days old *D. magna*, four instar *C. riparius* larvae or randomly selected adult *H. azteca* were sieved, washed with MilliQ water, snap frozen in liquid nitrogen and stored at −80 °C. In a single 1.5 mL vial, whole *D. magna* (40 organisms), whole *C. riparius* (5 organisms) larvae or whole *H. azteca* (40 organisms) were homogenized in ice cold homogenization buffer (Tot. vol. 1000 μL) containing phosphate buffer (0.13 M, pH 7.5), EDTA (1 mM), PMSF (1 mM), DTT (1 mM) and glycerol (10 % v/v). Homogenization was first performed with a tissue grinder (Econo Grind, Radnoti, U.S.) until the organisms were largely disrupted. After that, further homogenization was carried out with an ultrasonic stick (Digital Sonifier cell disruptor Model 450, Branson Ultrasonics, U.S.) on ice (3–5 pulses of 3, 10 s pause between pulses, 20 % amplitude). The homogenate was centrifuged (Optima TM MAX-XP Ultracentrifuge, Beckman Coulter, U.S.) at 10,000 g for 15 min at 4 °C; the supernatant was collected and further centrifuged at 100,000 g for 50 min at 4 °C. The resulting pellets containing the microsomes were re-suspended (mechanically using a micropipette and with the ultrasonic stick using 2–3 pulses of 3, 10 s pauses, 10 % amplitude) in 350 μL phosphate buffer (0.13 M, pH 7.5) and glycerol (10 % v/v) on ice. When more than 350 μL of microsomes were necessary, microsomes obtained from multiple vials were pooled into a single batch and stored at −80 °C.

Protein content

Protein content was determined according to Bradford (1976) by use of a Multi-mode microplate reader (SpectraMax M5 Microplate Reader, Molecular Devices, U.S.). The samples (5 μL) were incubated with 250 μL Bradford reagent at room temperature for 10–15 min. Absorbance was then measured at 595 nm and protein content quantified using a bovine serum albumin (BSA) standard dilution series (0–1.4 $mg_{protein}$ mL^{-1} BSA in phosphate buffer 0.13 M, pH 7.5 and glycerol 10 % v/v) as described in the Bradford Reagent—Sigma-Aldrich Technical bulletin (Sigma-Aldrich 2013).

In vitro cytochrome P450 activity assay

Based on an existing method (Bach and Snegaroff 1989) a procedure for the measurement of P450-dependent ECOD activity in vitro was developed and optimized (for details see Section 2 in Supporting Information). The assay consisted of re-suspended microsomes (>0.036 mg microsomal protein mL^{-1}, 50 μL) incubated with 150 μL of incubation medium containing phosphate buffer (0.13 M, pH 7.5), NADPH (0.0156 mM) and 7-ethoxycoumarin (0.6 mM) (Tot. vol. 200 μL). Blanks were made with all reagents except the substrate in order to account for

background fluorescence of NADPH (Aitio 1978) and with re-suspended microsomes (50 µL) in order to maintain the same density of the medium. Fluorescence increase due to 7-hydroxycoumarin formation (excitation: 380 nm, emission: 480 nm) was measured every 15 s for 30 min in a transparent microwell plate (Greiner Bio-one flat bottom 96-well plate, VWR) with a Multi-mode microplate reader (SpectraMax M5 Microplate Reader, Molecular Devices, U.S.) at 25 ± 1 °C for *D. magna* or *H. azteca* microsomes and at 30 ± 1 °C [as in Bach and Snegaroff (1989) and Fisher et al. (2003)] for *C. riparius* larvae microsomes.

In vitro P450 activity inhibition by endogenous compounds

In order to test whether cytochrome P450 responsible for ECOD activity was inhibited by endogenous compounds present in whole *D. magna*, *C. riparius* larvae or *H. azteca* and released at the first step of microsomes preparation (homogenization), ECOD activity of rat liver microsomes was measured after addition of invertebrate homogenates. Since this measure consists of two components: (a) the reduction of fluorescence due to a decrease in P450 activity and (b) the reduction of product fluorescence due to homogenate turbidity (scattering), the latter was measured in a separate experiment with constant product concentrations and increased macroinvertebrate homogenate concentration. Homogenates of the invertebrates were prepared in the same way as described under microsomes preparation, only tissue grinding and homogenization with the ultrasonic stick was performed. 50 µL of homogenate were added in different concentrations (0–0.24 mg protein mL^{-1}) to 50 µL rat liver microsomes (0.03 mg microsomal protein mL^{-1}). The mixture was then incubated with 150 µL incubation medium (as described under in vitro cytochrome P450 activity assay) (Tot. vol. 250 µL). Fluorescence (7-hydroxycoumarin; excitation: 380 nm, emission: 480 nm) was measured every 15 s for 30 min at 37 °C. In order to control for possible attenuation (scattering) of fluorescence intensity due to the turbid homogenates, the same dilution series of macroinvertebrate homogenate were mixed with the fluorescent product 7-hydroxycoumarin (230 mg mL^{-1} corresponding to 0.38 µM, 150 µL) and 50 µL rat liver microsomes (0.03 mg microsomal protein mL^{-1}) (Tot. vol. 200 µL). Fluorescence (7-hydroxycoumarin; excitation: 380 nm, emission: 480 nm) was measured every 15 s for 30 min at 37 °C.

Data treatment

The measured fluorescence at time zero of the samples in the in vivo experiments was always lower than the blanks (substrate dissolved in M7 medium without organisms). This was probably due to adsorption of the slightly fluorescent substrate molecule (Aitio 1978) to the organisms. To adjust that, the average fluorescence value at time point zero of the samples ($FLU_{sample,t=0}$) and of the blanks ($FLU_{blank,t=0}$) were subtracted to the fluorescence values over time of the samples and blank, respectively ($AdjustedFLU_{sample} = FLU_{sample} - FLU_{sample,t=0}$ and $AdjustedFLU_{blank} = FLU_{blank} - FLU_{blank,t=0}$). Subsequently, the adjusted values were corrected for changes in blank fluorescence over time by subtracting the average fluorescence value of the blanks ($CorrectedFLU_{sample} = AdjustedFLU_{sample}$– average $AdjustedFLU_{blank}$). The fluorescence measured in vitro over time was corrected by subtracting the average fluorescence measured in the blanks (microsomes + NADPH without substrate) over time ($CorrectedFLU_{sample} = FLU_{sample}$– $averageFLU_{blank}$). This was done to correct for background fluorescence due to NADPH (Aitio 1978). Cytochrome P450 activity (change in fluorescence per hour, flu h^{-1}) was determined as the slope of a linear regression fitted with the software Sigma Plot 12.5 in the linear range of fluorescent product formation (Fig. S4 in Supporting Information). Fluorescence was transformed into units of pmol product via calibration standards of 7-hydroxycoumarin in the range 0–140 pmol in MilliQ water for in vivo studies (Fig. S10 in Supporting Information) or 0–85 pmol in phosphate buffer 0.13 M for in vitro studies (Fig. S11 in Supporting Informatio). The rate of product formation, considered proportional to the P450 activity, was finally expressed relative to protein content (pmol h^{-1} mg microsomal $protein^{-1}$ mL) for the in vitro and per organism (pmol $organism^{-1}$ h^{-1}) for the in vivo assays.

Inhibition of ECOD activity of rat liver microsomes in presence of macroinvertebrate homogenate was expressed relative to ECOD activity of rat liver microsomes without addition of macroinvertebrate homogenate (100 % activity). Likewise, attenuation of fluorescence due to macroinvertebrate homogenate was compared to the fluorescence of 7-hydroxycoumarin without addition of macroinvertebrate homogenate (100 % fluorescence).

Statistic

Fluorescence measured over time in the samples and in the blanks were statistically compared with an analysis of covariance (ANCOVA) using the program R. Cytochrome P450 activities, defined as the slopes describing fluorescence over time of the samples were considered to be statistically significantly different from the blanks or from each other when $p < 0.01$.

Results and discussion

In vivo cytochrome P450 activities: substrate suitability

ECOD activity was measurable in vivo in *D. magna* (Fig. 1a) and *C. riparius* larvae (Fig. 1b) while no activity could be detected in *H. azteca* (Fig. 1c). ECOD activity in *D. magna* was 1.37 ± 0.08 pmol h^{-1} $organism^{-1}$ while for *C. riparius* larvae the activity was 2.2 ± 0.2 pmol h^{-1} $organism^{-1}$. On a dry weight base, ECOD activity in *D. magna* was 21 pmol h^{-1} mg_{dw}^{-1} (0.64 ± 0.01 mg dry weight, 10 organisms, $n = 3$) while for *C. riparius* larvae the activity was 3 pmol h^{-1} mg_{dw}^{-1} (2.3 ± 0.1 mg dry weight, 3 organisms, $n = 3$). These results showed that both *D. magna* and *C. riparius* larvae were able to convert in vivo the substrate 7-ethoxycoumarin to the fluorescent product 7-hydroxycoumarin. Cytochrome P450 isoforms with ECOD activity seem to be present in *D. magna* and *C. riparius* larvae, suggesting the suitability of the substrate 7-ethoxycoumarin for the measurement of cytochrome P450 activity in these species. The absence of detectable product formation for *H. azteca* might indicate that ECOD activity is too low to be detected and/or that 7-ethoxycoumarin may not be suitable for this particular species. Since the biomass of *H. azteca* (4.49 ± 0.44 mg dry weight, 10 organisms, $n = 3$) was seven-fold higher than the biomass of *D. magna* (0.64 ± 0.01 mg dry weight, 10 organisms, $n = 3$), it can be excluded that a too low biomass was the reason for undetected product formation in the case of *H. azteca*. It is generally known that cytochrome P450 activities found in crustaceans are low compared to that of other invertebrates (Livingstone 1998). This was partly confirmed by the P450 activities found in this study being one order of magnitude lower than the in vivo activities found for the freshwater snails *P. antipodarum* (23.18 ± 2.6 pmol h^{-1} org^{-1}, at 16 ± 0.5 °C) and *V. piscinalis* (21.06 ± 3.16 pmol h^{-1} org^{-1}, at 16 ± 0.5 °C) (Gagnaire et al. 2010). On the other hand, ECOD activity of *D. magna* found in the present study was an order of magnitude higher than the cytochrome P450 dependent ethoxyresorufin-O-deethylase (EROD) activity reported by David et al. (2012)(measured at 20 ± 1 °C). This agrees with the fact that the substrate 7-ethoxycoumarin is considered to be less specific toward human/crustacean CYP families as compared to 7-ethoxyresorufin thereby detecting a higher overall P450 activity (David et al. 2012; Waxman and Chang 2006). It is important to mention that besides cytochrome P450 catalyzed phase I metabolism 7-hydroxycoumarin formation measured in vivo is influenced by several other processes, like uptake, phase II metabolism and excretion (Gagnaire et al. 2010). Thus the different ECOD activities found among the species in in vivo studies may be affected by processes other than cytochrome P450 dependent oxygenation. Although a study has suggested that uptake rates may differ significantly between species (Rubach et al. 2010), it is generally believed that organic compounds are taken up passively (Livingstone 1998) and therefore different uptake rates may not be the main reason for the different ECOD activities found among the species in the present study. On the other hand, phase II metabolism could have an effect on detectable ECOD activities due to further biotransformation of 7-ethoxycoumarin to non-fluorescent compounds (Jones et al. 2010). Moreover, different excretion rates between species may also affect detectable ECOD activities (Hawker and Connell 1986).

It is generally accepted that a major detoxification pathway for pyrethroid insecticides is oxidation by

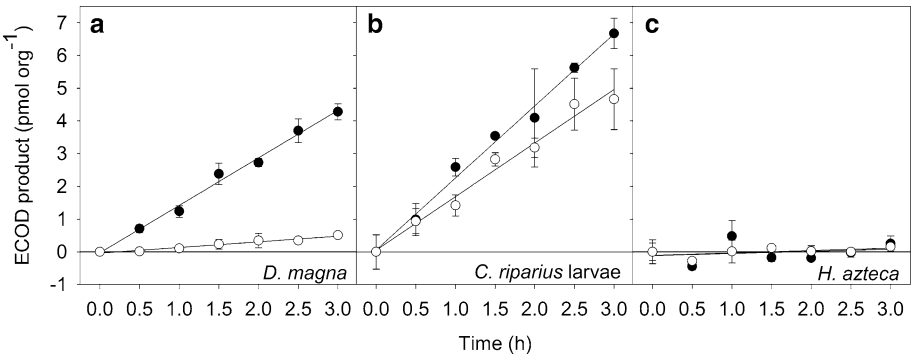

Fig. 1 In vivo 7-hydroxycoumarin formation of *D. magna* (**a**), *C. riparius* larvae (**b**) and *H. azteca* (**c**) as a function of time. 7-hydroxycoumarin formation by the organisms without and with simultaneous exposure to prochloraz (500 µg L^{-1}) is indicated with black and white circles, respectively. Data are mean ± S.E. ($n = 3$ biological replicates). Slopes of linear regression lines (pmol h^{-1} $organism^{-1}$ ± S.E.) for *D. magna*: 1.45 ± 0.08 $R^2 = 0.94$ (*black circles*), 0.17 ± 0.03 $R^2 = 0.55$ (*white circles*); *C. riparius* larvae: 2.2 ± 0.2 $R^2 = 0.84$ (*black circles*), 1.6 ± 0.2 $R^2 = 0.80$ (*white circles*); *H. azteca*: 0.0 ± 0.1 $R^2 = 0.02$ (*black circles*), 0.07 ± 0.06 $R^2 = 0.06$ (*white circles*). Background fluorescence (pmol h^{-1} org^{-1}) was equal to 0.00 ± 0.03, 0.0 ± 0.2 and 0.00 ± 0.03 for a, b and c, respectively (not shown)

cytochrome P450 monooxygenase (Anand et al. 2006; Godin et al. 2007; Komagata et al. 2010; Nillos et al. 2010; Stevenson et al. 2011; Yang et al. 2006; Zhang and Scott 1996). *D. magna* typically have LC_{50} values towards these pesticides in the range of μg L^{-1} [bifenthrin $LC_{50,48h}$: 0.32 μg L^{-1} (Mokry and Hoagland 1990); permethrin $LC_{50,48h}$: 1.25 μg L^{-1} (Mokry and Hoagland 1990)] while the sensitivity of *H. azteca* are two to three orders of magnitude higher showing LC_{50} values in the range of ng L^{-1} [cyfluthrin $LC_{50,96h}$: 1.3–4.8 ng L^{-1} (Weston et al. 2013), $LC_{50,10d}$: 2.9 ng L^{-1} (Brander et al. 2009), bifenthrin $LC_{50,96h}$: 6.5–8.3 ng L^{-1} (Weston et al. 2013), permethrin: $LC_{50,10d}$: 48.9 ng L^{-1} (Brander et al. 2009)]. If the organism's ability to detoxify pesticides is assumed to be a major determinant of an organisms sensitivity, the lack of cytochrome P450 (ECOD) activity in *H. azteca* found in our study may explain the high sensitivity towards pyrethroids of *H. azteca* compared to *D. magna*.

Inhibition of in vivo ECOD activity by the P450 inhibitor prochloraz

The effect of prochloraz exposure (500 μg L^{-1}, 21 h total) on the metabolism of 7-ethoxycoumarin differed among species. ECOD activity of *D. magna* was reduced by 90 % by the addition of prochloraz (from 1.37 ± 0.08 unexposed to 0.13 ± 0.04 pmol h^{-1} org^{-1} for exposed organisms, ANCOVA $p = 0.05$) (Fig. 1a) while ECOD activity in *C. riparius* was only slightly affected (not statistically different from unexposed, ANCOVA $p = 0.09$) (Fig. 1b). These large differences in the inhibition potential of prochloraz between species might be due to several factors. Cytochromes P450 of *C. riparius* may be capable of detoxifying prochloraz more effectively than cytochrome P450 present in *D. magna*. Thus, the concentration of prochloraz used in the present study may have been too low to significantly inhibit ECOD activity of *C. riparius*. Moreover, *C. riparius* may be able to relatively quickly *de novo* synthesize large amounts of P450 during pre-exposure and measurements. Both explanations could be likely for a sediment-dwelling organism such as *C. riparius* which is expected to be exposed to reduced toxicants present in the sediment at a larger degree than a water swimming organisms such as *D. magna*. On the other hand, even though both species presented ECOD activities, the P450 isoenzymes involved in the dealkylation of 7-ethoxycoumarin in *D. magna* and *C. riparius* might be diverse. The inhibition potential of pharmaceutical azole fungicides toward P450 is known to be different for different isoenzymes (Venkatakrishnan et al. 2000) thus different cytochromes P450 with different affinities for prochloraz may be present in *D. magna* as compared to *C. riparius*.

In vitro cytochrome P450 activities

ECOD activity was detectable in vitro in *C. riparius* larvae (Fig. 2b) but not in *D. magna* (Fig. 2a) and *H. azteca* (Fig. 2c). The optimization of the existing methods (see Section. 2 and 3 in Supporting Information) resulted in a very sensitive method for the measurement of in vitro activities of *C. riparius* larvae microsomes, with the lowest measurable activity equal to 2 pmol h^{-1} (ANCOVA, $p < 0.01$) originated from a concentration of microsomes as low as 0.036 mg microsomal protein mL^{-1}. Negative activities found for *D. magna*, *H. azteca* and *C. riparius* larvae (for microsomes <0.036 mg microsomal protein mL^{-1}) may be attributed to a decrease of NADPH and/or substrate background fluorescence over time (Aitio 1978). A change in pH may also be responsible for changes in fluorescence of the product 7-hydroxycoumarin. However, this is most probably not an issue in the present work since the system was buffered at pH 7.5.

In our study, *C. riparius* larvae activities ranged from approx. 500 to 1000 pmol h^{-1} mg microsomal $protein^{-1}$ (at 30 °C). The calculated activities are relative to all the fluorescence values higher than the lowest detectable activity of the method (for details see Section 2 in Supporting Information) but with microsomes concentrations no higher than 0.05 mg microsomal protein mL^{-1} (for details see Section 5 in Supporting Information). These values are one to two orders of magnitude higher than previously reported in vitro ECOD activities in *C. riparius* larvae (30–60 pmol h^{-1} mg microsomal $protein^{-1}$, at 25 °C) (Sturm and Hansen 1999). These differences may be attributed to differences between the two methods applied. In the investigation of Sturm and Hansen (1999) ECOD activity was assessed in organism homogenate at 25 °C, while our measurements were carried out at 30 °C on microsomes, where higher activities are expected. Furthermore, the optimization of the procedure in the present study might have yielded higher activities.

Different ECOD activity could be observed between different batches of *C. riparius* larvae microsomes (Fig. 2b, black and white circles) (217 and 108 pmol h^{-1} at 0.038 mg microsomal protein mL^{-1}). Moreover, thawing twice a single batch of *C. riparius* larvae microsomes was also found to alter its activity (Fig. 2b, white circles and white triangles) (108 pmol h^{-1} and 62 pmol h^{-1} at 0.038 and 0.036 mg microsomal protein mL^{-1}, respectively). It can be excluded that the lack of activities in *D. magna* and *H. azteca* as compared to *C. riparius* larvae was due to high temperature or mechanical stress during homogenization procedures, since the three different species were processed with the same optimized techniques (for details see Section 3 in Supporting Information). A

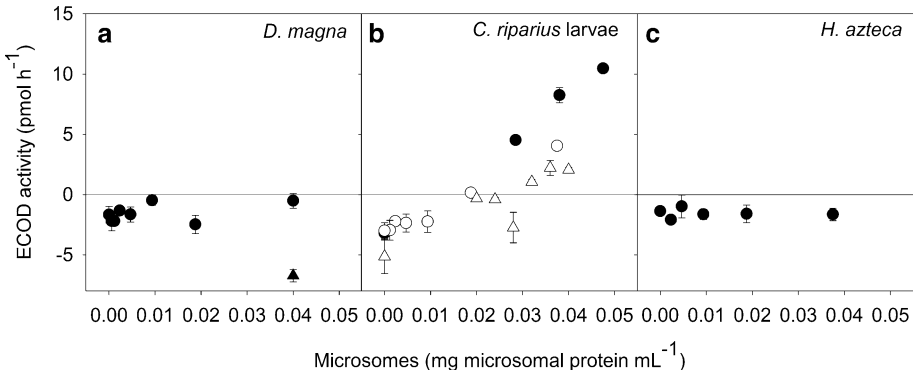

Fig. 2 In vitro ECOD activities of *D. magna* (**a**), *C. riparius* larvae (**b**) and *H. azteca* (**c**) relative to microsomes concentration. For *D. magna* two separate experiments were conducted with two different microsomes batches (*black circles* and *black triangles*). For *C. riparius* larvae three separate experiments were conducted with two different microsomes batches (*black and white circles*). The third experiment (*white triangles*) tested the effect of two thawing-cycles of the second microsomes batch. For *H. azteca* a single experiment was conducted with geometrical dilutions of a single microsomes batch (*black circles*). Data are mean ± S.E. (3 analytical replicates)

possible explanation for detectable activity of *D. magna* in vivo but not in vitro is discussed in the next section.

In vitro ECOD activity inhibition by endogenous compounds

As mentioned in the introduction, the absence of measurable cytochrome P450 activities in vitro in *D. magna* could be due to endogenous substances inhibiting cytochrome P450 activity released during the processing of the organisms and preparation of microsomes (Baldwin and Leblanc 1994; James and Boyle 1998). In order to investigate whether this was the case in our study, the inhibition of ECOD activity in rat liver microsomes by compounds present in *D. magna*, *C. riparius* and *H. azteca* homogenates was investigated. The homogenates indeed caused a decrease in ECOD activity present in rat liver microsomes, but to different degrees. The highest concentration of *D. magna* homogenate (0.24 mg protein mL^{-1}) (Fig. 3a) decreased rat liver microsomes activity to 45 ± 8 % while having no effect on detectable fluorescence (102 ± 2 %). In a similar manner, *C. riparius* homogenate (0.24 mg protein mL^{-1}) (Fig. 3b) decreased activity to 66 ± 4 % and detectable fluorescence to 85 ± 9 %. *H. azteca* homogenate (0.24 mg protein mL^{-1}) decreased activity to only 97 ± 3 % but surprisingly increased detectable fluorescence to 126 ± 10 % (Fig. 3c). The inhibition of cytochrome P450 activity due to endogenous substances has been shown for several insects (Brattsten and Wilkinson 1973; Gilbert and Wilkinson 1975; Orrenius et al. 1971; Valles and Yu 1996) and was hypothesized for *D. magna* (Baldwin and Leblanc 1994). The results of the present study confirmed the hypothesis that substances that inhibit cytochrome P450 activity may be released during homogenization. This was particularly evident for *D. magna*, which may explain the general lack of reported in vitro activities in the literature (Sturm and Hansen 1999). Inhibition by endogenous molecules was also present in *C. riparius* larvae although the majority of decreased fluorescence intensity could be attributed to fluorescence attenuation by the dark colour of the homogenate. The substances in the homogenate were responsible for approximately 20 % of the decrease in activity. On the other hand, *H. azteca* homogenate did not affect ECOD activity. In vitro measurement of microsomal activity in *H. azteca* might therefore be possible by further method optimization, e.g., testing different substrates. Although there is no obvious explanation for the increase in product fluorescence after addition of *H. azteca* homogenate, it can be hypothesized that fluorescent substances may be present in organisms and released during processing.

Strengths and limitations of the in vivo versus in vitro assays

In the present study, measurements of cytochrome P450 ECOD activities were carried out in the crustaceans *D. magna* and *H. azteca* and in the insect larvae *C. riparius*. The in vivo assay provided a tool to test the suitability of the substrate of choice, in vivo measurements of activity, unlike measurements carried out in vitro, are not subject to activity inhibition due endogenous compounds. The in vivo method was relatively easy to perform (fewer instruments needed) and was characterized by a low chemical consumption, fast fluorescence reading (endpoint instead of kinetic reading) and it did not require time-demanding preparation of microsomes. On the other hand, incubation times were relatively long (3 h) which meant a further stress for the organisms. This is particularly a problem when the assessment of activity needs to be carried out

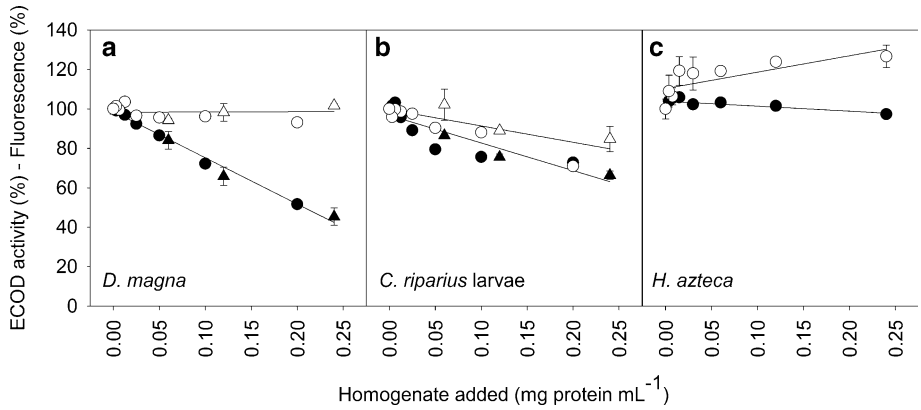

Fig. 3 The effect of *D. magna* (**a**), *C. riparius* larvae (**b**) and *H. azteca* (**c**) homogenates on rat liver microsomes ECOD activity (*black symbols*) and on the fluorescence of 7-hydroxycoumarin (*white symbols*). Experiments were conducted with geometrical dilutions of two homogenate batches (*circles and triangles*). Data are mean ± S.E. (3 analytical replicates) (When *bars* are not depicted, 1 analytical replicate)

after exposure to other chemicals or stresses. Moreover, as was previously mentioned, the activity measured in vivo is the result of several toxicokinetic processes (uptake, biotransformation, excretion) therefore the differences in activity seen among species might not only be due to differences in P450 activity.

The in vitro assay presents both advantages and disadvantages as compared to the in vivo assay. In general, since the assay is carried out on the microsomal fraction, which is derived from the endoplasmic reticulum, the resulting activities can be assumed to be representative of Phase I metabolism, in which cytochrome P450 plays the major role. Thus, the in vitro assay is better suited to analyse biochemical questions related to direct interactions with these enzymes. Although optimization of the in vitro assay was both time and resource consuming (see Sections 2 and 3 in Supporting Information), the final method allowed a faster assessment of activity (30 min incubation as compared to 3 h in vivo). The choice of kinetic instead of end point measurements provides a larger number of fluorescence measurements over time resulting in more robust slope (activity) determination. A higher measurable activity in vitro (1000 pmol h^{-1} mg microsomal $protein^{-1}$ on average converted to 38 pmol h^{-1} org^{-1} by using a conversion factor of 0.038 mg microsomal protein org^{-1} in microsomes) was found compared to the rate obtained in vivo (2.2 pmol h^{-1} org^{-1}) which suggests higher sensitivity of the in vitro assay as compared to in vivo. This is in accordance with the fact that microsomes generally contain a concentrated amount of readily accessible cytochrome P450, in contrast to in vivo where all P450 enzymes might not all be accessible to the substrate. In addition, the difference might also be attributed to the difference in incubation temperature (5 °C higher for in vitro) and the optimized amounts of substrate and cofactors for the in vitro assay as compared to the in vivo test. Differently from the in vivo setup, the in vitro method was characterized by snap-freezing and storage of the organisms at −80 °C. Snap-freezing permits to quickly sample the organisms and to better preserve the enzymatic activity at the time of sampling; in addition, it allows measurements of activity in organisms that are collected in short intervals (minutes) after a potential treatments proposed to affect P450 activity (for example exposure to another chemical). Hence, the rate of induction or inhibition of P450 activity in living organisms caused by other stressors can be more easily measured over time using the in vitro assay. Moreover, the in vitro assay can be simultaneously carried out on a large number of samples under similar conditions.

Conclusion

Taken together, the evaluated and optimized in vivo and in vitro ECOD assays could provide a strong and versatile tool for the measurement of cytochrome P450 activity in different aquatic invertebrates. In vitro and in vivo assays may be used as complementary methods, the choice of method being determined by the specific research question. The in vivo assay could be used to investigate cytochrome P450 activity at the organism level, although the measured activities might also be affected by processes other than phase I metabolism. A significant inhibition of in vivo ECOD activity of *D. magna* by the fungicide prochloraz was measured, suggesting the applicability of the in vivo assay to assess the effect of pesticides interacting with cytochrome P450 in *D. magna*. The method could be applied to a variety of research questions dealing with contaminant interactions and the physiological

mechanisms behind combined stressor effects. The in vitro assay was characterized by high sensitivity and it allowed investigating cytochrome P450 activity at the molecular level. However, it required a long optimization due to the high number of parameters and processes involved and the interference from endogenous substances released during microsomes preparation did not permit its application to all species. Thus, we suggest that inhibition due to endogenous compounds is a very important preliminary test to carry out prior to any investigation of in vitro activity in aquatic macroinvertebrates. Finally, since the present study compared in vitro and in vivo assay designed to measure ECOD activity, depending on the organisms or the different cytochrome P450 investigated, other substrates should be tested.

Acknowledgments The authors thank laboratory technician Anja Weibell for taking good care of the organisms' cultures and Post Doc Tomas Laursen for his suggestions on the cytochrome P450 in vitro assay as well as for solving practical issues.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Aitio A (1978) A simple and sensitive assay of 7-ethoxycoumarin in deethylation. In: Leonard BJ (ed) Toxicological aspects of food safety, vol 1., Archives of toxicologySpringer, Berlin, p 275. doi:10.1007/978-3-642-66896-8_53

Anand SS, Bruckner JV, Haines WT, Muralidhara S, Fisher JW, Padilla S (2006) Characterization of deltamethrin metabolism by rat plasma and liver microsomes. Toxicol Appl Pharmacol 212:156–166. doi:10.1016/j.taap.2005.07.021

Anderson TD, Zhu KY (2004) Synergistic and antagonistic effects of atrazine on the toxicity of organophosphorodithioate and organophosphorothioate insecticides to *Chironomus tentans* (Diptera : Chironomidae). Pestic Biochem Physiol 80:54–64. doi:10.1016/j.pestbp.2004.06.003

Bach J, Snegaroff J (1989) Effects of the fungicide prochloraz on xenobiotic metabolism in Rainbow trout: in vivo induction. Xenobiotica 19:1–9

Baldwin WS, Leblanc GA (1994) Identification of multiple steroid hydroxylases in *Daphnia magna* and their modulation by xenobiotics. Environ Toxicol Chem 13:1013–1021. doi:10.1897/1552-8618(1994)13[1013:iomshi]2.0.co;2

Belden JB, Lydy MJ (2000) Impact of atrazine on organophosphate insecticide toxicity. Environ Toxicol Chem 19:2266–2274. doi:10.1897/1551-5028(2000)019<2266:ioaooi>2.3.co;2

Binelli A, Ricciardi F, Riva C, Provini A (2006) New evidences for old biomarkers: effects of several xenoblotics on EROD and AChE activities in Zebra mussel (*Dreissena polymorpha*). Chemosphere 62:510–519. doi:10.1016/j.chemosphere.2005.06.033

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Brander SM, Werner I, White JW, Deanovic LA (2009) Toxicity of a dissolved pyrethroid mixture to *Hyalella azteca* at environmentally relevant concentrations. Environ Toxicol Chem 28:1493–1499

Brattsten LB, Wilkinson CF (1973) A microsomal enzyme inhibitor in the gut contents of the house cricket (*Acheta domesticus*). Comp Biochem Phys Part B Comp Biochem 45:59–70. doi:10.1016/0305-0491(73)90284-8

Cedergreen N (2014) Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. PLoS One 9:e96580. doi:10.1371/journal.pone.0096580

Cedergreen N, Kamper A, Streibig JC (2006) Is prochloraz a potent synergist across aquatic species? A study on bacteria, daphnia, algae and higher plants. Aquat Toxicol 78:243–252. doi:10.1016/j.aquatox.2006.03.007

Chalvet-Monfray K, Belzunces LP, Colin ME, Fleche C, Sabatier P (1996) Synergy between deltamethrin and prochloraz in bees: modeling approach. Environ Toxicol Chem 15:525–534. doi:10.1897/1551-5028(1996)015<0525:sbdapi>2.3.co;2

Daborn P, Boundy S, Yen J, Pittendrigh B, Ffrench-Constant R (2001) DDT resistance in drosophila correlates with Cyp6g1 over-expression and confers cross-resistance to the neonicotinoid imidacloprid. Mol Genet Genomics 266:556–563. doi:10.1007/s004380100531

David RM, Jones HS, Panter GH, Winter MJ, Hutchinson TH, Chipman JK (2012) Interference with xenobiotic metabolic activity by the commonly used vehicle solvents dimethylsulfoxide and methanol in zebrafish (*Danio rerio*) larvae but not *Daphnia magna*. Chemosphere 88:912–917. doi:10.1016/j.chemosphere.2012.03.018

EPA (2000) Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates, 2nd edn. United States Environmental Protection Agency, Washington, DC

Feyereisen R (1999) Insect P450 enzymes. Annu Rev Entomol 44:507–533. doi:10.1146/annurev.ento.44.1.507

Fisher T, Crane M, Callaghan A (2003) Induction of cytochrome P-450 activity in individual *Chironomus riparius* meigen larvae exposed to xenobiotics. Ecotoxicol Environ Saf 54:1–6. doi:10.1016/s0147-6513(02)00031-3

Gagnaire B, Geffard O, Noury P, Garric J (2010) In vivo indirect measurement of cytochrome P450-associated activities in freshwater gastropod molluscs. Environ Toxicol 25:545–553. doi:10.1002/tox.20515

Gilbert MD, Wilkinson CF (1975) An inhibitor of microsomal oxidation from gut tissues of the honey bee (*Apis mellifera*). Comp Biochem Phys Part B Comp Biochem 50:613–619. doi:10.1016/0305-0491(75)90099-1

Glockner R, Muller D (1995) Ethoxycoumarin O-deethylation (ECOD) activity in rat-liver slices exposed to beta-naphthoflavone (BNF) in vitro. Exp Toxicol Pathol 47:319–324

Godin SJ, Crow JA, Scollon EJ, Hughes MF, DeVito MJ, Ross MK (2007) Identification of rat and human cytochrome P450 isoforms and a rat serum esterase that metabolize the pyrethroid insecticides deltamethrin and esfenvalerate. Drug Metab Dispos Biol Fate Chem 35:1664–1671

Gonzalez-Mendoza D (2007) Enzymatic complex cytochrome P450 in plants. Rev Int Contam Ambient 23:177–183

Guengerich FP (2005) Human cytochrome P450 enzymes. In: de Ortiz Montellano P (ed) Cytochrome P450. Springer, New York, pp 377–530. doi:10.1007/0-387-27447-2_10

Hawker DW, Connell DW (1986) Bioconcentration of lipophilic compounds by some aquatic organisms. Ecotoxicol Environ Saf 11:184–197. doi:10.1016/0147-6513(86)90063-1

James MO (1984) Catalytic properties of cytochrome P-450 in hepatopancreas of the Spiny lobster, *Panulirus argus*. Marine Environ Res 14:1–11. doi:10.1016/0141-1136(84)90066-7

James MO, Boyle SM (1998) Cytochromes P450 in crustacea. Comp Biochem Phys C-Pharmacol Toxicol Endocrinol. 121:157–172. doi:10.1016/s0742-8413(98)10036-1

Johnson RM, Dahlgren L, Siegfried BD, Ellis MD (2013) Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*). PLoS One 8:e54092. doi:10.1371/journal.pone.0054092

Jones HS, Panter GH, Hutchinson TH, Chipman JK (2010) Oxidative and conjugative xenobiotic metabolism in zebrafish larvae *in vivo*. Zebrafish 7:23–30. doi:10.1089/zeb.2009.0630

Kelly SL, Kelly DE (2013) Microbial cytochromes P450: biodiversity and biotechnology Where do cytochromes P450 come from, what do they do and what can they do for us? Philosophical Trans R Soc B-Biol Sci 368:17. doi:10.1098/rstb.2012.0476

Koenig S, Fernandez P, Sole M (2012) Differences in cytochrome P450 enzyme activities between fish and crustacea: relationship with the bioaccumulation patterns of polychlorobiphenyls (PCBs). Aquat Toxicol 108:11–17. doi:10.1016/j.aquatox.2011.10.016

Komagata O, Kasai S, Tomita T (2010) Overexpression of cytochrome P450 genes in pyrethroid-resistant *Culex quinquefasciatus*. Insect Biochem Mol Biol 40:146–152. doi:10.1016/j.ibmb.2010.01.006

Kotze AC (2000) Oxidase activities in macrocyclic-resistant and -susceptible *Haemonchus contortus*. J Parasitol 86:873–876. doi:10.1645/0022-3395(2000)086[0873:oaimra]2.0.co;2

Kretschmann A, Ashauer R, Hitzfeld K, Spaak P, Hollender J, Escher BI (2011) Mechanistic toxicodynamic model for receptor-mediated toxicity of diazoxon, the active metabolite of diazinon, in *Daphnia magna*. Environ Sci Technol 45:4980–4987. doi:10.1021/es1042386

Livingstone DR (1998) The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comp Biochem Phys a-Mol Integr Phys 120:43–49. doi:10.1016/s1095-6433(98)10008-9

Mayer RT, Jermyn JW, Burke MD, Prough RA (1977) Methoxyresorufin as a substrate for fluorometric assay of insect microsomal O-dealkylases. Pestic Biochem Physiol 7:349–354. doi:10.1016/0048-3575(77)90038-4

Mokry LE, Hoagland KD (1990) Acute toxicities of five synthetic pyrethroid insecticides to *Daphnia magna* and *Ceriodaphnia dubia*. Environ Toxicol Chem 9:1045–1051. doi:10.1002/etc.5620090811

Moon JY, Lee DW, Park KH (1998) Inhibition of 7-ethoxycoumarin O-deethylase activity in rat liver microsomes by naturally occurring flavonoids: structure-activity relationships. Xenobiotica 28:117–126. doi:10.1080/004982598239623

Nillos MG, Chajkowski S, Rimoldi JM, Gan J, Lavado R, Schlenk D (2010) Stereoselective biotransformation of permethrin to estrogenic metabolites in fish. Chem Res Toxicol 23:1568–1575. doi:10.1021/tx100167x

Noury P, Geffard O, Tutundjian R, Garric J (2006) Non destructive in vivo measurement of ethoxyresorufin biotransformation by zebrafish prolarva: development and application. Environ Toxicol 21:324–331. doi:10.1002/tox.20184

OECD (2004) Test no. 202: *Daphnia* sp. Acute immobilisation test. In: OECD guidelines for the testing of chemicals, section 2: effects on biotic systems. Organisation for Economic Co-operation and Development

OECD (2010) Test no. 233: sediment-water chironomid life-cycle toxicity test using spiked water or spiked sediment. In: OECD guidelines for the testing of chemicals, section 2: effects on biotic systems. Organisation for Economic Co-operation and Development

OECD (2011) Test no. 235: *Chironomus* sp., acute immobilisation test. In: OECD guidelines for the testing of chemicals, section 2: effects on biotic systems. Organisation for Economic Co-operation and Development

OECD (2012) Test no. 211: *Daphnia magna* reproduction test. In: OECD guidelines for the testing of chemicals, section 2: effects on biotic systems. Organisation for Economic Co-operation and Development

Ong CE, Pan Y, Mak JW, Ismail R (2013) In vitro approaches to investigate cytochrome P450 activities: update on current status and their applicability. Expert Opin Drug Metab Toxicol 9:1097–1113. doi:10.1517/17425255.2013.800482

Orrenius S, Berggren M, Moldeus P, Krieger RI (1971) Mechanism of inhibition of microsomal mixed-function oxidation by gut-contents inhibitor of southern armyworm (*Prodenia-eridania*). Biochem J 124:427–430

Pilling ED, Bromleychallenor KAC, Walker CH, Jepson PC (1995) Mechanism of synergism between the pyrethroid insecticide lambda-cyhalothrin and the imidazole fungicide prochloraz, in the honeybee (*Apis-mellifera* L.). Pestic Biochem Physiol 51:1–11. doi:10.1006/pest.1995.1001

Rewitz KF, Styrishave B, Lobner-Olesen A, Andersen O (2006) Marine invertebrate cytochrome P450: emerging insights from vertebrate and insect analogies. Comp Biochem Phys C-Toxicol Pharmacol 143:363–381. doi:10.1016/j.cbpc.2006.04.001

Routti H, Letcher RJ, Arukwe A, van Bavel B, Yoccoz NG, Chu SG, Gabrielsen GW (2008) Biotransformation of PCBs in relation to phase I and II xenobiotic-metabolizing enzyme activities in ringed seals (*Phoca hispida*) from Svalbard and the Baltic sea. Environ Sci Technol 42:8952–8958. doi:10.1021/es801682f

Rubach MN, Ashauer R, Maund SJ, Baird DJ, Van den Brink PJ (2010) Toxicokinetic variation in 15 freshwater arthropod species exposed to the insecticide chlorpyrifos. Environ Toxicol Chem 29:2225–2234. doi:10.1002/etc.273

Sigma-Aldrich (2013) Bradford Reagent : Technical bulletin. http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/b6916bul.pdf

Snyder MJ (2000) Cytochrome P450 enzymes in aquatic invertebrates: recent advances and future directions. Aquat Toxicol 48:529–547. doi:10.1016/s0166-445x(00)00085-0

Stevenson BJ et al (2011) Cytochrome P450 6M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: sequential metabolism of deltamethrin revealed. Insect Biochem Mol Biol 41:492–502. doi:10.1016/j.ibmb.2011.02.003

Sturm A, Hansen PD (1999) Altered cholinesterase and monooxygenase levels in *Daphnia magna* and *Chironomus riparius* exposed to environmental pollutants. Ecotoxicol Environ Saf 42:9–15. doi:10.1006/eesa.1998.1721

Tian SM, Pan LQ, Zhang H (2014) Identification of a CYP3A-like gene and CYPs mRNA expression modulation following exposure to benzo a pyrene in the bivalve mollusk *Chlamys farreri*. Marine Environ Res 94:7–15. doi:10.1016/j.marenvres.2013.11.001

Timbrell JA (2008) Factors affecting toxic responses: metabolism. In: Principles of biochemical toxicology, 4th edn. CRC Press, pp 75–127. doi:10.3109/9781420007084-5

Uno T, Ishizuka M, Itakura T (2012) Cytochrome P450 (CYP) in fish. Environ Toxicol Pharmacol 34:1–13. doi:10.1016/j.etap.2012.02.004

Valles SM, Yu SJ (1996) German cockroach (Dictyoptera: Blattellidae) gut contents inhibit cytochrome P450 monooxygenases. J Econ Entomol 89:1508–1512

Vanderweiden MEJ, Tibosch HJH, Bleumink R, Sinnige TL, Vandeguchte C, Seinen W, Vandenberg M (1993) Cytochrome P450 1a induction in the Common carp (*Cyprinus carpio*) following exposure to contaminated sediments with halogenated polyaromatics. Chemosphere 27:1297–1309. doi:10.1016/0045-6535(93)90177-7

Venkatakrishnan K, von Moltke LL, Greenblatt DJ (2000) Effects of the antifungal agents on oxidative drug metabolism: clinical

relevance. Clin Pharmacokinet 38:111–180. doi:10.2165/00003088-200038020-00002

Waxman DJ, Chang TK (2006) Use of 7-ethoxycoumarin to monitor multiple enzymes in the human CYP1, CYP2, and CYP3 families. Methods Mol Biol 320:153–156. doi:10.1385/1-59259-998-2:153

Weston DP, Poynton HC, Wellborn GA, Lydy MJ, Blalock BJ, Sepulveda MS, Colbourne JK (2013) Multiple origins of pyrethroid insecticide resistance across the species complex of a nontarget aquatic crustacean *Hyalella azteca*. Proc Natl Acad Sci 110:16532–16537. doi:10.1073/pnas.1302023110

Yang Y, Chen S, Wu S, Yue L, Wu Y (2006) Constitutive overexpression of multiple cytochrome P450 genes associated with pyrethroid resistance in *Helicoverpa armigera*. J Econ Entomol 99:1784–1789

Zelnickova L et al (2013) Biochemical markers for the assessment of pollution of selected small streams in the Czech Republic. Neuroendocrinol Lett 34:109–115

Zhang M, Scott JG (1996) Cytochrome b5 is essential for cytochrome P450 6D1-mediated cypermethrin resistance in LPR House flies. Pestic Biochem Physiol 55:150–156