Author(s): Vehniäinen, Eeva-Riikka; Schultz, Eija; Lehtivuori, Heli; Ihalainen, Janne; Oikari, Aimo

Title: More accuracy to the EROD measurements-The resorufin fluorescence differs between species and individuals

More accuracy to the EROD measurements – the resorufin fluorescence differs between species and individuals

Eeva-Riikka Vehniäinen[1*], Eija Schultz[2], Heli Lehtivuori[1, 3], Janne A. Ihalainen[1, 3] and Aimo O. J. Oikari[1]

[1]University of Jyväskylä, Department of Biological and Environmental Sciences, P.O.Box 35, FI-40014 University of Jyväskylä, Finland
[2]Finnish Environment Institute SYKE, Laboratory, Hakuninmaantie 6, FI-00430 Helsinki, Finland
[3]Nanoscience Center, Department of Biological and Enviromental Sciences, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

*corresponding author e-mail: eeva-riikka.vehniainen@jyu.fi
phone: +358-400-73 5959
fax: +358-14-260 2321

Abstract
Ethoxyresorufin-O-deethylase (EROD) activity is a biomarker of exposure to planar aromatic hydrocarbons, and it is often measured from the S9 fraction. The effect of the liver S9 fraction of seven boreal freshwater fish species on the fluorescence of resorufin was studied. The S9 fractions diminished resorufin fluorescence by 40 – 80 %, and there were large differences between species. Thus, using a resorufin standard curve without the S9 fraction leads to a large underestimation of the EROD activity. Therefore a microwell plate EROD method was developed that takes into account the effect of each sample on resorufin fluorescence. At least two mechanisms were involved in the decrease of the fluorescence: opaqueness of the sample, and enzymes (DT-diaphorase and plausibly NADPH - CYP450 oxidoreductase) that reduce resorufin to a non-fluorescent form.

Keywords

EROD activity, resorufin, fluorescence, ethoxyresorufin-O-deethylase, S9 fraction

1 Introduction

The cytochrome P450 (CYP) was first discovered as a CO binding pigment in microsomes of pig and rat liver (Garfinkel, 1958; Klingenberg, 1958) and was later identified as a b-type CYP (Omura & Sato, 1964). The CYP enzymes metabolize a wide variety of endogenous and exogenous substances. Most of these enzymes are monooxygenases that make their substrates more polar via oxidation (Zeldin & Seubert, 2008; Omura, 2010). The most thoroughly studied CYP system is CYP1A1, which is induced by its substrates, e.g. planar aromatic hydrocarbons, such as chlorinated dioxins and furans, planar PCBs, and certain polynuclear aromatic hydrocarbons (PAHs). CYP1A1 induction is widely considered as a biomarker of exposure to these substances.

One of the ways to study CYP1A1 induction is to measure its enzymatic activity. This is often done either fluorometrically or spectroscopically by measuring the 7-ethoxyresorufin O-deethylation (EROD) activity of the sample (Burke & Mayer, 1974; Klotz *et al.*, 1985). CYP1A1 catalyzes the formation of resorufin from ethoxyresorufin. The fluorescence or absorbance of the produced resorufin is measured, and the amount of resorufin is calculated using a standard curve. The EROD activity is reported as pmol resorufin formed/min/mg protein.

CYP1A1 is located in the endoplasmic reticulum, and its induction by exogenous inducers is greatest in the liver, the kidney, and the gills. For EROD measurements, the organ or tissue is homogenized softly, and the homogenate is partly purified by centrifugation. First the homogenate is centrifuged at 10 000 x g for at least 10 min to get a post-mitochondrial fraction, the supernatant, also called the S9 fraction. This fraction can either be used for the EROD analysis, or purified more by ultracentrifugation (e.g. 100 000 x g, 60 min). The ultracentrifugation yields a pellet of microsomes that are collected for the analysis. The preparation of the S9 fraction is much easier, faster, and cheaper than that of microsomes, and therefore it is often used for EROD analysis especially if there are a lot of samples to analyze and if the analysis is done in the field.

Microwell plate methods have been developed to allow for the analysis of many samples at once (e.g. Grzebyk & Galgani, 1991).

We had noticed that the S9 fraction of fish livers greatly diminishes resorufin fluorescence, and that species (and to a smaller extent also individuals) differ in the magnitude of the decrease of the fluorescence. Using a resorufin standard curve without the S9 fraction thus leads to a large underestimation of the EROD activity, and even using a standard curve made with a randomly picked S9 fraction at the same concentration as in the reaction may not give accurate results, especially if the EROD activities are small. Therefore, a microwell plate EROD method was developed that takes into account the effect of each sample on resorufin fluorescence. This study shows the effect of the S9 fraction of seven boreal freshwater fish species on the fluorescence of resorufin, and shows that at least two different mechanisms are involved in the decrease of the fluorescence.

2 Materials and methods

2.1 Chemicals

All chemicals were purchased from Sigma, except where indicated otherwise.

2.2 Animals and sampling

Juvenile (0+) Atlantic salmon (*Salmo salar* (Linnaeus, 1758)), landlocked salmon (*Salmo salar* m. *sebago*), brown trout (*Salmo trutta* m. *trutta* and *Salmo trutta* m. *lacustris*), and whitefish (*Coregonus lavaretus*) were obtained from a local fish farm (Hanka-Taimen, Venekoski) in February 2011. Roach (*Rutilus rutilus*) and European perch (*Perca fluviatilis*) were caught by ice-fishing in Lake Konnevesi in March 2011. All fish were made unconscious with a quick blow on the head and then killed by cutting the neck. Due to season, visual sex determination was possible only for roach and perch. After measuring the weight of the fish, the livers were dissected, rinsed in neutral buffered saline, frozen in liquid nitrogen and preserved there until analysis.

2.3 EROD analysis and protein analysis

The livers were weighed, and homogenizing buffer (70 mM potassium chloride, 20 mM HEPES sodium salt, pH 7.5 overall buffer) was added in ratio 200 µl buffer to 30 mg sample. The livers were homogenized on ice with plastic rods that fit 1.5 ml centrifuge tubes, until homogeneous (approx. 35 rounds/sample). The homogenates were centrifuged at 10 000 x g for 20 min in +4 ºC. The supernatant (S9 fraction) was transferred to a new tube and used for analyses. The homogenates were not pooled, but kept separate for each individual in all assays.

The EROD activity was measured using black microwell plates (Greiner Bio-One Fluotrac™ flat bottom 96-well plates, VWR). The reaction consisted of 20 µl S9 fraction, 20 µl 2.5 µM ethoxyresorufin, 20 µl 5 mM NADPH and 140 µl reaction buffer (100 mM HEPES sodium salt, pH 7.8). The resorufin and ethoxyresorufin solutions were prepared fresh daily from stock solutions (1 mM in DMSO), and NADPH solution was prepared fresh just before use. The reaction was started by adding the NADPH solution, and fluorescence was measured in a Labsystems Fluoroskan fluorometer with excitation at 540 nm and emission at 584 nm for 4 min every 30 s. Standard curve containing 0 to 128 pmol resorufin per well in reaction buffer was run daily. The standard containing the same amount of sample as the reaction mixture was prepared by pipetting 20 µl sample, 80 µl reaction buffer, and 100 µl resorufin so that the concentration in the well was 0, 2, 16, 32 or 64 pmol. After the linearity was checked in a few samples, only 0 and 64 pmol resorufin standards were used for drawing the sample-specific standard curves. Total protein concentrations of the supernatants were analyzed by modified Lowry method (BioRad DC) according to the instructions of the manufacturer, using bovine serum albumin as standard.

The EROD activity was calculated as pmol resorufin/min/mg protein. The linearity of the increase in the concentration of resorufin was confirmed, and only the linear portion of the reaction was used for calculation. The amount of resorufin in the reaction well was calculated in the beginning and in the end of this linear rise in concentration, and based on these the amount of resorufin formed per minute was calculated. The actual enzyme

activity was calculated by taking into account the amount of protein present in the reaction well.

2.4 Spectroscopic measurements

2.4.1 Steady-state absorbance and fluorescence measurements

The absorbance of undiluted S9 fractions at 540 and 584 nm was measured on a Beckman DU-640 cuvette spectrophotometer. All the other absorbance and fluorescence determinations were made by diluting the samples and resorufin with the homogenization buffer (70 mM potassium chloride, 20 mM HEPES sodium salt, pH 7.5 overall buffer).

The absorbance of different S9 fraction concentrations (0.5, 1, 5, and 10 %, v/v) at 540 nm was measured in four samples per species with a microwell plate reader (Labsystems iEMS Reader MF photometer). The same S9 fraction concentrations were used for measuring the fluorescence of 100 pmol resorufin on black 96-well plates (Greiner Bio-One Fluotrac™ flat bottom 96-well plates, VWR) with Labsystems Fluoroskan fluorometer (ex 540 nm, em 584 nm). The total volume in all measurements was 200 µl. The fluorescence was measured within 30 s of the adding of the resorufin (0 min), and after 60 min incubation in the dark in room temperature. Background absorbance and fluorescence of the samples was determined in all measurements and this background was subtracted.

2.4.2 Time-resolved fluorescence

The fluorescence quenching could be due to electron or energy transfer, enhanced intersystem crossing, or enhanced internal conversion. To establish the mechanism of the quenching, the time-resolved optical spectroscopy measurements were performed with a commercial PicoQuant HydraHarp 400 time-correlated single photon counting (TCSPC) data acquisition system. The excitation source comprised of a PicoQuant PDL 800-D pulsed diode laser driver with 485 nm (spectral FWHM 4 nm) diode laser head LDH-P-C-485. The repetition rate of the excitation pulses was set to 10 MHz in all measurements and the output power of the laser was 0.60 mW for 483 nm excitation. Time resolution of the experiment was determined to be approximately 110 ps (FWHM of the instrument

response function). Measurements were carried out at room temperature and ambient conditions. The samples were 100 pmol resorufin in buffer and 100 pmol resorufin with 0.8 % (v/v) roach or trout *S. trutta* m. *trutta* S9 fraction.

2.5 Inhibitor experiments

To test if the decrease of resorufin fluorescence to its non-fluorescent form is caused by DT-diaphorase or NADPH cytochrome P450 oxidoreductase, the effect of specific inhibitors dicumarol and diphenyleneiodonium, respectively, were studied. The reactions and measurements were performed on black 96-well plates. The reaction mixture (total volume 200 µl) consisted of 20 µl S9 fraction, 10 µM dicumarol or diphenyleneiodonium (DPI), and 100 pmol resorufin. Each plate contained six wells with 100 pmol resorufin in buffer. The S9 fraction was incubated with the inhibitor for 5 min at room temperature, resorufin was added to the wells, and fluorescence was measured within 30 s of the adding of the resorufin (0 min) with Labsystems Fluoroskan fluorometer (ex 540 nm, em 584 nm). The plate was incubated in the dark in room temperature for 60 minutes, after which the fluorescence was measured again. The results were calculated as the percentage of fluorescence relative to 100 pmol resorufin in buffer in the respective plate.

2.6 Statistics

The effect of fish size and sample protein content on sample resorufin fluorescence was tested using Pearson correlation. *t*-test was used for testing the effect of sex on resorufin fluorescence. Kruskall-Wallis was used to test the effect of species on resorufin fluorescence, and after that Dunnett T3 was used to define similar groups. The effect of inhibitors on resorufin fluorescence in different species was tested with ANOVA after log-transformation of the fluorescence readings, and Tukey's test was used to define similar groups. PASW Statistics 18 was used for the statistical analyses.

3. Results

Fish weights, S9 fraction protein contents, S9 fraction EROD activities, and absorbances at resorufin excitation (540 nm) and emission (584 nm) wavelengths are shown in Table 1.

The fluorescence of resorufin standards differed markedly between species and individuals. All fish liver S9 fractions reduced the fluorescence of resorufin when compared to resorufin in buffer (Fig. 1). The smallest fluorescences were measured in roach samples, in which the fluorescence was almost completely diminished.

Resorufin fluorescence did not correlate with sample protein content (n = 138, Pearson correlation -0.39, p = 0.646). There was no effect of sex nor size of the animal on resorufin fluorescence (t-test for sex p > 0.05, correlation between weight and resorufin fluorescence p > 0.05).

Figure 2 presents the emission decays of resorufin, resorufin with roach and resorufin with trout (*S. trutta* m. *trutta*). As can be seen in the from the decay curves as well as from the Table 2, there was no decrease in the lifetime of the resorufin when roach or *S. trutta* m. *trutta* S9 fractions were added. This means that there must occur chemical changes in the resorufin molecule that make it non-fluorescent at the excitation wavelength.

Figure 3a shows the absorbance at 540 nm as a function of the concentration of the S9 fractions in different species. The typical protein concentration in microplate EROD assays is around 1 mg/ml, which corresponds to the S9 fraction concentration of 10 % used in this work (e.g. Grzebyk & Galgani, 1991, ISO/TS 23893-2:2007). The more S9 fraction there was, the more it absorbed light – the more opaque the sample was. The roach samples absorbed less than other species. However, the resorufin fluorescence decreased to a greater extent in roach than in other species with similar S9 fraction concentrations (Fig. 3b). After 60 min incubation both roach and whitefish S9 fractions decreased the resorufin fluorescence more than the other species (Fig. 3c).

Inhibition of DT-diaphorase by dicumarol diminished the decrease of resorufin fluorescence at 0 min in whitefish, *S. trutta* m. *lacustris*, and *S. trutta* m. *trutta*, and at 60 min in whitefish, perch, and roach (Fig. 4). Addition of diphenyleneiodide affected the

decrease of fluorescence at 0 min in whitefish, *S. trutta* m. *trutta* and *S. trutta* m. *lacustris*, and at 60 min in perch, *S. trutta* m. *lacustris*, whitefish, and roach (Fig. 4). Both of the inhibitors increased the fluorescence in nearly all individual samples, but due to large variations between individuals the statistical tests failed to show statistical significance.

4. Discussion

Using the resorufin standard curve prepared only in buffer in the calculation of EROD activity leads to underestimation by 40 – 80 %. There are large differences between species and individuals, and thus using a random sample S9 fraction in the resorufin standard measurements may lead to inaccurate results. The safest way is to make resorufin standards separately for each sample.

The method described in the methods section is suitable for fluorometric plate readers without a dispenser. If the fluorometer is equipped with a dispenser, the ideal way to make the resorufin standards is to prepare wells otherwise identical to the reaction wells but replacing NADPH with resorufin (e.g. 20 pmol/well). The fluorescence is measured just after adding the substrate, before adding the resorufin standard, then resorufin or NADPH is added to the well with the dispenser, and the fluorescence is measured immediately and every 30 s for at least 4 min. The reason this method is probably not suitable for the fluorometers without a dispenser is that all S9 fractions contain some NADPH, and cause some formation of resorufin from ethoxyresorufin. Thus the standard wells would contain both a known amount of the resorufin standard and resorufin formed from substrate. This may lead to underestimation of the EROD activity especially in samples with high activity, if the time between the pipetting of the ethoxyresorufin and measuring the fluorescence is not minimal.

We assumed that the decrease of resorufin fluorescence by the roach samples would result from the absorbance either at the excitation or emitting wavelength of resorufin (540 and 584 nm, respectively). This was not the case. On the contrary, roach samples had the smallest absorptions of all species at both wavelengths.

The absorbance measurements showed that at least two mechanisms are involved in the decrease of fluorescence by fish S9 fraction. The first mechanism is an un-specific one: all samples decrease the fluorescence because they absorb at both the excitation and the emission wavelengths of resorufin. The existence of a second, more species-specific mechanism is revealed in Figs. 3a and 3b. Though the absorbance (the opaqueness) of all samples grows in the same way when the concentration grows (the curves in Fig. 3a are linear), the resorufin fluorescence is diminished to a greater extent by roach S9 fraction, and at 60 min also by whitefish S9 fraction (the curves in Fig. 3b and 3c have a different slope, those of roach and whitefish being steeper). Thus the decrease of fluorescence is not due to opaqueness.

The decrease of the fluorescence by the roach S9 fraction could be either due to quenching of the fluorophore (resorufin) or due to a chemical change of the fluorophore to a non-fluorescent form. In order to establish whether quenching of the fluorescence takes place we measured fluorescence lifetimes of resorufin in different environments. This method implies that if the fluorescence properties (i.e. spectral position or lifetime) of the fluorophore, change in respect to the environment (in this case in roach S9 fraction vs. trout S9 fraction), quenching of the fluorescence occurs. The fluorescence lifetime of resorufin was in good agreement with the value found by Bueno et al. (2002), who observed the lifetime to be 2.9 ns. The lifetime of the resorufin did not change when roach or trout liver S9 fractions were added. This leads to the conclusion that the resorufin molecules still present at the time of the experiment show exactly the same fluorescence properties as the control sample. In addition, fluorescence spectra remained at the same position in all species (data not shown). Then, chemical changes must occur in the resorufin molecule that lead to decrease of the fluorescence.

Diminished resorufin fluorescence could result from chemical reduction to non-fluorescent form. It has been shown that at least two enzymes are able to reduce resorufin: DT-diaphorase (EC1.6.99.2) and NADPH – CYP450 oxidoreductase (CYPOR, EC 1.6.2.4) (Nims *et al.*, 1984; Dutton *et al.*, 1989). DT-diaphorase (also termed NAD(P)H:quinone oxidoreductase 1, nicotinamide quinone oxidoreductase 1, quinone

reductase type 1, or menadione reductase) is a soluble enzyme that is inhibited by dicumarol (Hosoda *et al.*, 1974; Dinkova-Kostova & Talalay, 2010). CYPOR is situated in the endoplasmic reticulum, and it can be inhibited by diphenyleneiodonium (DPI) (Tew, 1993). DPI inhibits also other flavin-containing enzymes and has been shown to interfere with cell redox metabolism (Riganti *et al.*, 2004). As DT-diaphorase is a flavoprotein, the addition of DPI probably inhibited both DT-diaphorase and CYPOR.

The inhibitors affected the fluorescence of resorufin in nearly all individual samples in all fish species, though the statistical testing failed to show significance due to large variation. It can be assumed that, to some extent, resorufin is enzymatically reduced to a non-fluorescent form in all species, and that the variation in the decrease of fluorescence between samples may be caused by different amounts or activities of the enzymes that reduce resorufin. The variation was greatest in the roach: dicumarol increased the fluorescence to 114 – 147 % of the fluorescence without inhibitor at 0 min and to 166 – 247 % at 60 min; and DPI to 173 – 220 % at 0 min and to 203 – 541 % at 60 min.

The decrease of resorufin fluorescence by the roach liver S9 fractions most probably results from both DT-diaphorase and CYPOR reducing resorufin to a non-fluorescent form. The DT-diaphorase can be inhibited with dicumarol, or microsomal fractions can be used for EROD assay, so the largest part of this enzyme can be removed by ultracentrifugation. The reduction of resorufin by CYPOR makes the kinetic EROD assay difficult for roach S9 samples, because this enzyme provides electrons to the CYP1A1, and is thus needed for the EROD reaction to take place. Moreover, because the CYPOR is a membrane-bound protein, also microsomal fractions contain this enzyme. We have observed that the roach liver microsomes indeed diminish the fluorescence of resorufin less than the S9 fraction of the same individuals, but still remarkably more than the microsomal fraction of other species (Vehniäinen, unpublished). This corroborates the conclusion that both soluble and membrane-bound enzymes are involved in the process in roach.

The EROD activity measurements of roach S9 samples could be done as endpoint measurements, where the reaction is ended after a certain time e.g. with methanol or some other organic solvent that inhibit the reducing action of the enzymes and allow the resorufin to autooxidate back its fluorescent form (Lubet *et al.*, 1985). However, it has been shown that microsomal CYPOR is capable of reducing ethoxyresorufin as well, and this may interfere with the EROD reaction (Dutton *et al.*, 1989). Therefore, probably the most reliable way to measure CYP1A1 induction in species where resorufin is reduced by CYPOR is to use either immunological methods (immunoblotting or ELISA) that measure the amount of CYP1A1 protein, or qPCR that measures the mRNA level.

It must be born in mind that our results were obtained from animals held in clean reference waters. Exposure to AhR agonists leads to induction of DT-diaphorase (Nioi & Hayes, 2004). Thus the S9 fractions of animals with induced EROD activity are likely to reduce resorufin even more than those from reference sites, so it is advisable to include dicumarol in the EROD reactions to count for this difference.

In conclusion, we recommend using sample-specific resorufin standards in EROD measurements for more accurate results. Resorufin standards made separately for each S9 sample of fish liver also reveal if the sample reduces resorufin to a non-fluorescent and non-absorbing form and if further actions should be taken to ensure reliable results.

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Tables
Table 1.

Fish weights, S9 fraction protein contents, EROD activities, and absorbances at resorufin excitation (540 nm) and emission (584 nm).

Data shows mean ± 1 standard deviation (SD).

species and strain	fish weight, g	S9 fraction protein concentration, mg/ml	S9 fraction EROD activity, pmol/min/mg prot	S9 fraction absorbance at 540 nm	S9 fraction absorbance at 584 nm
Atlantic salmon *Salmo salar* (Linnaeus, 1758), Neva strain	19 ± 4	24 ± 4	18 ± 6	2.27 ± 0.14	2.42 ± 0.16
landlocked salmon *Salmo salar* m. *sebago*	56 ± 3	24 ± 5	11 ± 5	2.41 ± 0.20	2.52 ± 0.13
brown trout *Salmo trutta* m. *trutta* (Linnaeus, 1758), Isojoki strain	12 ± 2	25 ± 4	20 ± 6	2.15 ± 0.30	2.28 ± 0.22
brown trout *Salmo trutta* m. *lacustris*	9 ± 2	26 ± 4	27 ± 9	2.13 ± 0.25	2.26 ± 0.29
whitefish *Coregonus lavaretus*	20 ± 3	17 ± 7	10 ± 8	2.05 ± 0.14	2.01 ± 0.22
roach *Rutilus rutilus*	22 ± 3	16 ± 2	2 ± 1	1.74 ± 0.35	1.46 ± 0.38
European perch *Perca fluviatilis*	25 ± 9	14 ± 3	20 ± 7	1.74 ± 0.21	1.46 ± 0.23

Table 2. Decay fit results. τ is the lifetime, and the weighted mean square deviations (χ^2) obtained from ordinary fitting procedure.

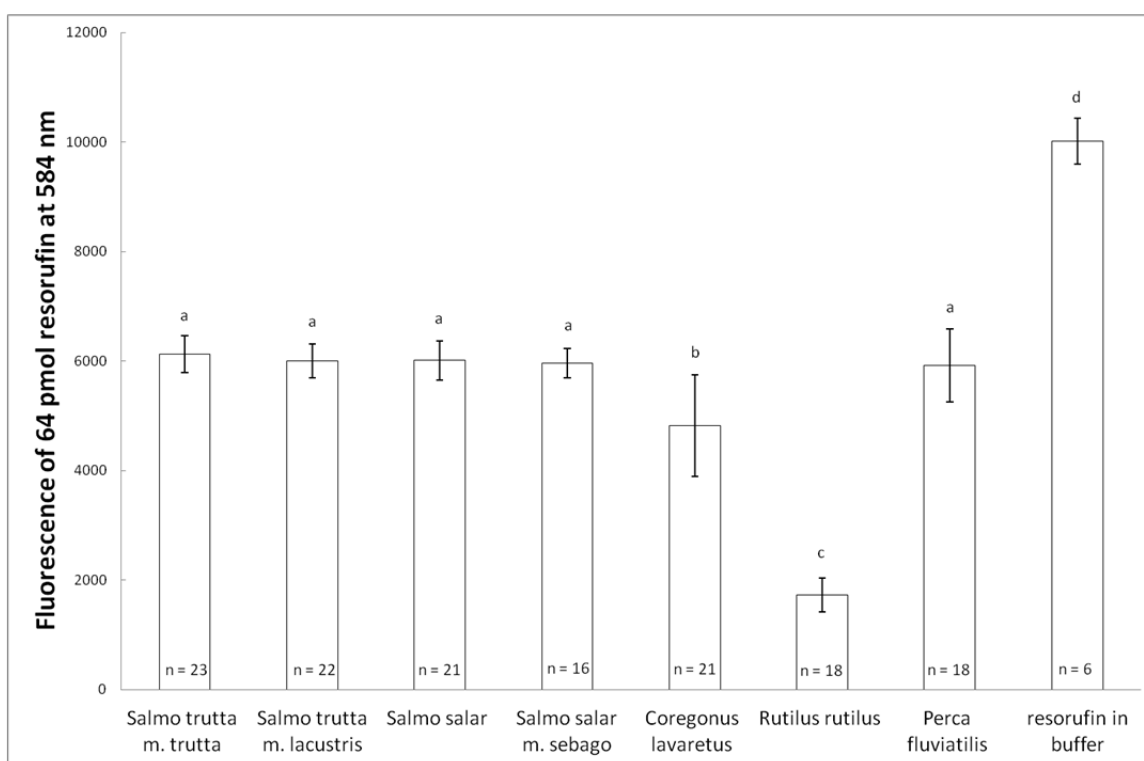| sample	τ, ns	χ^2
resorufin	2.80±0.04	1.026
resorufin and roach	2.81±0.04	1.192
resorufin and trout	2.81±0.04	1.161

Figure 1. Fluorescence of 64 pmol resorufin with liver S9 fractions (10% v/v) from different fish species. Bars show mean ± SD. Samples denoted with the same letter do not differ significantly from each other ($p>0.05$).
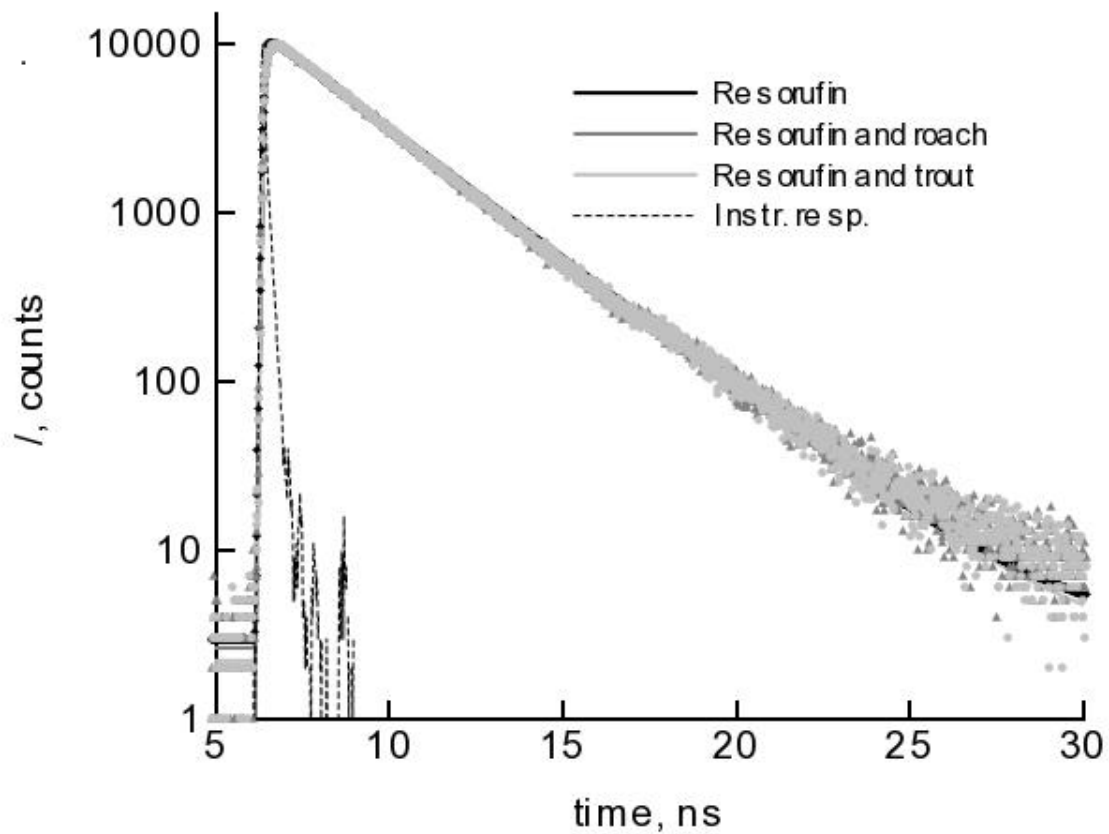
Figure 2. Time-resolved fluorescence decays for resorufin, resorufin with roach as well as resorufin with trout at the monitoring wavelength of 584 nm. The solid curves correspond to the mono-exponential fits. Rate parameters obtained from the fitting are shown in Table 2. Excitation wavelength was 485 nm. Trout denotes *S. trutta* m. *trutta.*
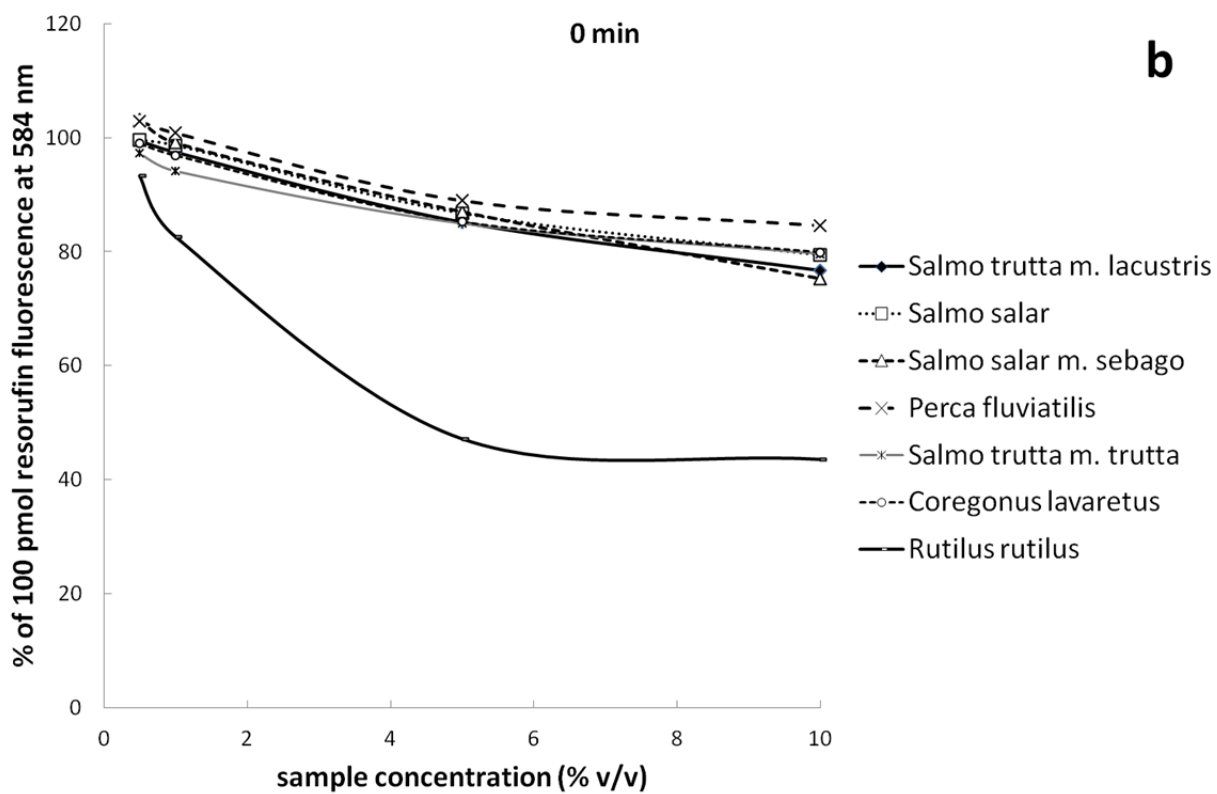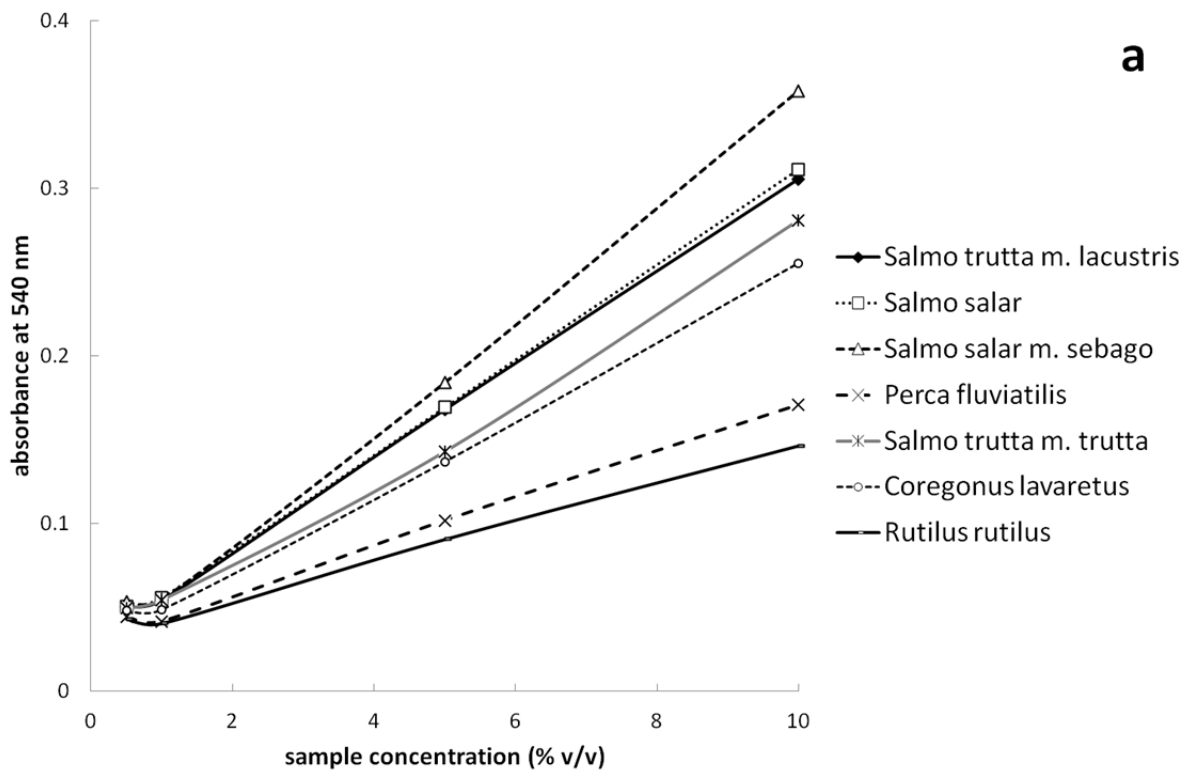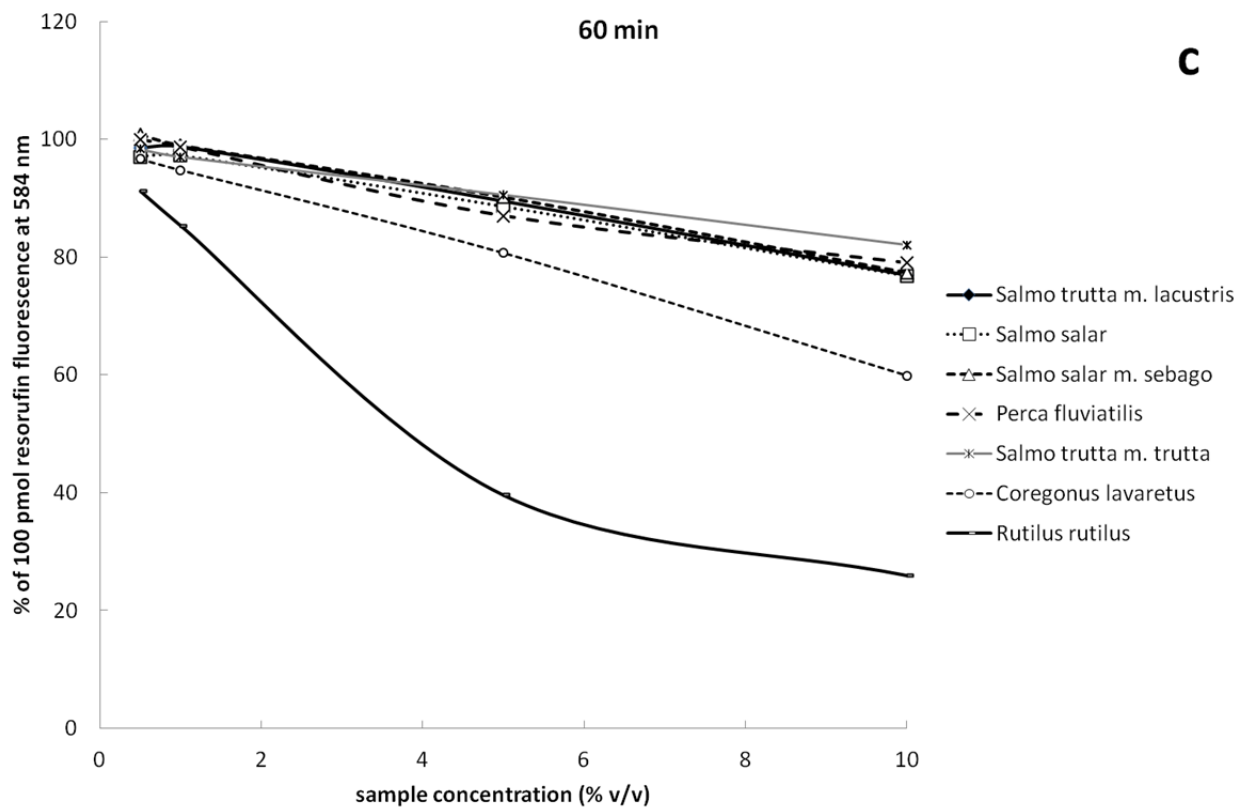
Figure 3. Sample absorbance and fluorescence of 100 pmol resorufin as a function of sample concentration (% v/v) in different species, n = 4 animals / species. a) Absorbance of the sample at 540 nm, b) fluorescence of 100 pmol resorufin (ex 540 nm, em 584 nm) with the sample right after the addition of resorufin (0 min), c) fluorescence of 100 pmol resorufin (ex 540 nm, em 584 nm) with the sample after 60 min incubation.
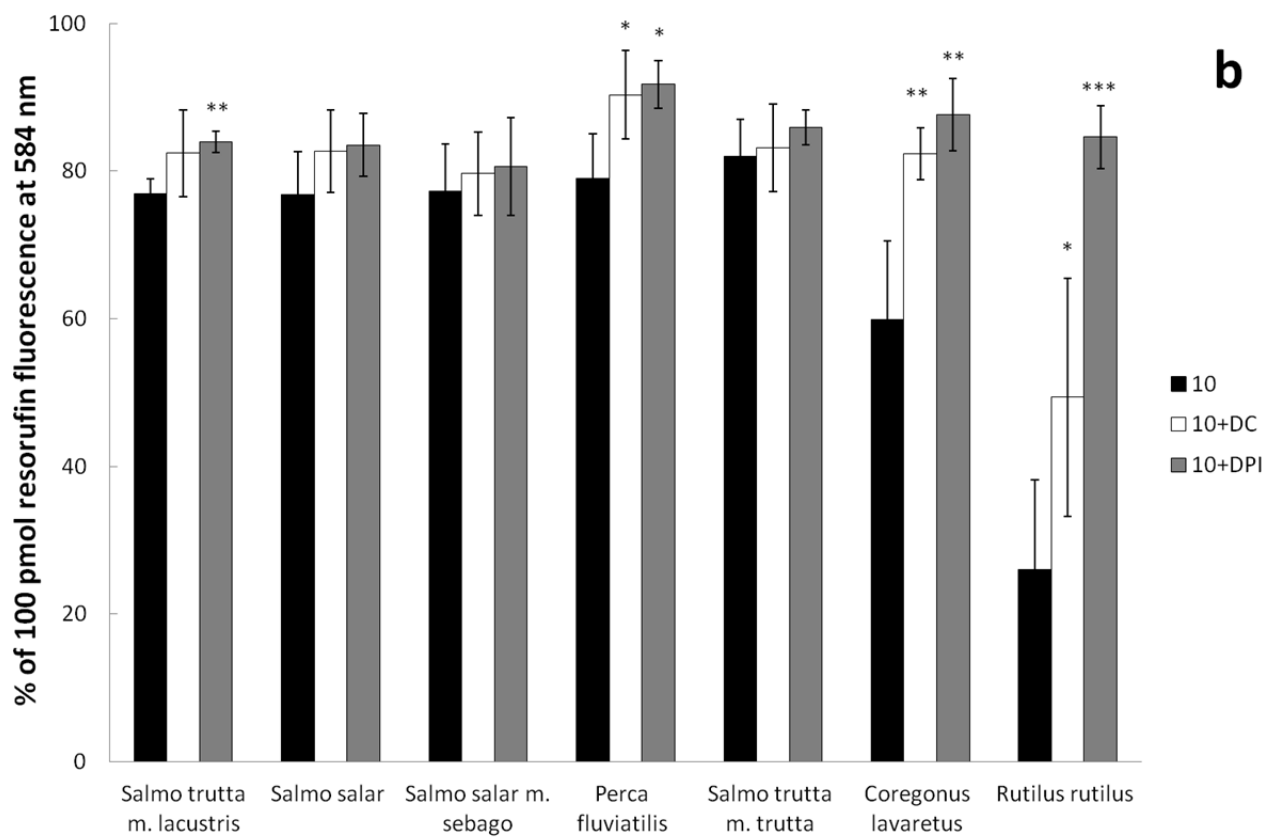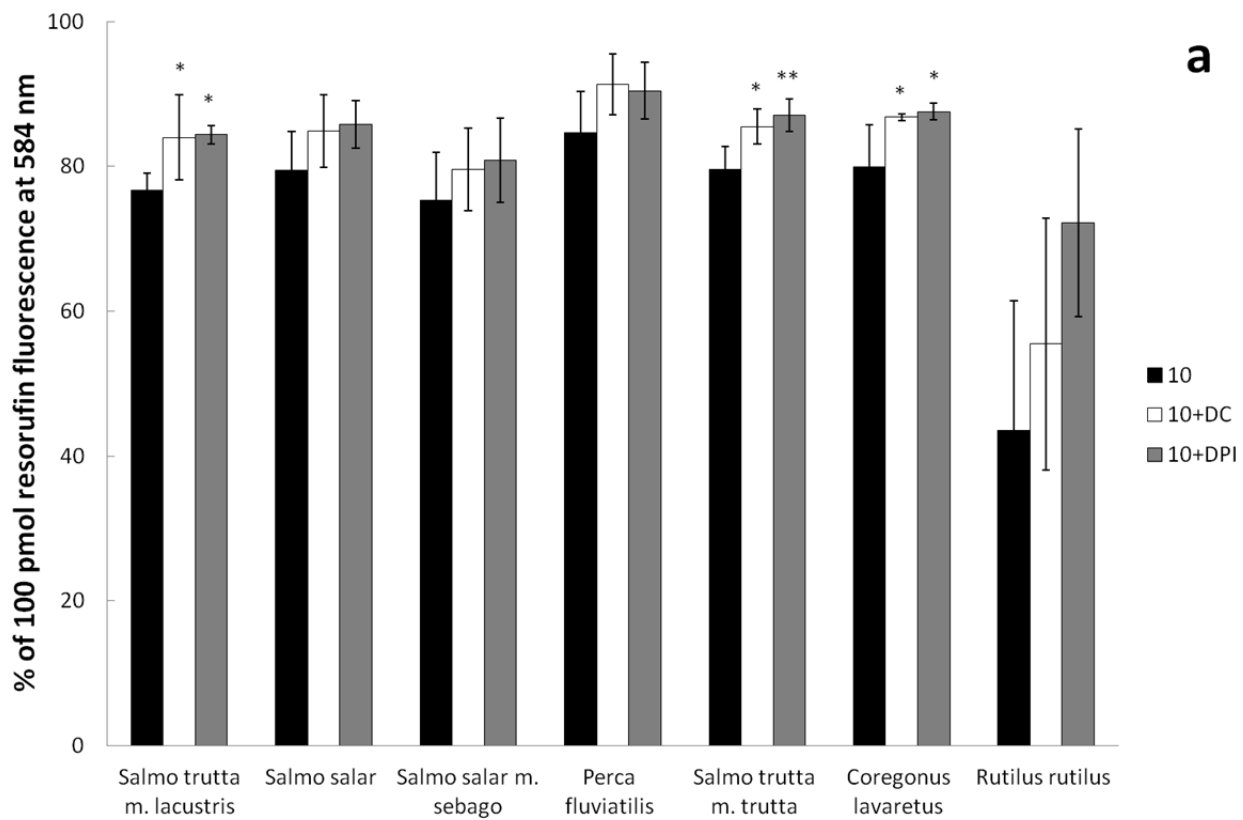
Figure 4. The effect of inhibitors of DT-diaphorase (DC) and NADPH-CYP450 oxidoreductase (DPI) on the resorufin fluorescence (ex 540 nm, em 584 nm) in samples from different species. All treatments contained 100 pmol resorufin and 10 % (v/v) liver S9 fraction. DC = 10 µM dicumarol, DPI = 10 µM diphenyleneiodonium. Bars represent mean ± SD, n= 4 animals / treatment / species. a) 0 min, b) 60 min incubation.