In vivo measurement of ethoxyresorufin biotransformation by zebrafish prolarva to evaluate cytchrome P450 1A induction: application to fresh waters and environmental sediment extracts

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Introduction

Fish EROD activity is widely used to assess the exposure of organisms to CYP1A inducer in marine water, fresh water and sediment

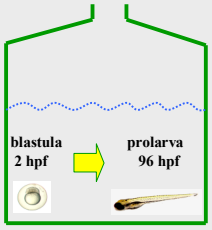
Recently we developed a non « destructive » method (1) based on the biotransformation activity of the ethoxyresorufin substrate in the pro-larvae of the fish *Danio rerio*. EROD enzymatic activity induction, is detected by measuring the fluorescence increase in the test medium due to the excretion of the resorufin by the fish, after pre-exposure to chemicals or to environmental samples.

Therefore, the proposed methodology allows a simple, sensitive and reproducible micro biotest to be performed for the detection of sublethal concentrations of AhR chemical inducers in environmental samples such as natural waters and organic extracts of sediments. Here we present the results obtained with several water and sediment samples from French river upstream and downstream urban area. *D. rerio* embryos and prolarvae were either exposed to water samples or to organic extracts of sediment.

(1) Noury P, Geffard O, Tutundjian R, Garric J. 2006. Non destructive in vivo measurement of ethoxyresorufin biotransformation by zebrafish prolarva: Development and application. Environmental Toxicology 21(4):324-331.

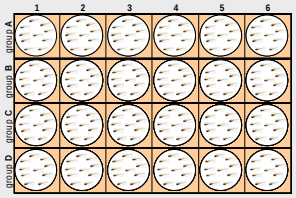
Assay protocol

1st Pre-exposure to environmental samples from 2 to 96 hours post fertilization (hpf)



- 100 embryos by experimental group : together in 300 ml of test medium
- Static exposure in glass bottle
- Undeveloped embryos removed at 24 h
- Temperature = 26° C
- Hatching from 72h to 96 h

2nd Ethoxyresorufin exposure of prolarvae in microplates and kinetic measurements of fluorescence emission

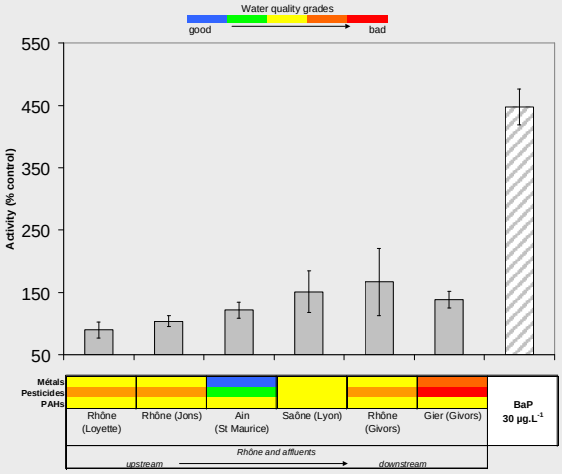


- 10 larvae by well (2 ml) ; 6 wells by experimental group
- Temperature = 26°C
- Ethoxyresorufin : 1.5 µmol.L^{-1} (DMSO 0.1 %)
- fluorescence measurement every hour during 5-6h
- excitation : 545 nm
- emission : 587 nm (resorufin); 565 nm (baseline)

Application to fresh waters

Responses to static pre-exposure to surface fresh water from the Rhône river and affluents (Ain, Saône, Gier), upstream and downstream suburbs of Lyon and Saint Etienne ; comparisons with coded physico-chemical data from « Reseau National de Bassin ».

mean and 95 % confidence limits (N=6 wells ; 10 larvae/well)



Pre-exposure and assay

- 6 freshwater samples collected from several French rivers (Ain, Saône, Gier and Rhône)
- 100 embryos pre-exposed together in 300 ml of sample; negative and positive control performed simultaneously.
- After 96 h: prolarvae are moved in microplates (10 prolarvae x 6 wells) and fluorescent kinetic are performed

Results

- No significant difference between control (test medium) and reference sites (Ain).
- Significant differences (0.001<p<0.04) between the reference site and sites downstream of suburbs of Lyon (Saône, Givors) and Saint Etienne (Gier).
- Toxicity of the Givors sample involved a high variability for the results of the surviving larvae.
- The presence of inducers is suspected downstream. PAHs seems not involved (no PAHs gradient detected. Others inducers (PCBs could be present).

Application to organic extracts of sediments

In the Framework of ongoing French National Program « Programme National de Recherche sur les Perturbateurs Endocriniens » (PNRPE), sediments were sampled from French rivers upstream and downstream urban areas to assess both their Ah receptor and oestrogenic inducing activity . Drôme river at Saillans is a low impacted site in the Rhone Alpes region, Rhône river at Givors is impacted by the Lyon city area and chemicals industries. On Lez river, five sites are studied , from the source to the ponds downstream Montpellier city; finally Blanquefort river, close to Bordeaux city, is a small river impacted by urban and motorway effluent as well as diffuse pollution from market gardening. Here are presented preliminary results dealing with AhR inducing activity of the sediment organic extracts

Material and method

Extracts preparation

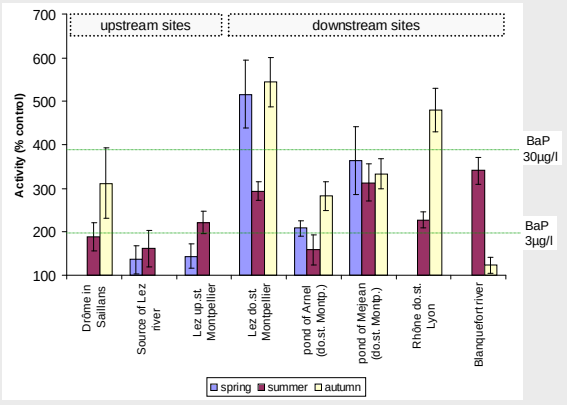
- On site: sediments sampling and sieving (< 2 mm)
- Storing and freezing (-20 °C) in aluminium box
- Lyophilisation
- Extraction by ASE of 5 g of dry sediment (solvant mixture : 50 % heptan - 50% aceton)
- Evaporation by nitrogen flow
- Dry extract collected in 1 ml of methanol (MeOH)
- Conservation in brown vial at ambient temperature

Zebrafish embryos pre-exposure and assay

- 19 organic extracts tested within 7 assays (february to may 2007); same batch of genitors;
- For each extract : 100 embryos pre-exposed together in 300 ml of test medium (static exposure)
- Test medium = 30 µl extract + 300ml water = 500mg/l equivalent of dry sediment
- For each assay negative and positive controls : Water, Solvent MeOH (1/10000), BaP 3 µg/l, BaP 30 µg/l
- After 96 h pre exposure : prolarvae are moved in microplate (10 prolarvae x 6 wells) and fluorescent kinetic are performed

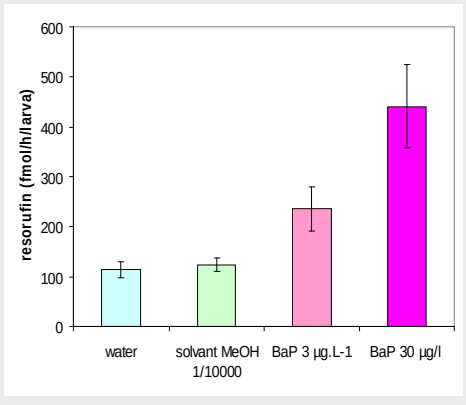
Résults

EROD in vivo induction caused by sediment extracts
Mean and standard deviation (6 microplates wells)



- No significant mortality observed (results not shown)
- Minimum significant induction = 1.4 x basal activity (lez up.st., spring)
- Induction max. reaches to 5.4 x the basal activity (lez do.st., automn)
- Upstream stations show low to moderate activity induction
- Downstream stations show low to high activity induction
- Lez river : the up/downstream gradient induction is higher in spring than in summer

Inter assay variability of the controls :
Mean and standard deviation (7 assays)



- Moderate inter assay variability (from 10 % to 18 %)
- No difference between solvent controls and water controls
- basal activity = 114 +- 16 fmol/h/larva
- BaP 3 µg/l effect is about 2 x basal activity
- BaP 30 µg/l effect is about 4 x basal activity

Conclusion

The first results obtained are encouraging as this microbiotest allows to discriminate between low or moderate and contaminated sites downstream urban areas. Moreover, the inter assays comparison shows a low variability of the negative and positive controls, which allows reliable comparisons between sites and time of sampling. This microbiotest, can be a valuable screening approach to quickly assess the presence of CYP1A inducers in environmental samples,as water, effluent and sediment.

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