

Validation of hepatic EROD-activity in Rainbow Trout as a biomarker to assess effluent toxicity of a sewage treatment plant.

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Abstract

Rainbow trout eggs and juveniles (*Oncorhynchus mykiss*) were exposed to effluent dilutions of an industrial sewage treatment plant. Both drinking water, as well as filtered water of the receiving river were used as controls. In a first study growth and biomass reduction turned out to be equally sensitive endpoints for the early life stages, as the hepatic ethoxyresorufin O-deethylase (EROD) activity for the adults.

For the interpretation and validation of the increased detoxifying (EROD) -activity in juvenile fish, phase II enzyme activities (glutathione S-transferases and UDP-glucuronosyl transferases), histopathological endpoints, as well as the formation of DNA-adducts were investigated in subsequent studies.

Increased EROD-activity was accompanied by histopathological changes in liver, kidney and gill, such as irreversible alterations of the nucleus and necrosis. The biochemical studies showed formation of DNA-adducts. Based on the results of the various parameters studied, EROD-activity was a sensitive biomarker of effluent exposure. In addition, the results of the various parameters allowed the interpretation and validation of the biomarker and the assessment of the potentially adverse longterm effects on the trout population.

Introduction

Hepatic mixed function oxidase (MFO) activity, as indicated by 7-ethoxyresorufin O-deethylase (EROD) and other measurements of cytochrome P450IA1, is a sensitive indicator of the ability of fish to detoxify certain xenobiotics. MFO-activity can be related quantitatively to the extent of xenobiotic exposure and can therefore be used as biomarker. For the assessment and monitoring of the longterm effluent toxicity of an industrial sewage treatment plant on rainbow trout, EROD-activity was chosen as biomarker. Its interpretation and validation therefore played a key role in the present study.

Materials and Methods

Exposure of Trout to Effluent Dilutions of an Industrial Sewage Treatment Plant

Rainbow trout eggs and juveniles (*Oncorhynchus mykiss*) were exposed to effluent dilutions 1:40, 1:160 and 1:640 of varying composition for periods of 24 days to 5 months. Both carbonfiltered tap water (TW), as well as gravel- & sandfiltered water of the receiving river (FW) were used as controls. The latter was also used as dilution water.

Sampling of Organs

Samples of the organs were taken immediately after the fish were sacrificed and stored in liquid nitrogen (for enzyme activities) and/or fixed in Bouin's solution (for histopathology).

Subcellular Trout Liver Fractions

The microsomal and cytosolic liver fractions were prepared according to the method of Burke and Mayer (1974).

Endpoints

Effect parameters used included growth, biomass and survival of early life stages and juveniles, as well as several blood, biochemical and histopathological parameters:

Measurement of Biochemical Parameters

- Protein contents of the cytosolic liver fractions: Smith et al. (1985) and Lowry et al. (1951).
- Ethoxyresorufin O-deethylase: Burke and Mayer (1974).
- Microsomal UDP-glucuronosyltransferase activities: Mulder and van Doorn (1975).
- Cytosolic glutathione S-transferase activities: Habig et al. (1974).

DNA Adducts

[³²P]postlabelling assay: Gupta 1985 and Beach et al. 1992.

Histopathology

Various histopathological effects on the gill, liver and kidney were used as endpoints. Tissues were embedded in paraffin; 4-6µm slices of paraffin were stained with Haematoxylin & Eosin.

Statistical Analysis

For the determination of the No Effect Concentration (NEC), individual analysis of each fish and endpoint was performed using a linear regression approach model with quadratic and general Lack of Fit (Tukey et al, 1985).

Results and Discussion

- in a first part of the study, **growth and biomass reduction** of the early life stages (Fig. 1) turned out to be equally sensitive endpoints as the up to about four-fold **increased hepatic ethoxyresorufin O-deethylase (EROD) activity** of the adults (Fig. 2).
- the NEC's of each of these endpoints corresponded to a similar effluent dilution range of 1:640 to 1:160.

For further interpretation and validation of the increased EROD-activity in relation to the control (FW), phase II enzyme activities, histopathological endpoints, as well as the formation of DNA-adducts were investigated subsequently:

From the results of the **phase II enzyme activities** studied (Fig. 3), it is concluded that:

- the effluents during the May 92 experiment contained substances with 3-methylcholanthrene **3MC-type inducing properties** (risk of mutagenicity and carcinogenicity).
- the effluents during the February 92 experiment displayed a weaker 3MC inducing potency but,
- in addition, appeared to contain constituents which elicit a phase II enzyme induction profile comparable to a phenobarbitone **PB-type induction** of foreign compound metabolizing enzymes in rats (regarded as a reversible adaptive response with little, if any, toxic consequences in mammals).

The **histopathological investigation** revealed:

- adverse effects in the gill, kidney and liver.
- the size of the effect increased with exposure time and effluent concentration (Fig. 4).
- severe **irreversible alterations** included nucleus anomalies in the kidney and necrosis in the liver (Fig. 5) (down to highest effluent dilution of 1:640 after prolonged exposure).
- cancerogenic effects could not be shown histologically (exposure too short).

Formation of a **covalent binding with the DNA**:

- trout exposed to the lowest dilution 1:40 of the effluent revealed at least **four DNA adducts** in the liver and in the gill (Fig. 6).
- the overall DNA adduct level was relatively low (1 adduct per 108 nucleotides, which indicated only a minimal cancer risk for the exposed fish).
- in trout exposed to control water (FW), no DNA adducts were detectable neither in the liver nor in the gill (Fig. 6).

Fig. 1
Biomass reduction in early life stages of Rainbow Trout (83 days exposure)

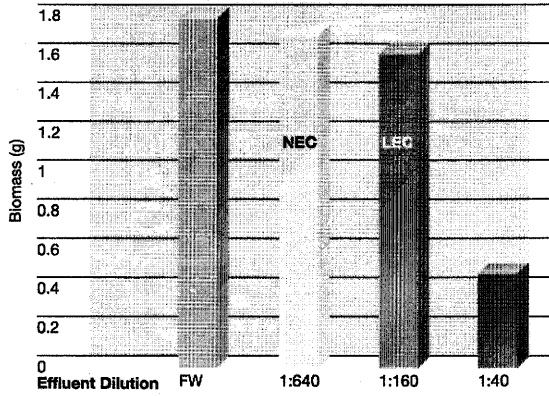


Fig. 2
Hepatic EROD-activity in Rainbow Trout (24 days exposure)

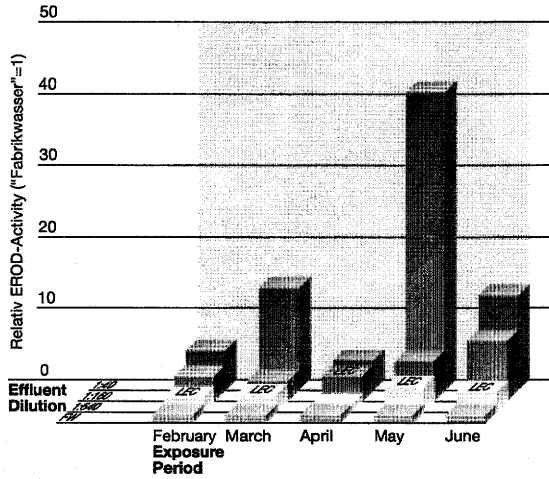


Fig. 3
EROD- and phase II enzyme activities in Rainbow Trout (48 days exposure 1:40)

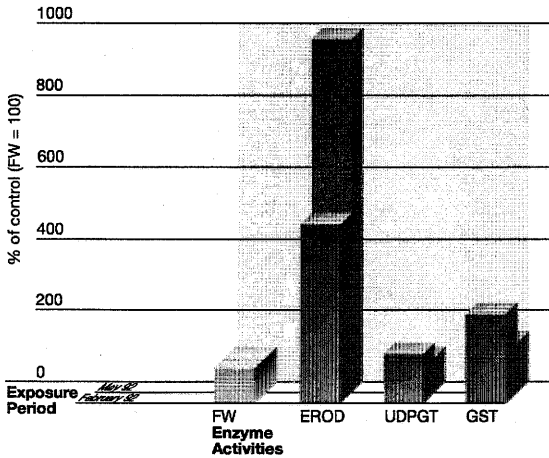


Fig. 4
Scored histopathological effects in 1993 (1-5 months exposure)

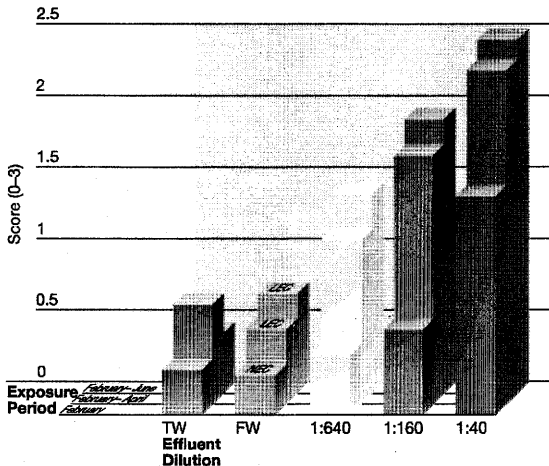


Fig. 5
Disseminated focal necrosis in the liver of rainbow trout (24 days exposure 1:40)

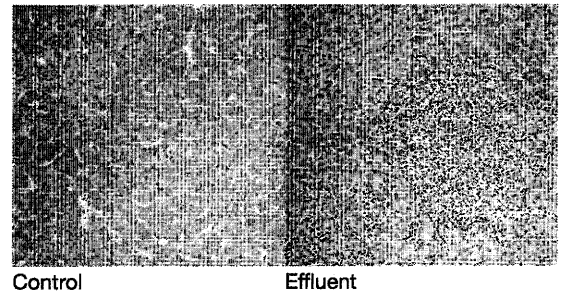
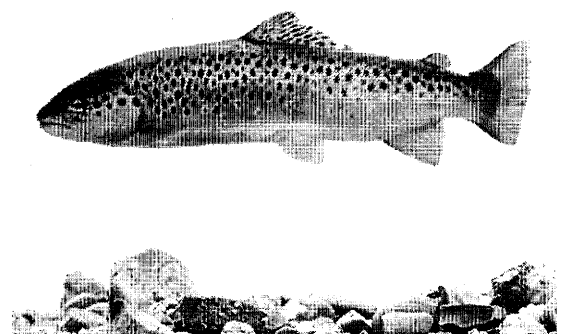
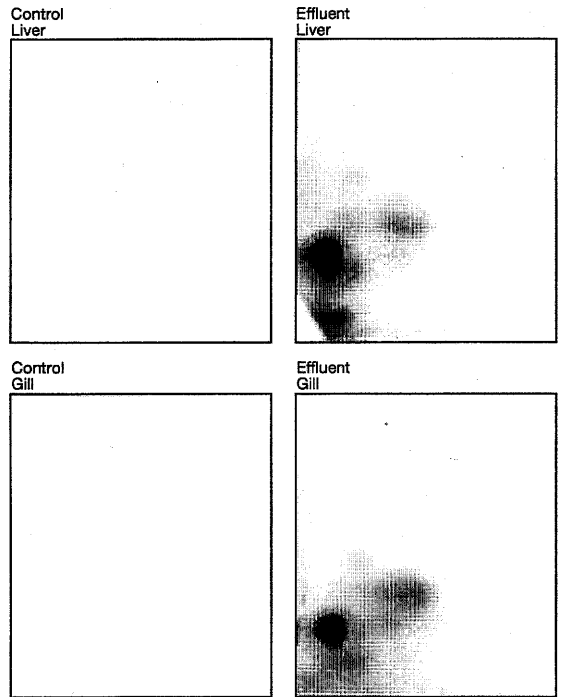


Fig. 6
DNA Adducts of Rainbow Trout (3 months exposure 1:40)



Conclusions

The results showed, that:

- the hepatic **EROD-activity** was not only a biomarker to monitor the quality of the effluent with respect to the inductive potency for foreign compound metabolizing enzymes in trout liver, but,
- it was also the **most sensitive parameter** among more than 30 endpoints tested.
- the interpretation and validation of these results in regard to higher levels of biological organisation required additional investigations and the **link to biological effects**.
- of the additional effects studied, the results of
 - 1.) survival & growth (in particular of the early life stages)
 - 2.) histopathological studies (gill, kidney and liver) and
 - 3.) formation of DNA-adducts (liver and gills)contributed the most in regard to the interpretation and validation of the EROD-activity, thus allowing the assessment of the potentially adverse longterm effects on the trout population.

Abbreviations

3MC	3-methylcholanthrene
PB	phenobarbitone
UDPGT	UDP-glucuronosyltransferase
GTP	glutathione S-transferase
EROD	7-ethoxyresorufin O-deethylase
FW	water of the receiving river: sand- & gravel filtered
TW	tap water
MFO	hepatic mixed function oxidase or mono-oxygenase
NEC	No Effect Concentration
LEC	Lowest Effect Concentration

Literature

- Beach AC, Gupta RC (1992) Human biomonitoring and the [32P] postlabelling assay. *Carcinogenesis* 13: 1053-1074
- Burke, M.D., Mayer, R.T., 1974: Ethoxyresorufin: Direct fluorimetric assay of microsomal O-deethylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Disp.* 2: 583-588.
- Gupta RC (1984) Nonrandom binding of the carcinogen N-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA in vivo. *Proc Natl Acad Sci USA* 81: 6943-6947
- Gupta RC (1985) Enhanced sensitivity of [32P]postlabelling analysis of aromatic carcinogen:DNA adducts. *Cancer Res* 45: 5656-5662
- Habig, W.H., Pabst, M.J., and W.B. Jakoby, 1974: Glutathione S-transferases. The first step in mercapturic acid formation. *Biol. Chem.* 249, 7130-7139.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall R.J., 1951: Protein measurement with the folin phenol reagent. *J. Bio. chem.* 193: 265-275.
- Mulder, G.J., van Doorn, A.B.D., 1975: A rapid NAD⁺-linked assay for microsomal uridine diphosphate glucuronosyltransferase of rat liver and some observations on substrat specificity of the enzyme. *Biochem. J.* 151, 130-140.
- Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A. K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C., 1985: Measurement of protein using bicinchonionic acid. *Anla. biochem.* 150, 76-85.
- Tukey, J.W., Cimidera, J.L. and Heyse, J.F., 1985: Testing the statistical certainty of a response to increasing doses of a drug, *Biometrics*, 41.