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Application of P450 reporter gene system (RGS) in the analysis of sediments near pulp and paper mills

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Cytochrome P4501A (CYP1A) induction in fish and other animals has been reported following exposure to pulp and paper mill effluent. Dioxins and furans as well as polycyclic aromatic hydrocarbons (PAH) are known inducers of CYP1A and have been found in sediments near pulp and paper mills. Retene (7-isopropyl-1-methylphenanthrene), an alkyl-substituted phenanthrene, has been recently associated with effluent and found to induce CYP1A in fish. This study utilized an *in vitro* assay, P450 Reporter Gene System (RGS), to assess the transcriptional activation of human CYP1A by retene after short (6 h) and long (16 h) exposures. Retene was as potent as benzo[*a*]pyrene in inducing RGS, but was not as readily biotransformed by the cells. Extracts of sediments collected near a pulp and paper mill were analysed, and RGS-derived toxic equivalencies (TEQ) were strongly correlated with Chemical TEQ analysis of dioxins and furans determined by EPA Method 8290 using high-resolution gas chromatography/mass spectrometry. RGS 6-h responses indicated the presence of PAH in the extracts, which was confirmed by GC/MS analysis. Retene was detected at considerably higher concentrations than other PAH. These data support the use of the RGS assay to detect the presence of CYP1A-inducing compounds, including retene as well as dioxins and furans, in sediments near pulp mills.

Keywords: Pulp and paper, retene, CYP1A, dioxins.

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Introduction

Exposure to pulp and paper mill effluents has been found to cause various biochemical responses in fish, including induction of hepatic cytochrome P4501A (Kloepper-Sams and Benton 1994) and associated enzymes (Hodson *et al.* 1992, Munkittrick *et al.* 1994). Munkittrick *et al.* (1992) reported that installation of secondary treatment did not remove CYP1A-inducing compounds from bleached kraft mill effluent (BKME). Further, mill shutdown resulted in rapid (2 weeks) reduction of CYP1A activity in the liver of white sucker (*Catostomus commersoni*) collected near the mill, indicating that induction was not related to the presence of persistent compounds such as dioxins and furans. Rather, multiring polycyclic aromatic hydrocarbons (PAH) with alkyl substitutions and not chlorine substitutions, found in secondary-treated BKME, were found to cause induction of CYP1A activity (Burnison *et al.* 1996).

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Resin acids, including abietic and dehydroabietic acid, are natural plant products from the softwood of conifers found in some pulp mill effluents (Morales *et al.* 1992). Retene (7-isopropyl-1-methylphenanthrene) is an alkyl-substituted phenanthrene derived from the anaerobic metabolism of these acids, and has been found in unbleached kraft pulp and paper mill effluent (Wakeham *et al.* 1980, Zender *et al.* 1994). Fragoso *et al.* (1998, 1999) reported that chronic exposure to retene caused sustained induction of CYP1A activity and protein in rainbow trout (*Oncorhynchus mykiss*), and that retene was metabolized by the liver.

In vitro cell assays have been developed using several different species and employed as rapid, sensitive and inexpensive alternatives to *in vivo* assays and analytical methods for determining the presence of compounds which induce CYP1A in environmental samples (Tillitt *et al.* 1991, Garrison *et al.* 1996, Kennedy *et al.* 1996, Richter *et al.* 1997, Willett *et al.* 1997). One such assay utilizes human hepatoma cells (101L) stably transfected with a luciferase reporter gene downstream of human CYP1A promoter sequences (Postlind *et al.* 1993). This assay, known as the P450 Reporter Gene System (RGS), has been used extensively to derive toxic equivalencies that are highly correlated with levels of CYP1A-inducing PAH, coplanar PCBs, and dioxins and furans in the tested extracts (Kim *et al.* 1997, Anderson *et al.* 1999a–c). Because PAH are readily biotransformed by the RGS cell, but chlorinated compounds are not, optimal induction of CYP1A by PAH occurs earlier than by chlorinated compounds. Thus, the use of two exposure periods can enable the characterization of CYP1A-inducing compounds as PAH and/or chlorinated compounds (Jones and Anderson 1998, Jones *et al.* 2000).

In this study, RGS was used to assess the transcriptional activation of CYP1A by retene, which was compared with benzo[*a*]pyrene (B[*a*]P). Short (6 h) and long (16 h) exposures were tested to determine the degradation of retene by the 101L cell, compared with a standard mixture of PAH. RGS was also used to analyse extracts of 28 sediment samples collected by the US Fish and Wildlife service for Region 4 of the US EPA from riverine system in Florida near a pulp and paper mill. RGS-derived toxic equivalency (TEQ), a measure of the induction potency of a sample compared with a standard mixture of dioxins and furans, was determined from testing at 16 h after exposure, and then correlated with analyses of dioxins and furans in the samples determined by EPA Method 8290 using high-resolution gas chromatography/mass spectrometry (Anderson *et al.* 2000). Next, RGS testing at both 6 and 16 h was used to determine the contribution of PAH in selected samples. PAH were then measured analytically in two of the extracts using GC/MS SIM. The Method 8290 analyses were conducted by an EPA contract laboratory, and the TEQ data file for the 28 samples was provided by Region 4 EPA in Atlanta, GA.

Materials and methods

Test toxicants

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and B[*a*]P were obtained from Ultra Scientific (North Kingstown, RI, USA). The dioxin/furan mixture (table 1) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). The PAH mixture (table 2) was obtained by Supelco, Inc.

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Table 1. Composition of standard mixture of dioxins and furans.

Analyte	Concentration (ng ml^{-1})	TEF*	Product
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	40	1	40
1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin	200	0.5	100
1,2,3,4,7,8-Hexachlorodibenzo-*p*-dioxin	200	0.1	20
1,2,3,6,7,8-Hexachlorodibenzo-*p*-dioxin	200	0.1	20
1,2,3,7,8,9-Hexachlorodibenzo-*p*-dioxin	200	0.1	20
1,2,3,4,6,7,8-Heptachlorodibenzo-*p*-dioxin	200	0.01	2
Octachlorodibenzo-*p*-dioxin	400	0.001	0.4
2,3,7,8-Tetrachlorodibenzofuran	40	0.1	4
1,2,3,7,8-Pentachlorodibenzofuran	200	0.05	10
2,3,4,7,8-Pentachlorodibenzofuran	200	0.5	100
1,2,3,4,7,8-Hexachlorodibenzofuran	200	0.1	20
1,2,3,6,7,8-Hexachlroodibenzofuran	200	0.1	20
1,2,3,7,8,9-Hexachlorodibenzofuran	200	0.1	20
2,3,4,6,7,8-Hexachlorodibenzofuran	200	0.1	20
1,2,3,4,6,7,8-Heptachlorodibenzofuran	200	0.01	2
1,2,3,4,7,8,9-Heptachlorodibenzofuran	200	0.01	2
Octachlorodibenzofuran	400	0.001	0.4

*Toxic equivalency factors from Safe (1990).
TEQ, toxic equivalency quotient (sum of products) = 400.8ng ml^{-1}.

Table 2. Composition of PAH mixture.

PAH compound	Concentration (μg ml^{-1})
Benzo[*k*]fluoranthene	13.7
Dibenzo[*a*,*h*]anthracene	4.7
Benzo[*b*]fluorathene	13.7
Ideno[1,2,3-*cd*]pyrene	10.5
Benzo[*a*]pyrene	21.2
Benzo[*a*]anthracene	14.8
Chrysene	15.9
Benzo[*g*,*h*,*i*]perylene	13.5
Acenaphthene	1.7
Acenaphthylene	4.2
Anthracene	5.3
Benzo[*e*]pyrene	16.4
Biphenyl	14.7
Dibenzothiophene	2.3
Fluoranthene	16.1
Fluorene	3.8
Naphthalene	15.0
Perylene	22.4
Phenanthrene	13.6
Pyrene	29.6
1-Methylnaphthalene	45.7
2-Methylnaphthalene	33.5

(Bellefonte, PA, USA). Retene (7-isopropyl-1-methylphenanthrene), was obtained from Sigma (St Louis, MO, USA), and described as 81.6% pure. Additional analysis showed a purity of 79.8%, with other alkylated phenanthrenes present. When a retene standard was purified to the 93% level, there was a reduction in the response of the RGS assay to levels below that of B[*a*]P. All solutions were

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prepared in HPLC-grade dichloromethane (DCM) or dimethylsulphoxide (DMSO) (Fisher, Pittsburgh, PA, USA).

Environmental samples

Sediments samples (40 g) were extracted following EPA Method 3550 by sonication in dichloromethane (DCM) and taken to a final volume of 1 ml.

Cell culture and application of test toxicants

Human hepatoma cells stably transfected with a luciferase reporter gene downstream of human CYP1A promoter sequences were grown as monolayers in an atmosphere of 5% CO_2 and 100% humidity at 37°C in Eagle's minimum essential media (Mediatech, Herndon, VA, USA), supplemented with 10% foetal bovine serum, 2% L-glutamine, 1% sodium pyruvate, and 0.4 mg ml^{-1} Geneticin (all Sigma). Cells were trypsinized upon reaching a maximum of 90% confluence and discarded after 24 passages.

In preparation for testing, cells were subcultured into 6-well plates at a density of 2.5×10^5 cells/well and grown for 72 h in the environment described above to reach a density of $\sim 10^6$ cells/well. Standard solutions and environmental sample extracts were applied at 2–10 µl to replicate wells containing 2 ml culture media. The concentrations of these extracts in the media never exceeded 0.5% (v/v). When both 6- and 16-h exposures were tested, duplicate plates were dosed, so that one plate was incubated for 6 h and the other for 16 h.

Luciferase assay

The detailed methodology used here was described elsewhere (APHA 1998, ASTM 1999). After 6- or 16-h incubation with the test solutions, the cells were washed with Hank's balanced salt solution (Mediatech), and lysed with 200 µl buffer containing 1% Triton, 25 mm Tricine, pH 7.8, 15 mM $MgSO_4$, 4 mM EDTA, and 1 mM dithiothreitol (DTT). Cell lysates were centrifuged at 6000 rpm for 10 s, and 50 µl supernatant was applied to a 96-well plate, followed by 100 µl 0.1 M potassium phosphate buffer, pH 7.8, containing 5 mM ATP and 10 mM $MgCl_2$. Reactions were initiated by injection of 100 µl luciferin, dissolved in 0.1 M potassium phosphate buffer, pH 7.8. Luminescence in relative light units (RLU) was measured using a ML2250 Luminometer (Dynatech Laboratories, Chantilly, VA, USA). Luciferase assay buffers were purchased from PharMingen (San Diego, CA, USA).

With each test run, a solvent blank (using a volume of DCM equal to the sample volume being tested) and a reference toxicant (2,3,7,8-TCDD at concentrations of 0.005 and 1 ng ml^{-1}) were also applied to replicate wells. Mean fold induction of the solvent blank was set = 1, and the fold induction of each standard solution and TCDD were determined by dividing the mean RLU produced by that solution by the mean RLU produced by the solvent blank. The per cent coefficient of variation among replicates did not exceed 20% for any sample extract or the reference toxicant.

Equivalency calculations

The dioxin/furan mixture used in this study is composed of 17 analytes (table 1). Using the concentration of each analyte and its toxic equivalency factor (TEF), a chemical toxic equivalency (Chem TEQ) was calculated for the mixture (Safe 1990). From the concentration–response curve of this mixture, the 16-h RGS fold induction response was approximately equal to the mixture Chem TEQ (pg ml^{-1}). Based on this, an RGS TEQ (termed so because it is based on the RGS response, not the chemical concentration) for environmental samples was calculated as follows:

$$\text{RGS TEQ} = (\text{fold induction}/1000) * ((V_e/V_a)/W_d),$$

where V_e is the total extract volume, V_a is the volume of extract applied to cells and W_d is the dry weight of sample. A factor of 1000 yields RGS TEQ in ng g^{-1}.

Chemical analyses of sediment samples

Analyses of dioxins and furans in all 28 sediment samples were performed by Region 4 of the US EPA using Method 8290. PAH and retene were determined in two extracts by Columbia Analytical Services (Kelso, WA, USA) following EPA Method 8270 for solid waste and soils using GC/MS in the selected ion monitoring (SIM) mode (EPA 1986).

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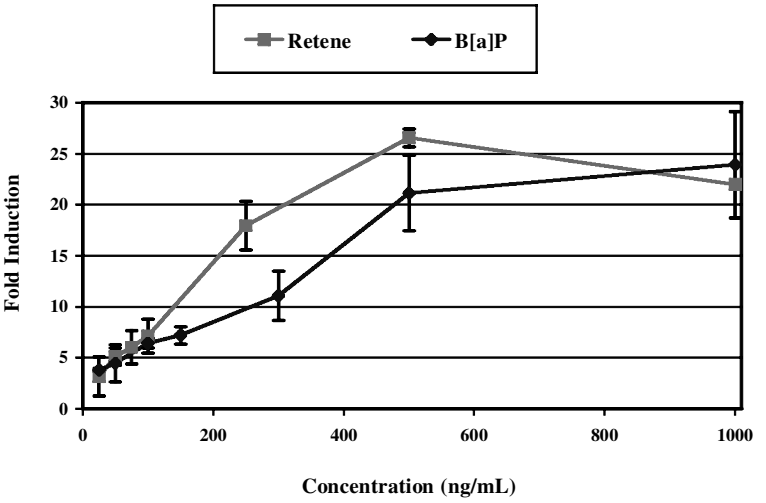

Figure 1. Induction of CYP1A by various concentrations of benzo[*a*]pyrene (B[*a*]P) and retene as measured by luciferase production in the RGS assay demonstrates similar potency. Each datum point represents the mean of at least two analyses, with bars showing SD.

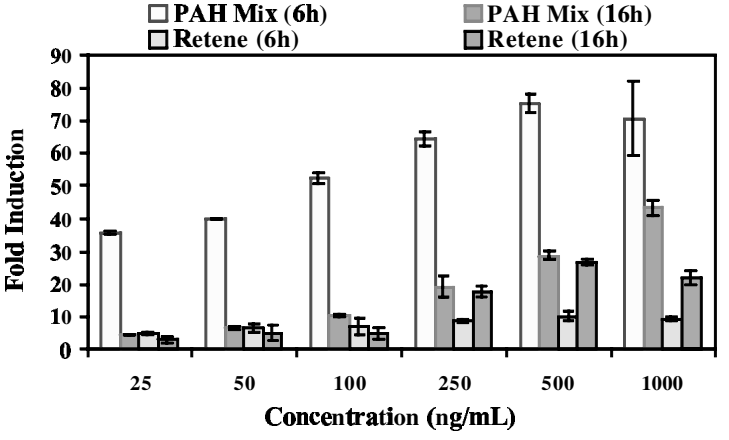

Figure 2. Induction of CYP1A measured by luciferase production in the RGS assay at 6 and 16 h following exposure to increasing concentrations of a standard PAH mixture and retene. Each column represents the mean of at least two analyses, with bars showing SD.

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Results

RGS response to retene

Concentration–response curves of retene and B[*a*]P were used to compare the potency of these two inducers (figure 1), which was quite similar. The resulting standard curves were the typical monotonic shape, where the fold induction response increased with concentration up to some maximal value, where a plateau was reached. This maximal concentration was the same for retene and B[*a*]P

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($\sim$500 ng ml^{-1}). Further analysis at two exposure times indicated that, like a mixture of PAH, retene produced higher responses at 6 than at 16 h at low concentrations ($\leq$ 100 ng ml^{-1}; figure 2). However, at concentrations $>$ 100 ng ml^{-1}, the 16-h response to retene was greater than the 6-h response, which is more typical of chlorinated compounds that are not as readily biotransformed by the RGS cell (Jones *et al.* 2000). In addition, the decrease in RGS response from 6 to 16 h at lower concentrations of retene was not as substantial as that exhibited by the PAH mixture or other individual PAH tested earlier.

RGS and chemical analyses of sediment extracts

Sixteen-hour fold induction responses were used to calculate RGS TEQ for 28 sediment extracts as described above. Regression analysis of RGS TEQ and

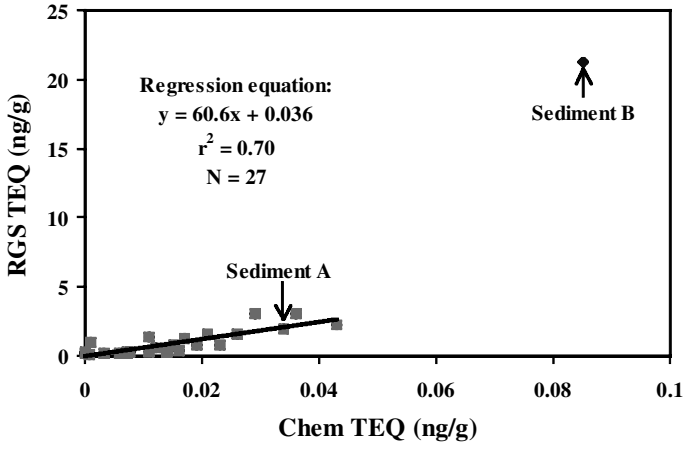

Figure 3. Regression analysis of RGS assay-derived TEQ and Chemical (Method 8290) TEQ of 27 sediment extracts. The outlier datum point (Sediment B) was removed from the regression analysis.

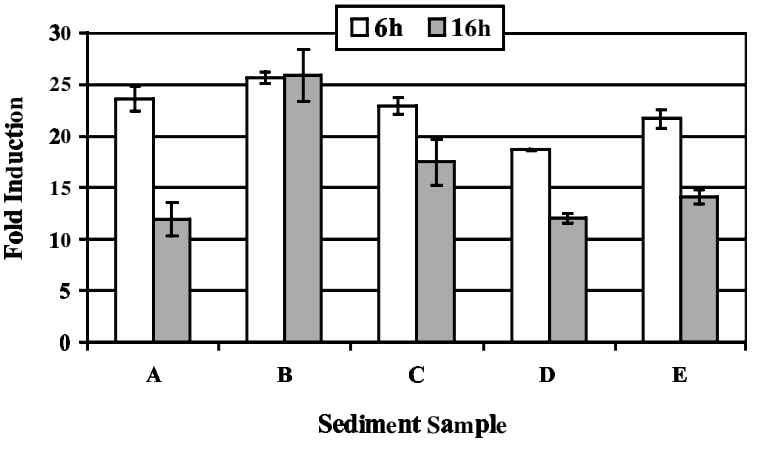

Figure 4. Induction of CYP1A measured by luciferase production in the RGS assay at 6 and 16 h following exposure to five sediment extracts collected from a river receiving input from a pulp and paper mill. Each column represents the mean of at least two analyses, with bars showing SD.

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Table 3. Concentrations ($ng\,g^{-1}$) of polycyclic aromatic hydrocarbons (PAH) in two sediment extracts determined by GC/MS SIM.

Analyte	Sediment A	Sediment B
Naphthalene	3	1
2-Methylnaphthalene	1	3
1-Methylnaphthalene	0.7	1
Biphenyl	ND	ND
2,6-Dimethylnaphthalene	1	5
Acenaphthylene	1	3
Acenaphthene	ND	ND
Dibenzofuran	1	2
2,3,5-Trimethylnaphthalene	2	7
Fluorene	1	0.7
Dibenzothiophene	1	ND
Phenanthrene	9	3
Anthracene	ND	ND
Carbazole	ND	ND
1-Methylphenanthrene	2	ND
Fluoranthene	8	3
Pyrene	18	9
Benz(*a*)anthracene	2	1
Chrysene	1	2
Benzo(*b*)fluoranthene	ND	1
Benzo(*k*)fluoranthene	ND	0.7
Benzo(e)pyrene	0.8	2
Benzo(*a*)pyrene	1	2
Perylene	ND	2
Indeno(1,2,3-*cd*)pyrene	0.7	1
Dibenz(*a*,*h*)anthracene	1	0.7
Benzo(*g*,*h*,*i*)perylene	2	1.6
Total PAHs	57.2	51.7
Retene	410	450

Total PAH is the sum of all analytes detected, excluding retene.

Analytes shown in **bold** are CYP1A-inducers by the RGS assay.

ND: less than the method detection limit for the specific analyte.

Chemical TEQ (EPA Method 8290) yielded a correlation ($r^2 = 0.70$, $n = 27$; figure 3). The highest data point (labelled Sediment B) was removed from the regression analysis as an outlier, due to its extremely high TEQ (> 3 SD above the mean), relative to the remaining 27 data points. From the slope of the regression equation, RGS TEQ were ~60 times higher than Chemical TEQ, indicating the presence of other CYP1A-inducing compounds contributing to the RGS response but not accounted for by 8290 analysis. For this reason, the five extracts producing the highest TEQ were diluted 1:10 and analysed again using RGS at both 6 and 16 h to determine the relative contribution of PAH (figure 4). Six-hour responses were > 16-h responses in four of five samples. Two extracts (labelled Sediments A and B in figure 3) were then analysed using GC/MS to confirm the presence of PAH and retene in these sediments (table 3). The concentrations of PAH, and particularly those known to induce RGS, were relatively low (~$50\,ng\,g^{-1}$) and not expected to produce significant responses. However, the concentrations of retene found in these two sample extracts (410 and $450\,ng\,g^{-1}$) were considerably higher, and very likely contributed substantially to the RGS responses. Neither the

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analyses of dioxins and furans nor the levels of retene observed explained the very high RGS response to the extract of Sediment B. It must be concluded that unidentified chemicals present in the extract contributed to the enhanced induction observed, and much more extensive analytical chemical methods would need to be employed to characterize these chemicals. In the same regard, compounds other than retene (other alkylated phenanthrenes) were recently found in the standard solution used to conduct this testing (communication from NCASI). When the per cent of impurities was reduced from ~20 to ~7% by NCASI, the response of the RGS assay decreased to approximately one-half that of B[*a*]P. This should serve as a caution to those utilizing standards that are not nearly 100% pure.

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Discussion

Relative potency of retene

The highest concentration of total dioxins and furans in the 28 sediment samples was 2.6 ng g^{-1} (Chemical TEQ = 0.085 ng g^{-1}; Sediment B), with all other samples containing <1.3 ng g^{-1} (Chemical TEQ = 0.043 ng g^{-1}) (figure 3). In contrast, retene was found at concentrations ~400 ng g^{-1}. Like B[*a*]P, retene is between three and four orders of magnitude less potent to RGS than the dioxin/furan mixture (Jones and Anderson 1999). Even so, the concentrations of retene found in these sediments (400 ng g^{-1}) equates to exposure concentrations of >100 ng ml^{-1} in the RGS assay (before dilution), which was sufficient to produce a significant response (near 5-fold induction) from the amount of extract tested. This may help to explain why the RGS-derived TEQ of the 28 sediment samples were higher than the 8290-derived TEQ.

RGS testing of retene over a wide range of concentrations indicated that, compared with a mixture of PAH containing a range of low and high molecular weight compounds, retene appeared to be less readily biotransformed by the RGS cells at higher concentrations (figure 2). This may indicate differences in the course of cellular events occurring during and after Ah receptor-ligand binding, such as interactions with cellular proteins and binding to the dioxin-responsive elements of the DNA, and/or differences in the rate of metabolism and degradation based on physico-chemical properties (Whitlock 1990).

Application of RGS in analyses of dioxins and furans in sediments

The RGS assay has been used extensively in projects conducted for the National Oceanic and Atmospheric Administration (NOAA), with a database of >700 sediment analyses from coastal areas of the USA (Anderson *et al.* 1999c). In these studies, the primary organic contaminants of concern have generally been PAH, and the RGS-derived equivalencies correlate well with subsequent chemical analyses using GC/MS.

In certain regions, especially near pulp and paper mills, there is concern for the presence of dioxins and furans, which are much more potent inducers of the RGS assay than PAH (Jones and Anderson 1999). The present study supports the use of RGS to determine the presence and relative amounts of dioxins and furans in sediments. RGS-derived TEQ were closely correlated with Chemical (8290) TEQ of dioxins and furans in extracts of 28 sediment samples collected near a Florida

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pulp mill. The fact that RGS TEQ were at least 60 times greater than the Chemical TEQ indicates the presence of other CYP1A-inducing compounds in the extracts. Indeed, further RGS analyses of dilutions of the extracts indicated the presence of PAH-type inducers, since most 6-h responses were greater than 16-h responses. Because no known sources of PAH were present near the collection site, retene was suspected, and detected at concentrations 8 times greater than the total of all other PAH using GC/MS. While Method 8290 yielded important confirmation of the actual concentrations of dioxins and furans in the sediments, the RGS analysis provided a measure of the total CYP1A-inducing content of the extract, and ultimately the discovery of retene.

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Conclusion

The results of this study support previous studies demonstrating that sediments near pulp and paper mills may contain CYP1A-inducing compounds other than dioxins and furans (Munkittrick *et al.* 1992, Burnison *et al.* 1996). Retene is one such compound that may occur at concentrations much greater than other PAH or dioxins and furans in sediments near mills. Leppanen and Oikari (1999) reported concentrations of retene as high as $1600\,\mu g\,g^{-1}$ in sediments from a lake receiving effluents form three pulp and paper mills. Further, they concluded that retene was bioavailable, as it was found in the bile of certain fish species. Similarly, Fragoso *et al.* (1998, 1999) reported rapid depuration of retene from rainbow trout (*Oncorhynchus mykiss*) in laboratory exposures, as well as metabolism in the liver.

This study utilized an *in vitro* assay, P450 Reporter Gene System (RGS), to assess the transcriptional activation of human CYP1A by retene after short (6 h) and long (16 h) exposures. The concentrations of retene found in sediments analysed in this study (table 3) present cause for concern, given that the potency of retene in inducing RGS luciferase production is similar to that of the known carcinogen, B[*a*]P. Future monitoring efforts and toxicological studies should address the presence and toxic potential of retene in the environment.

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