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Environmental Toxicology and Chemistry, Vol. 22, No. 1, pp. 59–66, 2003

Printed in the USA
0730-7268/03 $12.00 + .00

SCREENING PYRENE METABOLITES IN THE HEMOLYMPH OF DUNGENESS CRABS (*CANCER MAGISTER*) WITH SYNCHRONOUS FLUORESCENCE SPECTROMETRY: METHOD DEVELOPMENT AND APPLICATION

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(*Received* 9 *November* 2001; *Accepted* 5 *July* 2002)

Abstract—The present study examined the metabolic pathways of pyrene in dungeness crabs (*Cancer magister*) in the laboratory and the potential of using synchronous fluorescence spectrometry (SFS) to determine pyrene metabolite concentrations in the hemolymph of crabs exposed to polycyclic aromatic hydrocarbons (PAHs) in the field. Pyrene was metabolized by crabs mainly to 1-hydroxypyrene and pyrene-1-glucoside. Both pyrene metabolites could be detected by SFS in the hemolymph of crabs. A nondestructive hemolymph collection procedure was developed and used in conjunction with the SFS assay to assess the exposure of crabs to PAHs in Kitimat Arm (British Columbia, Canada). Our results showed that crabs obtained near the source of PAH contamination had the highest level of pyrene-related fluorescence in the hemolymph, whereas concentrations were lower at other sites downstream from the pollution source. In a separate study, the hepatopancreases of crabs were analyzed for parent PAHs by using gas chromatography–mass spectrometry. Pyrene-associated fluorescent responses of the hemolymph were found to correlate positively with the concentration of total PAHs in the hepatopancreas ($r = 0.39$, $p < 0.05$).

Keywords—Dungeness crab Polycyclic aromatic hydrocarbons Synchronous fluorescence spectrometry Biomonitoring

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of ubiquitous environmental pollutants. They are generated from the incomplete combustion of organic matter but are ultimately stored in the aquatic environment. Some of these PAHs are potent carcinogens for both aquatic and terrestrial animals. However, the carcinogenicity of PAHs seems to vary with the chemical composition of the mixture, the associated matrix, and the bioavailability of the PAHs to animals [1–3]. Despite the large number of studies on the environmental fate and effects of PAHs, very few biomarkers of PAH exposure have been reported for invertebrates in the marine environment.

Ariese et al. [4] first used synchronous fluorescence spectrometry (SFS) as a biomonitoring tool to screen for pyrene-1-glucuronide in fish bile. In recent years, SFS also has been used increasingly to screen for PAHs in various sample matrices including seawater [5], fish bile [4,6,7], terrestrial isopods [8], and crab hemolymph [6,9]. A spectrofluorometric assay may be conducted either by scanning both the excitation and emission monochromator of a spectrofluorometer simultaneously with a constant wavelength difference (the SFS approach) or by scanning the emission monochromator and keeping the excitation wavelength fixed (the fixed-wavelength approach) [10]. The SFS method usually results in a much simpler fluorescence spectrum than the fixed wavelength method because SFS has the ability to reduce the spectral interferences from other chemicals with similar excitation and emission wavelengths and provides a single peak for quantification [4]. In this case, a constant wavelength difference of $\lambda = 37$ nm is selective for pyrene and its metabolites, and other potentially interfering compounds or PAHs do not produce spectral bands in the resulting spectra. Therefore, by using the SFS method adapted in our laboratory, we have been successful in obtaining separate fluorescent peaks to determine pyrene and pyrene metabolites in the hemolymph of crabs without relying on chromatographic separation.

Gas chromatography–mass spectrometry (GC-MS) is a highly sensitive and selective instrument for PAH separation, detection, and analysis. However, a laborious and expensive extraction step often is required to cleanup tissue samples before GC-MS analysis. Synchronous fluorescence spectrometry has several advantages over the more traditional GC-MS method in analyzing PAH-related chemicals in the tissues of aquatic organisms. For example, our SFS assay uses the hemolymph of crabs to screen for both pyrene and pyrene metabolites. The volume of hemolymph in crabs is large and can be easily obtained with a syringe from the arthroidal membrane of a leg joint. Sample preparation is fast, requiring only a short centrifugation treatment rather than a long extraction and cleanup process. Therefore, a large number of hemolymph samples can be processed quickly. Scanning of hemolymph samples by SFS also is rapid. In our laboratory, a prepared hemolymph sample can be analyzed in only 2 to 3 min. Finally, the SFS method can be used together with a nondestructive hemolymph collection method, which allows the release of crabs back into the environment if they are not required for further study.

The SFS method also has several advantages over the high-performance liquid chromatography–fluorescence (HPLC-F) method developed by Krahn et al. [11] for monitoring PAHs in fish bile. The HPLC-F method requires an enzyme hydrolysis and extraction step to prepare the samples [4,11], whereas enzyme hydrolysis is not used in this SFS method. Moreover,

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the HPLC-F method involves the summation of chromatographic peaks at different wavelength pairs for individual PAHs. Note that the majority of the HPLC-F peaks are unidentified because they are assumed to represent a particular parent PAH or PAH metabolite based on retention time of the chromatogram. Because chemicals other than the target PAH may fluoresce at the monitoring wavelengths in the HPLC-F [11], total fluorescence is just an arbitrary number if it is not calibrated or validated with an independent standard. This can make interlaboratory comparison of HPLC-F results difficult because of dependence on sample treatment and instrument parameters. In contrast, the SFS method is selective for pyrene and its metabolites and is calibrated with 1-hydroxypyrene as an independent standard.

The objectives of this study were to develop an SFS method to screen for PAH-exposed dungeness crabs (*Cancer magister*) and to apply the SFS method in detecting pyrene and its metabolites in the hemolymph of crabs caught in Kitimat Arm, a site known to be contaminated by PAHs.

Kitimat Arm is located at the end of Douglas Channel on the north coast of British Columbia, Canada (see Fig. 1 in Eickhoff et al. [12]). This area currently is the subject of various studies to assess the impact of PAH contaminants on aquatic biota. The aluminum smelter is alleged to be the primary source of PAH input into the aquatic environment through release of contaminated effluent, surface runoff, and atmospheric transport [13]. Polycyclic aromatic hydrocarbons also may be released through loss of green coke during transport to the smelter, airborne emissions of PAHs from smelter pots, and release of PAHs into waterborne effluents. Crabs were collected at three sites in Kitimat Arm at fixed distance from the PAH source and two clean reference sites, Kildala Arm and Kitkiatka Inlet (see Fig. 1 in Eickhoff et al. [12]). The study area is thought to be unique because PAHs are the major contaminants in the local environment and their effects on biota would not be confused with those of other organic, persistent contaminants such as dioxins and polychlorinated biphenyls, which often are present in industrially impacted areas.

In this study, we adapted the SFS method [4] to monitor pyrene and its metabolites in the hemolymph of PAH-exposed crabs. To validate the method, we exposed crabs to pyrene in the laboratory. Enzymatic hydrolysis experiments were conducted and HPLC-F analysis was used to identify the pyrene metabolites in the hemolymph. The hemolymph samples also were analyzed by the SFS method and HPLC-F to quantitate the concentration of pyrene metabolites and parent pyrene, respectively, in the hemolymph. Once validated by the laboratory-generated samples, the SFS assay was applied to the analysis of field samples obtained from Kitimat Arm.

MATERIALS AND METHODS

Chemicals

Pyrene and 1-hydroxypyrene were purchased from Aldrich Chemical (Milwaukee, WI, USA). Sulfatase (arylsulfatase, aryl-sulfate sulfohydrolase, phenolsulfatase; EC 3.1.6.1) from *Aerobacter aerogenes*, β-glucosidase (EC 3.2.1.21), β-glucuronidase (type B-10 from bovine liver), Trizma® buffer, *p*-nitrophenyl sulfate potassium salt, salicin, trichloroacetic acid, sodium acetate trihydrate, potassium biphthalate sodium hydroxide buffer, and sodium citrate buffer were obtained from Sigma Chemical (St. Louis, MO, USA). All chemicals were used without prior purification. The HPLC-grade ethanol and water were obtained from Fisher Scientific (Vancouver, BC, Canada). These solvents were used to prepare 66% and 50% ethanol solutions for spectrofluorometric measurements.

Laboratory studies

Treatment of crabs in the laboratory. Three adult male dungeness crabs (mean weight 0.886 kg) were obtained locally at the Dollarton Crab Shack (North Vancouver, BC, Canada). The crabs were kept in a large refrigerated tank at 10°C in 27‰ seawater at Simon Fraser University (Burnaby, BC, Canada) and fed chopped fish for a week before experimentation. After each crab was administered a single dose of pyrene (2 mg/kg) intravascularly via the arthroidal membrane of a leg joint, they were held in separate aquaria in a temperature-controlled room at 10 ± 1°C in 27‰ seawater. Seawater was changed daily. Hemolymph samples (1 ml) were taken via the arthroidal membrane of a different leg joint from that used for dosing, with 1-ml disposable syringes at time intervals up to 100 h after dosing. The experiment was terminated at 100 h, at which time the crabs were bled by sectioning a walking leg and 15 ml of hemolymph was collected before the crabs were euthanized.

Identification of glucuronic acid– and sulfate-conjugated metabolites. Aliquots (2 ml) of the hemolymph were extracted with 8 ml of hexane to remove unchanged pyrene and unconjugated pyrene metabolites. Trichloroacetic acid (5 μl) was added to break down gel formation at the solvent interface. The hexane layer was removed. A further 4 ml of hexane was added to the remaining aqueous phase and extracted as above. The hexane layer was again removed. Two extraction steps were found to be sufficient to remove all unchanged pyrene and unconjugated pyrene metabolites. The remaining aqueous layer was blown gently with N_2 to remove any hexane that might still be remaining. Water-soluble, conjugated pyrene metabolites in the aqueous layer were hydrolyzed with each specific enzyme in 5 ml of sodium citrate buffer (pH 6.8) as follows. To hydrolyze the glucuronic acid–conjugated metabolites, a 1-ml aliquot of β-glucuronidase enzyme resuspended in HPLC-grade water was added to the sodium citrate–buffered solution [14]. This mixture was incubated at 37°C for 21 h. Sulfate-conjugated pyrene metabolites were hydrolyzed by mixing an aliquot (5 ml) of the sodium citrate buffered solution with 27.5 units of sulfatase in 1.5 ml of potassium biphthalate sodium hydroxide buffer (0.05 M, pH 5.0). The mixture was incubated for 23 h at 37°C according to methods supplied with the enzyme.

Identification of glucoside-conjugated metabolites. An aliquot (5 ml) of hemolymph was deproteinized with 66% ethanol (15 ml) in a 50-ml polypropylene centrifuge tube. The mixture was refrigerated for 20 min at 4°C and centrifuged at 9,000 rpm at 4°C for 20 min with a Sorvall RC-5B refrigerated superspeed centrifuge (Sorvall Instruments, Wilmington, DE, USA). The supernatant was decanted and dried under a Jouan RC10 centrifugal evaporator (Canberra Packard Canada, Mississauga, ON, Canada). The residues were resuspended in 3 ml of 100 mM sodium acetate buffer (pH 5.0). To hydrolyze the glucose-conjugated metabolite(s), an aliquot (3 ml) of the sodium citrate–buffered solution was mixed with 10 units of β-glucosidase in 1 ml of buffer. The mixture was incubated at 37°C for 24 h.

Extraction of incubated mixture. At the conclusion of the incubation, the enzymatic reactions were stopped with an equal volume of hexane containing benz[*a*]anthracene, the internal

standard. The reaction mixture was mixed on a mechanical shaker and the hexane layer was removed. The extraction was repeated once because two extraction steps were found to be sufficient to remove all unchanged pyrene and unconjugated pyrene metabolites. The hexane extracts were combined and evaporated with N_2. The residues were redissolved in acetonitrile (2 ml) and analyzed by HPLC.

High-performance liquid chromatography. Both conjugated and unconjugated metabolites of pyrene were qualitatively identified initially by comparing their retention time with those of the authentic chemicals with a Hewlett-Packard 1050 high-performance liquid chromatograph (Hewlett-Packard Canada, Mississauga, ON, Canada). The high-performance liquid chromatograph was equipped with a Hypersil 5 ODS 100-mm × 4.6-mm column (Phenomenex, Torrence, CA, USA), a Hypersil 5 C18 30-mm × 4.6-mm guard column, and an Hewlett-Packard 1046A fluorescence detector. The excitation and emission wavelengths of the detector were set at 230 nm and 400 nm, respectively. A gradient mobile phase was used to resolve pyrene from its metabolites. Solvent A was composed of HPLC-grade water containing 0.005% acetic acid and solvent B was 100% methanol. The initial solvent ratio was 95% A and 5% B. The proportion of solvent B was increased at a linear rate over 15 min to 100%, held for 10 min, and returned to initial solvent proportions over 5 min.

Field studies

Sampling location and date. Legal-size male dungeness crabs were caught at a depth of 15 to 40 m from Kitimat Arm and Douglas Channel with round, stainless steel, commercial crab traps (Ladner Crab Traps, Ladner, BC, Canada). The following are specific sites of sampling: Hospital Beach (53°59.60‘N, 128°41.58′W), Kitamaat Village (53°59.22′N, 128°39.25′W to 53°59.15′N, 128°39.30′W), and Wathlsto Creek (53°57.09′N, 128°39.64′W) (see Fig. 1 in Eickhoff et al. [12]). The two reference sites were Kildala Arm (53°50.52′N, 128°30.32′W) and Kitkiatka Inlet (53°38.1′N, 129°15.5′W). The crabs were collected on four separate occasions; March 1994, May 1995, May 1996, and October 1996. Because of sampling and time constraints, and inequalities in catch success, all sites were not sampled equally. Table 1 in the previous paper [12] lists the locations, dates, and catch results. Whenever possible, at least 15 crabs were collected from each site for statistical analysis.

Collection of hemolymph. The crabs were weighed (mean ± standard error, 744 ± 10 g) and the carapace width (between the tips of the 10th antennal spines) was measured (mean ± standard error, 17.6 ± 0.1 cm) before the removal of hemolymph for subsequent analysis. The crabs were bled individually by sectioning a walking leg at the segment above the dactyl tip. Hemolymph samples were collected from each crab separately in 22-ml glass scintillation vials. The crabs were dissected and hepatopancreas tissues were removed and stored individually in glass vials. All tissues were stored at −20°C for subsequent SFS or GC-MS analysis [12]. The study by Ariese et al. [4] stated that the pyrene metabolite conjugates are quite stable at −20°C. Our own studies showed that prepared samples were stable for a couple of weeks when stored at 4°C, but decomposition of the conjugates took place after approximately one month of storage.

Synchronous fluorescence spectrometry

An aliquot (1 ml) of hemolymph sample from pyrene-exposed crabs in the laboratory or from crabs caught in Kitimat Arm was deproteinated with 3 ml of prechilled ethanol (66%) in a 10-ml polypropylene centrifuge tube. The mixture was kept at 4°C for 20 min before being centrifuged at 9,000 rpm at 4°C for 20 min in a Sorvall RC-5B refrigerated superspeed centrifuge. The supernatant was decanted into a 7-ml glass vial and refrigerated at 4°C before analysis.

The sample extracts were warmed to room temperature before analysis. Each extract was vortexed thoroughly before being decanted into a 1-cm quartz cuvette and scanned on a Perkin-Elmer LS 50 Luminescence Spectrometer (Perkin-Elmer, Norwalk, CT, UK). Both monochromators were scanned synchronously at a fixed wavelength difference ($\Delta\lambda$) of 37 nm from 300 to 400 nm. Excitation and emission slit widths were both set at 5 nm. Net peak area was integrated from 330 to 351 nm by using background subtraction to quantify unchanged pyrene and pyrene metabolites in the hemolymph extract. Peak integration was performed with the FL Data Manager software (Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) on an IBM PS2 computer, model 50Z (IBM, Armonk, NY, USA). Authentic pyrene and 1-hydroxypyrene were used as fluorescence standards. A standard curve was prepared for the spectrofluorometer by using 1-hydroxypyrene standard concentrations ranging from 1.2×10^{-9} to 4.9×10^{-8} M. Fluorescence units were expressed as nanograms of 1-hydroxypyrene equivalents per milliliter of hemolymph. The detection limit was 0.02 ng of 1-hydroxypyrene equivalents per milliliter of hemolymph. The detection limit was determined as three times the baseline net peak area or baseline noise of a blank sample.

Statistical analysis

Spectrometric results from different sites or dates were compared. Differences in 1-hydroxypyrene equivalents were analyzed with SAS® (Ver 6.12 for Windows, SAS Institute, Cary, NC, USA). Both the mixed-model analysis of variance (ANOVA, Proc Mixed, SAS Institute) and the multiple comparison tests (Tukey–Kramer least squares means) were used to examine the differences in hemolymph 1-hydroxypyrene equivalents between sites or dates. Mixed-model ANOVA was used to resolve the differences in sample size and collection date of the sites. The contrast test was used to determine if a gradient in concentrations between sites was present. Correlation analysis was performed with Proc Corr and Microsoft Excel 97 SR-2 (Microsoft, Redmond, WA, USA). Data were log transformed before analysis because both 1-hydroxypyrene equivalent values and hepatopancreas PAH concentrations were found to be log normally distributed.

RESULTS

Synchronous fluorescence spectrometry analysis of the hemolymph of crabs exposed to pyrene in the laboratory revealed a number of peaks in the spectrum representing unchanged pyrene, 1-hydroxypyrene, and an unknown pyrene metabolite(s). As expected, the pyrene metabolite profiles in the hemolymph were found to vary with the postdosing time. For example, within 1 h after dosing, only one spectrographic peak represented by pyrene was found by SFS in the spectrum at 355 nm. However, at 100 h after dosing, an unknown metabolite peak that had maximum fluorescence by SFS at 344 nm was observed in the spectrum. An overlay of the unknown metabolite peak in relation to the pyrene and 1-hydroxypyrene peaks in 50% ethanol is shown (Fig. 1a). Pyrene, the unknown metabolite, and 1-hydroxypyrene had maximum fluorescence

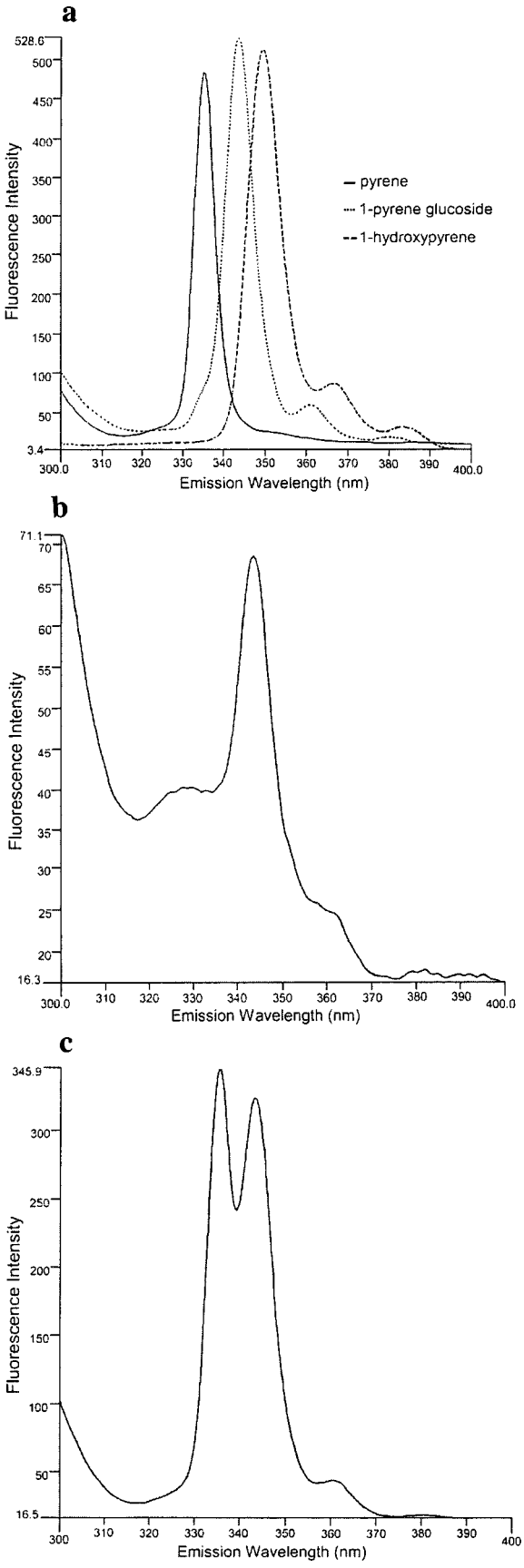

Fig. 1. Synchronous fluorescence spectrographs of crab hemolymph extracts. (**a**) Overlay showing pyrene, 1-OH pyrene, and the unknown conjugated metabolite; (**b**) field sample showing pyrene-1-glucoside peak; and (**c**) laboratory-dosed crab showing pyrene and pyrene-1-glucoside.

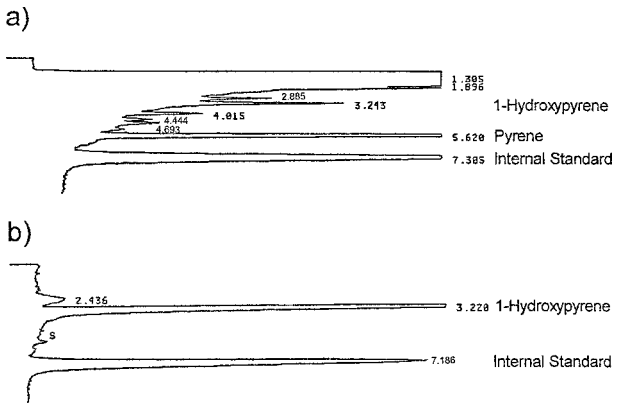

Fig. 2. Chromatograph of pyrene metabolites extracted from hemolymph (**a**) before and (**b**) after glucosidase treatment.

in the SFS spectrum at 335 nm, 344 nm, and 349 nm, respectively. We also obtained fish bile from pyrene-exposed flounder, which contained mainly pyrene-1-glucuronide [4,15] in the bile and had maximum fluorescence at 342.5 nm in the SFS assay. Based on the results of these studies, the unknown pyrene metabolite in the hemolymph of crabs was not 1-hydroxypyrene or pyrene-1-glucuronide.

The identity of the unknown metabolite in the hemolymph was characterized further with a reverse-phase high-performance liquid chromatograph equipped with a fluorescence detector. A typical HPLC chromatogram of the hemolymph extracts shows four peaks: a large polar peak representing the unknown metabolite(s), the benz[*a*]anthracene internal standard peak, and two smaller peaks coeluting with the 1-hydroxypyrene and pyrene standards (Fig. 2a). Pretreatment of hemolymph with β-glucosidase reduced or eliminated the unknown metabolite peak (retention time 1–1.9 min) with a concomitant increase in the size of the 1-hydroxypyrene peak (retention time 3.2 min; Fig. 2b). Pretreatment of the hemolymph with sulfatase produced a much smaller yield of 1-hydroxypyrene, and β-glucuronidase pretreatment had no effect on the unknown pyrene metabolite (data not shown). These results show that the dungeness crabs preferentially conjugate 1-hydroxypyrene with glucose to form the glucoside conjugate(s) and that the SFS assay is able to distinguish the carbohydrate moiety in the conjugated metabolite.

The SFS assay was used to monitor pyrene metabolites in the hemolymph of crabs exposed to pyrene in the laboratory. The uptake and elimination of pyrene measured by HPLC and the increase in metabolite concentrations measured by SFS were monitored in three crabs dosed intravascularly with pyrene at 2 mg/kg (Fig. 3). The pyrene hemolymph concentration versus time curve showed an uptake phase and an elimination phase, although pyrene was administered intravascularly. Perhaps this is due to the relatively slow flow rate of hemolymph in crabs, and the time required for pyrene to move from the site of injection to the pericardial sac of the crab, where it is then distributed to the rest of the tissues by the heart and vascular system.

The SFS assay also was used to screen for pyrene metabolites in the hemolymph of PAH-exposed crabs in Kitimat Arm. A typical scan of the hemolymph (Fig. 1b) shows the pyrene-1-glucoside peak. The concentration of pyrene metabolite in the hemolymph of these crabs generally was the highest at Hospital Beach, the site closest to the aluminum smelter, although the level of pyrene-related fluorescence was quite

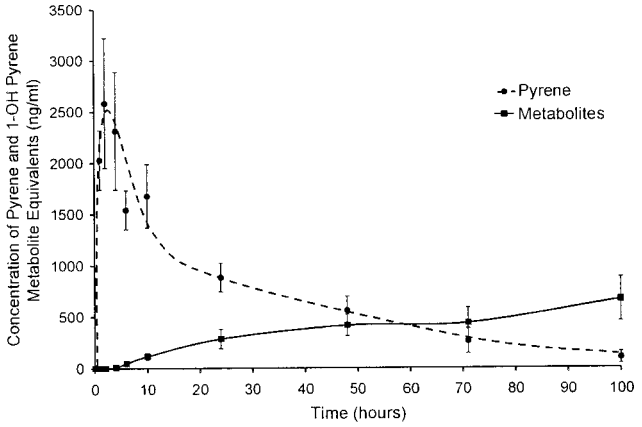

Fig. 3. Concentration of pyrene determined by high-performance liquid chromatography (ng pyrene/ml) and pyrene metabolites determined by synchronized fluorescence spectroscopy (ng 1-OH pyrene equivalents/ml) in hemolymph over time.

variable and appeared to change with time, particularly at the Kitamaat Village site (Fig. 4a). Indeed, significant differences were found in the 1-hydroxypyrene equivalent concentrations over time (ANOVA, $p < 0.05$). The mean concentration of 1-hydroxypyrene equivalents in the hemolymph of crabs at each site (Fig. 4b) showed a marked difference in concentration (ANOVA, $p < 0.05$) from a high level near the source of pollution to a lower level at various distances from the source. Multiple comparison tests with the Tukey–Kramer least squares means analysis confirmed that two significantly different ($p < 0.05$) levels of 1-hydroxypyrene equivalent concentrations occurred, denoted as A and B in Figure 4b. The error bars in Figure 4a and b represent the standard error of the mean, and show the variability in SFS measurements. Therefore, variability in 1-hydroxypyrene equivalents is the highest in Hospital Beach samples and decreases with distance from the smelter (Fig. 4b).

When the pyrene-associated fluorescent response of the hemolymph was plotted against the concentration of individual PAHs in the hepatopancreas of each crab [12], the fluorescence responses in individual crabs were found to correlate positively with the GC-MS–determined pyrene level in the hepatopancreas ($r = 0.30$, $p < 0.05$; Fig. 5a). A somewhat stronger positive correlation was found between the fluorescence response of the hemolymph in individual crabs with the total PAH (TPAH) concentration in the hepatopancreas ($r = 0.39$, $p < 0.05$; Fig. 5b). Note that TPAH is a sum of the phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and benzo[*a*]pyrene concentrations in the hepatopancreas of each crab.

DISCUSSION

Results of the present study indicate that dungeness crabs are capable of metabolizing pyrene to 1-hydroxypyrene and pyrene-1-glucoside, which can be detected semiquantitatively in the hemolymph by SFS. These results are consistent with a previous report that the American lobster (*Homarus americanus*) converts benzo[*a*]pyrene to benzo[*a*]pyrene-1-glucoside [16]. Pyrene and other PAHs, such as fluoranthene, often are found at relatively high concentrations in PAH-contaminated water or sediments [17]. Therefore, pyrene serves as a good indicator for the presence of other PAHs in the aquatic

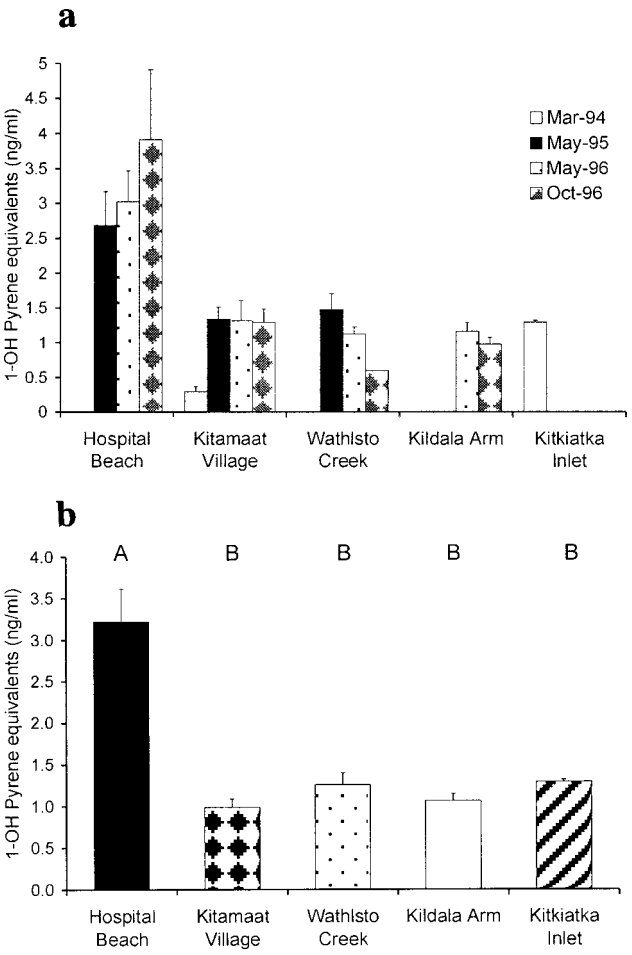

Fig. 4. Mean fluorescence values of hemolymph extracts from sampling sites in Kitimat Arm, (**a**) with respect to sampling time and (**b**) mean values including all sampling times for each site. Error bars indicate standard error of the mean.

environment and is easier to monitor than other PAH indicators such as fluoranthene because pyrene has high fluorescence and is more readily detectable by fluorescence analytical techniques.

The SFS method of Ariese et al. [4] has been used to screen for pyrene metabolites in the bile of fish. However, this SFS assay could not be adapted directly without modification to analyze the hemolymph of crabs because fish and crabs are different physiologically and in the pathways of pyrene biotransformation. Because of these differences, the following modifications were made to the SFS fish bile assay. First, the monitoring wavelengths were modified. The wavelengths used to quantify the fluorescence peak were modified to reflect the difference in the type and number of pyrene metabolite peaks produced in crabs and fish. Previous studies have shown that different carbohydrate substrates are used to conjugate 1-hydroxypyrene metabolite in fish and invertebrates. For example, English sole (*Parophrys vetulus*), flounder (*Platichthys flesus*), and starry flounder (*Platichthys stellatus*) conjugate 1-hydroxypyrene primarily with uridine diphosphate glucuronic acid [4,15,18]. In contrast, invertebrates such as the American lobster [16] and other crustaceans [19] use uridine diphosphate glucose cosubstrates to form glucoside metabolites. The current study has shown that dungeness crabs also use uridine diphosphate glucose to form pyrene-1-glucoside from 1-hydroxypyrene. Because pyrene-1-glucoside and pyrene-1-glu-

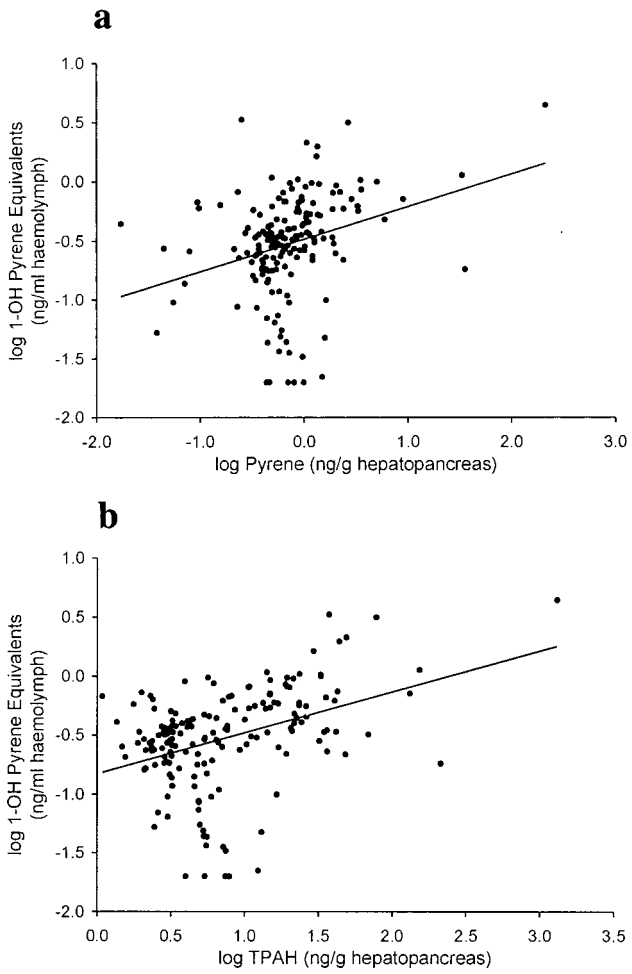

Fig. 5. (**a**) Correlation of log concentration of 1-hydroxypyrene equivalents in hemolymph and log pyrene concentrations in the hepatopancreas. (**b**) Correlation of log concentration of 1-hydroxypyrene equivalents in hemolymph and log total PAH (TPAH) concentrations in the hepatopancreas.

curonide had maximum fluorescence at different wavelengths (344 nm and 342.5 nm, respectively), the SFS assay for the hemolymph was modified accordingly. In addition, although only one SFS peak represents pyrene-1-glucuronide in the bile of flounder [4], several SFS peaks representing pyrene and pyrene metabolites may be present in the hemolymph of crabs (Fig. 1c). Because unchanged pyrene fluoresces maximally at 335 nm in the SFS assay, we scanned crab hemolymph from 330 to 351 nm instead of the reported 335 to 356 nm for fish bile [4].

Second, the calibration unit also has been modified. As mentioned before, little or no unchanged pyrene is found in the bile of pyrene-exposed fish [4,15]. Therefore, the fluorescent response of fish bile is calibrated against 1-hydroxypyrene standard and the results are expressed as pyrene-1-glucuronide units after correcting for the difference in molecular weight and fluorescence yield of 1-hydroxypyrene and pyrene-1-glucuronide. In contrast, both unchanged pyrene and pyrene metabolites are found in the hemolymph of crabs and the fluorescent peaks of pyrene and pyrene metabolites cannot be resolved completely (Fig. 1c). Therefore, the SFS assay for the hemolymph of crabs is calibrated against the 1-hydroxypyrene standard and the results are reported as 1-hydroxypyrene equivalent units.

Finally, a modification has been made to sample dilution and preparation. The flounder converts pyrene rapidly to pyrene-1-glucuronide and excretes the metabolites into the bile [15]. We have observed that crabs metabolize pyrene relatively slowly, therefore, both unchanged pyrene and pyrene metabolites can be found in the hemolymph. The volume of bile is usually less than 1 ml in fish. Therefore, pyrene metabolites can be detected readily after diluting the bile by as much as 1:500. In contrast, the volume of the hemolymph in crabs is very large; it is estimated at 35.7% of the dungeness crab's body weight [20]. Because pyrene metabolite concentrations in the hemolymph are likely to be very low, a much smaller dilution factor is used in the SFS assay (1:4 as compared to 1:500 in fish bile). Furthermore, to prepare an optically transparent solution for fluorescence spectrometry, hemolymph proteins and macromolecules are precipitated with chilled ethanol [21]. We used 66% ethanol to deproteinize the hemolymph so that the final ethanol concentration is 50%, the same percentage used in the fish bile assay [4].

In our laboratory, the SFS assay provides a simple, rapid, and effective method for monitoring both pyrene and pyrene metabolites in the hemolymph of crabs exposed to pyrene. We have also shown that this assay is capable of resolving the difference between pyrene, 1-hydroxypyrene, and conjugated pyrene metabolites such as pyrene-1-glucuronide and pyrene-1-glucoside. We have demonstrated that concentrations of pyrene metabolites can be monitored in the hemolymph of crabs exposed to pyrene. Thus, this assay can be a powerful tool to study the toxicokinetics of pyrene in crabs or other aquatic invertebrates.

Results of our field study show that SFS is an effective tool for monitoring exposure of dungeness crabs to PAHs. The pyrene-associated fluorescent response in the hemolymph of crabs is found to decrease to a lower level with distance from the aluminum smelter, the alleged source of PAH pollution in Kitimat Arm and Douglas Channel. Our results are in agreement with those of a previous SFS study on juvenile salmon in Kitimat Arm, which showed the highest pyrene-related fluorescent response in bile from fish collected close to the aluminum smelter [22]. Similarly, the concentrations of individual PAH analytes in the sediments were high near the smelter but are at lower, but similar concentrations at sites further down the Arm [10,13], as seen in the hemolymph samples. A recent study near an aluminum smelter in Norway also reported a similar pattern of PAH concentrations in sediments and indicator organisms [23]. Similar concentration profiles of individual PAH analytes have also been found in the hepatopancreas of crabs at Kitimat Arm [12]. Apparently, the concentrations of PAHs in the crabs are representative of the concentrations of PAHs in the sediments, and movement of crabs between sampling areas is unlikely. Therefore, the crab represents an effective biomonitor for PAHs in contaminated sediments.

We also observed a large variability in fluorescent responses among crabs collected from Hospital Beach, the site closest to the aluminum smelter (Fig. 4a and b). The variability in fluorescent response is consistent with the hypothesis that Kitimat Arm is contaminated by random deposition of PAH in soot, coal dust, and tar balls or the accidental spillage of pitch [24]. Therefore, organisms such as the crab experience varying levels of exposure to PAH-contaminated materials.

A high concentration of PAH analytes has been observed in the hepatopancreas tissue of crabs collected near the alu-

minum smelter [12]. The variability in the hepatopancreas PAH data seems to parallel that of the pyrene-associated fluorescent responses in the hemolymph. Correlation analysis was performed, and compared either the concentrations of pyrene or TPAHs in the hepatopancreas with the concentration of 1-OH pyrene equivalents in the hemolymph for individual crabs collected from all sites. A positive but low correlation was found between pyrene-associated fluorescence in the hemolymph and the concentration of pyrene in the hepatopancreas, whereas the correlation with TPAHs in the hepatopancreas was stronger (Fig. 5a and b). An explanation for the low correlation between the SFS and GC-MS results is not readily available but may be related to a comparison of different PAHs within the tissues of individual crabs, rather than a comparison of the tissue concentrations related to site-specific exposure levels for each group of crabs. Further, the correlation between SFS and GC-MS results may be low because of the differences in the toxicokinetics of individual PAHs in crabs and the target analytes of the analytical method. The toxicokinetics of PAHs in crabs are rather complicated and involve the interplay of PAHs with the biochemistry and physiology of crabs. Because of the high K_{OW} of PAHs and the relatively high lipid content of the hepatopancreas, PAHs are readily absorbed by crabs and accumulate in the hepatopancreas. However, because the mixed-function oxidase activities in the hepatopancreases of crabs are relatively low, pyrene is metabolized slowly to 1-hydroxypyrene and pyrene-1-glucoside. As a result, a relatively high concentration of unchanged pyrene is found in the hepatopancreas, because pyrene concentration is determined mainly by uptake, and absorption from water and food, metabolism, and elimination rates of pyrene, and also may be affected by feeding and respiration rates. Conversely, only low levels of pyrene metabolites are present in the hemolymph, and these levels are determined by the rate of pyrene metabolism in the hepatopancreas and the rate of metabolite elimination in the antennal gland, including the urinary bladder. Because the rates of absorption, metabolism, and elimination could vary with the individual PAH and with the postexposure time, it is not surprising that a relatively low correlation exists between pyrene-related fluorescence in the hemolymph and the concentration of pyrene or TPAHs in the hepatopancreas. Correlation with TPAH levels may be stronger because pyrene may be eliminated faster than other PAHs in the hepatopancreas, whereas other PAHs may remain longer. Therefore, the SFS analysis of pyrene and metabolites may be a better indicator of short-term exposure, whereas analysis of more persistent parent compounds by GC-MS may be a better monitor for long-term exposure. Finally, differences exist in target analytes and tissues used for analysis between the two analytical methods. The SFS analysis measures the combined fluorescent responses of pyrene and pyrene metabolites in the hemolymph, whereas the GC-MS method was used to determine the concentration of individual parent PAH compounds in the hepatopancreas.

However, despite the apparent weak statistical correlation between the levels of pyrene metabolites measured by SFS and pyrene and TPAHs determined by GC-MS in individual crabs, the SFS method is an effective method to monitor crabs exposed to PAHs in the marine environment. The SFS assay is equally capable of differentiating between different levels of PAH exposure within Kitimat Arm when compared with the GC-MS method, but at a lower cost in time and effort.

The field study has focused mainly on sites along the eastern side of Kitimat Arm such as Kitamaat Village and Walthsto Creek (see Fig. 1 in Eickhoff et al. [12]). These sites have been chosen based on our limited knowledge on the subpopulations of crabs in this area, the habitat of crabs, distance from the smelter, and proximity to human settlement. Further studies should be conducted with crabs on the western side of Kitimat Arm. Waterborne PAH-laden particles and atmospheric emissions tend to be deposited along the western side by prevailing currents and winds [13]. Unfortunately, our past attempts to catch crabs in this area have been hampered by rocky terrain, which is unsuitable habitat for the dungeness crab. We have subsequently identified some potential sites on the western side of Kitimat Arm for crab sampling.

The dungeness crab seems to be an excellent indicator species for monitoring PAH pollutants in Kitimat Arm or other geographical locations on the Pacific coast for the following reasons. First, crabs can be readily caught at many sites in the Arm. Second, detectable levels of pyrene and pyrene metabolites are present in the hemolymph. Finally, the crab is a benthic invertebrate with economic importance that does not move about from one area to another as quickly as most fish species. Therefore, pyrene-related fluorescent response in the hemolymph of crabs may reflect the bioavailability of PAHs at a particular site.

The SFS method may be used to monitor the exposure of crab species other than the dungeness crabs in other geographic locations. These crab species may include the snow crab (*Chionoecetes opilio*) and spider crab (*Hyas coarctatus*), which are important species in both the northern Atlantic and Pacific oceans to support fisheries in Alaska (USA), the Bering Sea, and the Gulf of St. Lawrence [25]. Similarly, the edible crab (*Cancer pagurus*) is an important fisheries species on the Atlantic coast of Europe, and the blue crab (*Callinectes sapidus* Rathbun) frequently has been used as a biomonitor on the Atlantic and Gulf coasts [26–28].

We have recently conducted a tag and release study to examine the motility of crabs in Kitimat Arm. We hypothesize that subpopulations of crabs are localized in small areas with favorable sandy or muddy bottoms surrounded by rocky areas throughout this fjord. Therefore, we have tagged and recaptured crabs to determine if the tagged crabs have moved from one site to another. At the same time, we examined PAH exposure on the western side of the Arm. Synchronous fluorescence spectrometry was used to monitor the exposure of crabs to PAHs for this purpose. Hemolymph samples were removed without endangering the health of animals, which were released and can be resampled upon recapture. This will provide a unique opportunity to assess the temporal levels of PAH exposure in individual animals.

In summary, we have presented a rapid screening method for monitoring exposure of dungeness crabs to PAHs with SFS. This rapid screening assay is capable of resolving different exposure levels between geographically distinct sites in a PAH-contaminated environment. Also, the pyrene-associated fluorescent response in the hemolymph of crabs generally parallels the concentration profile of PAHs in crab tissues determined by GC-MS. Therefore, SFS is an effective semiquantitative method for biomonitoring PAH exposure in the crab.

Acknowledgement—We would like to thank W. Cretney (The Institute of Ocean Sciences, Department of Fisheries and Oceans, Sidney, BC, Canada) and the crews of the *CFAV Endeavor* and *CCGS Vector*, who provided invaluable assistance in obtaining field samples. Thanks also to G. Harris (British Columbia Ministry of Environment and

Parks) for help with hauling crab traps, and to R. Nicholson for providing the spectrofluorometer in his laboratory for this work. This research was supported by a grant from Alcan Smelters and Chemicals and an Natural Science and Engineering Research Council strategic grant.

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