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DETERMINATION AND OCCURRENCE OF RETINOIC ACIDS AND THEIR 4-OXO METABOLITES IN LIAODONG BAY, CHINA, AND ITS ADJACENT RIVERS

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Abstract—Retinoic acids (RAs) and their metabolites play an important role in abnormal morphological development and are speculated to be a possible cause for the increased rates of deformities in wild frog populations. In the current study, a method using ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry was developed for simultaneously analyzing all-*trans*-RA (at-RA), 13-*cis*-RA (13c-RA), 9-*cis*-RA (9c-RA), and their 4-oxo metabolites, all-*trans*-4-oxo-RA (at-4-oxo-RA), 13-*cis*-4-oxo-RA (13c-4-oxo-RA), and 9-*cis*-4-oxo-RA (9c-4-oxo-RA) in wastewaters and surface waters. Method detection limits were matrix dependent, ranging from 0.02 to 0.37 ng/L. The method was used to investigate the occurrence of RAs and 4-oxo-RAs in Liaodong Bay and its adjacent rivers. Of these six retinoids, at-RA, 13c-RA, at-4-oxo-RA, and 13c-4-oxo-RA were detected in river waters at detection frequencies of 100%, 92%, 48.6%, and 21.6%, and concentrations of 0.05 to 1.23 ng/L, less than 0.03 to 0.41 ng/L, less than 0.02 to 1.00 ng/L, and less than 0.06 to 0.81 ng/L, respectively. Retinoic acids were detected for the first time in the aquatic environment and were found to be more persistent than 4-oxo-RAs. The hazard quotient for mortality of frog embryos caused by induction by retinoids detected in the current study was then estimated, and the value was calculated to be 0.09. No retinoid was detected in seawaters. Environ. Toxicol. Chem. 2010;29:2491–2497. © 2010 SETAC

Keywords—Retinoic acid 4-Oxo-retinoic acid Liquid chromatography-tandem mass spectrometry Solid-phase extraction River water

Inappropriate retinoid signaling is thought to play an important role in abnormal morphological development and thus may be responsible for the increasing incidence of frog malformations observed in North America [1]. Retinoid signaling is transduced by two classes of receptors, the retinoic acid receptors (RARs) and the retinoid X receptors, both of which belong to the nuclear receptors superfamily and act as ligand-activated transcription factors [2,3]. The natural ligands for RARs are all-*trans*-retinoic acid (at-RA) and its stereoisomers 13-*cis*-RA (13c-RA) and 9-*cis*-RA (9c-RA), whereas retinoid X receptors are activated by 9c-RA only [4]. In addition to the three RA isomers, several at-RA metabolites such as 4-oxo-RA, 4-OH-RA, 18-OH-RA, and 5,6-epoxy-RA [4,5] and some other retinoids including 4-oxo-retinaldehyde (4-oxo-RAL) [6] and 4-oxo-retinol (4-oxo-ROL) [7] also have been reported to be RAR ligands. Although retinoids are essential for the life of all chordates, an excess of retinoids causes teratogenesis in humans and animals [8]. Exposure to a high amount of at-RA can induce various patterns of deformities in different organisms, such as deformities of the brain, central nervous system, and tail in zebrafish [9,10], deformities in the lower jaw, caudal fin, and vertebrae in the larvae of Japanese flounder [11], malformations of the skeleton and of the neural tube in mice [12], as well as hindlimb and eye deformities in *Xenopus laevis* [13]. With respect to 13c-RA, a large number of studies have demonstrated that it can act as a potent teratogen as at-RA [14]. Other retinoids such as 9c-RA, at-4-oxo-RA, and 13c-4-oxo-RA are likewise teratogenic and can cause specific malformations in zebrafish [10]. Therefore, the potential occurrence of retinoids in the environment is of concern.

The natural retinoids are derivatives of all-*trans*-retinol (vitamin A), which is first metabolized into RAs via retinal, then further into a variety of oxidative metabolites [15], and finally excreted from the body by humans and animals. A previous study reported that at-RA and 13c-RA were present in human urine under normal physiological conditions [16]. Besides the endogenous origin of retinoids, at-RA and 13c-RA are also used in clinical treatment of dermatosis such as cystic acne and hyperkeratotic disorders [17] and at-RA has been proved to be effective in treatment of cancer like acute promyelocytic leukemia [18,19]. Through domestic sewage discharge these chemicals could be present in the aquatic environment and may have adverse effects on the aquatic organisms. Recently, at-4-oxo-RA and 13c-4-oxo-RA, two metabolites of RAs were identified as the main causal chemicals inducing RAR activity in sewage treatment plants (STPs) by a toxicity identification evaluation procedure [20]. However, no information on the occurrence of other retinoids such as RAs was reported in the environment because of a lack of appropriate analytical method, although environmental retinoids have received considerable attention [1,21–23].

During the last decade, high-performance liquid chromatography–mass spectrometry (HPLC-MS) has been used for the determination of retinoids in various biological samples [24,25]. Recently, tandem mass spectrometry (MS/MS) has also been applied for analyzing endogenous retinoids to achieve better selectivity, lower background levels, and higher sensitivity [26–31]. As for environmental samples, an effective enrichment method and cleanup procedure in sample preparation needs to be developed because of the trace levels of RAs and their metabolites in the environment and the complex matrix components.

All Supplemental Data may be found in the online version of this article.

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In the current study, we established a sensitive method for simultaneously analyzing three RAs, at-RA, 13c-RA, 9c-RA, and their corresponding 4-oxo metabolites, at-4-oxo-RA, 13c-4-oxo-RA, and 9c-4-oxo-RA in wastewaters and surface waters using solid-phase extraction and ultraperformance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) analysis, where a silica cartridge was used in the sample cleanup. The target retinoids were chosen because they had been detected previously [20] or potentially could be present in the environment. This method then was used to investigate the occurrence of these retinoids in water samples from Liaodong Bay (North China) and its adjacent rivers.

MATERIALS AND METHODS

Chemicals and materials

All-*trans*-RA, 13c-RA, 9c-RA and acitretin were purchased from Sigma. All-*trans*-4-oxo-RA, 13c-4-oxo-RA, 9c-4-oxo-RA, and at-RA-d_5 were obtained from Toronto Research Chemicals. Structures of the analytes and acitretin are shown in the Supplemental Data (Fig. S1). Methanol, acetonitrile, ethyl acetate, hexane, and methylene chloride were of HPLC grade and purchased from Fisher Chemical. HPLC-grade formic acid and acetic acid were provided by Dima Technology. Ultrapure water was obtained by a Milli-Q Synthesis water purification system.

Sample collection

The locations of the sampling sites are presented in Figure 1. The Xiaoling, Daling, Shuangtaizi, and Daliao rivers are the four main rivers in Liaodong Bay and ultimately flow into the Bo Sea. Thirty-seven samples of water from these four rivers and their tributaries, as well as 36 samples of coastal water from the Bo Sea, were collected from the subsurface zone (30–50 cm depth). One sample per site was collected. To explore the potential sources of retinoids in river waters, triplicate grab samples of the influent and the effluent from an STP located in the densely populated region of Panjin and a lagoon wastewater sample from one of several duck farms located in the suburban area of Panjin also were taken in this sampling campaign. All samples were collected during dry weather in May 2009, and no measurable precipitation was recorded at the sampling locations in the 24-h period preceding the sampling events. All of the samples were collected in amber bottles and were extracted within 6 h from the time of collection. Samples were vacuum filtered through a 1.2-μm glass fiber filter GF/C (Whatman) before extraction.

Sample preparation

After filtration, water samples (250 ml influent, 2 L effluent, river water, and sea water) spiked with 100 ng of at-RA-d_5 and 20 ng of acitretin were passed through the Oasis HLB cartridges (6 ml, 500 mg, Waters), previously conditioned with 6 ml ethyl acetate, 6 ml methanol, and 12 ml ultrapure water, at a flow rate of 5 to 8 ml/min. Sample vessels were rinsed with another 10 ml ultrapure water, and the rinses were passed through the solid-phase extraction cartridges. The cartridges were then dried with nitrogen gas. Analytes were eluted with 7 ml ethyl acetate containing 0.5% formic acid, which were allowed to drip through the cartridges under gravity. The extracts were evaporated to dryness under a gentle stream of nitrogen gas at room temperature.

The dry residues were redissolved in 0.2 ml methylene chloride, and then 2 ml hexane was added. The mixed solutions were applied to silica cartridges (6 ml, 500 mg, Waters), which

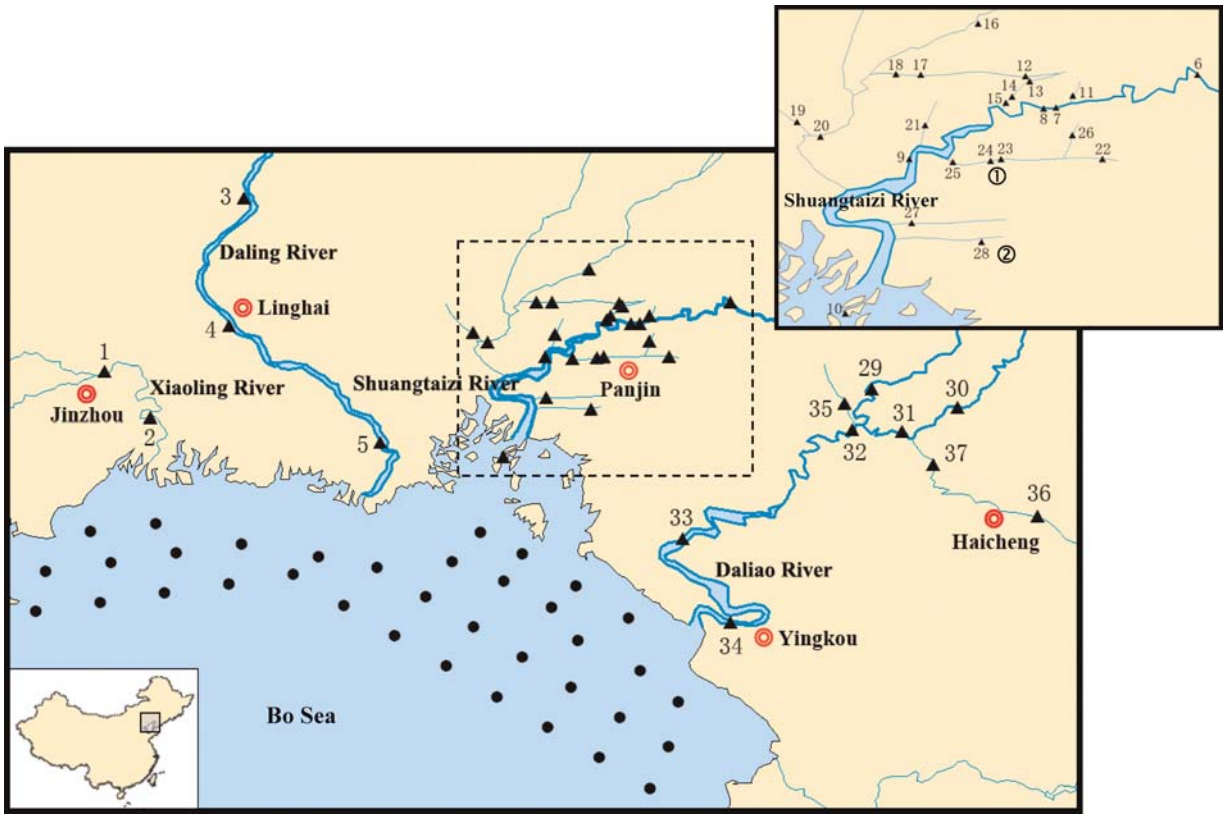

Fig. 1. Locations of sampling stations: black circles = sea water sampling sites; black triangles = river water sampling sites; 1 = the sewage treatment plant in Panjin, China; 2 = the duck farm. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

were preconditioned with 6 ml hexane. This procedure was repeated twice. After the cartridges were washed with 2 ml hexane/methylene chloride (1:1, v/v) and 2 ml methylene chloride, the analytes were eluted with 8 ml hexane/methylene chloride/isopropanol/acetic acid (87:10:1:2, v/v). The extracts were dried under a gentle nitrogen stream and reconstituted with 0.2 ml acetonitrile. To remove possible solid particles, all samples were filtered through polytetrafluoroethylene filters (13 mm, 0.2 μm, PALL) before injection to the UPLC-MS/MS system.

UPLC-MS/MS analysis

In this experiment, a Waters Acquity UPLC system consisting of binary solvent manager and sample manager was used. Chromatographic separation of compounds was performed at 40°C, using Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm). Mobile phase A was acetonitrile, and mobile phase B was 0.01% acetic acid in ultrapure water. The following gradient was used: 0 to 0.5 min, 10% A to 60% A; 0.5 to 4 min, 60% A to 62% A; 4 to 6 min, 62% A to 85% A; 6 to 8 min, 85% A; 8 to 9 min, 100% A; 9 to 11 min, re-equilibrate with 10% A. The flow rate of the mobile phase was kept constant at 0.3 ml/min, and the sample volume injected was 5 μl.

The analyses were performed using a Waters Micromass Quattro Premier XE detector equipped with an electrospray ionization source. Data acquisition was performed in the negative ion mode, and the optimized parameters were as follows: source temperature, 110°C; desolvation temperature, 350°C; capillary voltage, 2.8 kV; cone voltage, 30 V; desolvation gas flow, 600 L/h; cone gas flow, 50 L/h; and multiplier voltage, 650 V. Argon (99.999%) was used as the collision gas, and argon pressure in the collision cell was kept at $3.5e^{-3}$ mbar. Quantitative analysis was performed in the multiple reaction monitoring mode. The optimal conditions for MS/MS analysis are listed in Table 1. All data were acquired and processed using MassLynx 4.1 software.

Quantitation and quality control

All equipment was rinsed with methanol to avoid sample contamination. An operational blank was run with every batch. At-RA-d_5 and acitretin were used as surrogate standards for analyzing RAs and 4-oxo-RAs, respectively. A recovery experiment was used to assess the accuracy of the method, and the relative standard deviation was used to evaluate the precision. Confirmation of the target analytes in the environmental samples was accomplished by comparing the retention time (<2%) and the ratio (within 20%) of the selected multiple reaction monitoring (MRM) ion transition with those of standards. Because retinoids are much more stable under yellow light than under natural light conditions [32], all handling of water samples was done carefully in dark rooms under dim yellow light, and amber containers were used whenever possible to prevent photoisomerization and photodegradation [33]. Additionally, all of the solid-phase extraction and cleanup cartridges were wrapped in aluminum foil.

Yeast assay for RARα-mediated activity

The yeast two-hybrid assay described in previous paper [20,34] was applied to evaluate the RARα-mediated activity of 13c-RA, 9c-RA, and 9c-oxo-RA.

RESULTS AND DISCUSSION

Chromatographic separation

Good chromatographic separation is crucial in simultaneous determination of the three RA isomers and three 4-oxo-RA isomers, because of the possibility that these isomers are present simultaneously in an environmental sample. In the current study, two organic mobile phases (methanol and acetonitrile) and several additives in aqueous mobile phase (basic and acidic compounds) with Acquity UPLC BEH C18, C8 and Phenyl (Waters) as the stationary phase were tested to optimize chromatographic separation and ESI ionization. Baseline separation of all isomers in the same run was achieved when using Acquity UPLC BEH C18 as the separation column and acetonitrile and water containing acetic acid or formic acid as the mobile phase (Fig. 2, left panels). Basic additives such as ammonia, ammonium acetate, and ammonium bicarbonate are known to promote protonation of acidic molecules and resulting in an increase of signal in negative ESI detection. However, when using basic elution conditions, the complete separation of all isomers was sacrificed because the ionic compounds were weakly retained in reverse-phase chromatography. Acidic additives, conversely, can increase retention of RAs and 4-oxo-RAs through suppression of their ionization, which allowed for an interaction of analytes with the hydrophobic stationary phase and resulted in better retention and subsequent separation. Because acetic acid as the additive was found to provide better analyte sensitivity than formic acid, it was chosen for further experiments. To reduce the effects of acidic conditions on the signal intensities of analytes, the content of acetic acid was

Table 1. Optimized multiple reaction monitoring (MRM) conditions for the analysis of retinoic acids (RAs), 4-oxo-RAs, and surrogate standards by ultra-performance liquid chromatography-tandem mass spectrometry

	MRM					
	Quantification		Confirmation I		Confirmation II	
Analyte	(m/z)	CV[a]/CE[b]	(m/z)	CV/CE	(*m/z*)	CV/CE
All-*trans*-RA	299.2 > 118.9	34/28	299.2 > 255.2	34/16		
13-*cis*-RA	299.2 > 118.9	34/28	299.2 > 255.2	34/16		
9-*cis*-RA	299.2 > 118.9	34/28	299.2 > 255.2	34/16		
All-*trans*-4-oxo-RA	313.2 > 254.2	30/18	313.2 > 163.1	30/20	313.2 > 118.7	30/25
13-*cis*-4-oxo-RA	313.2 > 254.2	30/18	313.2 > 163.1	30/20	313.2 > 118.7	30/25
9-*cis*-4-oxo-RA	313.2 > 254.2	30/18	313.2 > 163.1	30/20	313.2 > 118.7	30/25
All-*trans*-RA-d_5	304.2 > 119.9	34/28	304.2 > 260.2	34/18		
Acitretin	325.2 > 266.2	34/18	325.2 > 251.1	34/25		

[a] CV = cone voltage (V).
[b] CE= collision energy (eV).

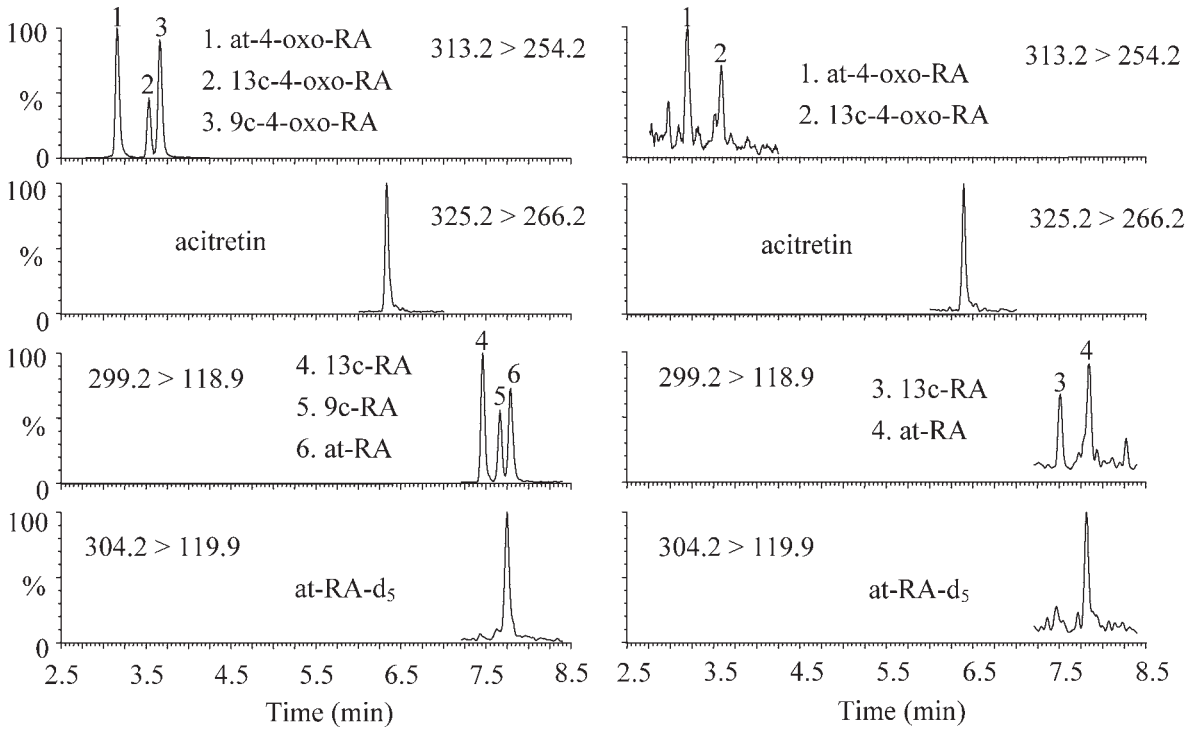

Fig. 2. Ultra-performance liquid chromatography–tandem mass spectrometry chromatograms of a standard solution (left panels) and a river water sample (right panels).

optimized to be 0.01% in aqueous mobile phase from the view of separation and sensitivity.

Quantitation and quality control

Electrospray ionization is susceptible to matrix effects, which would result in signal suppression or isobaric interference and therefore reduce the sensitivity of the assay [30,35]. To compensate for the matrix-induced signal suppression in ionization, the analyte losses during sample preparation, and variations in the instrument response from injection to injection in UPLC-ESI-MS/MS analysis, surrogate standards were used to determine the target compounds. Deuterated at-RA was used to determine RAs, and acitretin, which has been used as internal standard for the analysis of endogenous retinoids [32,33], was chosen for analyzing 4-oxo-RAs, because the isotope of 4-oxo-RA was difficult to obtain. The effects of the composition of mobile phases on the ionization of 4-oxo-RAs and acitretin in the ESI source were evaluated because a gradient elution was applied. The retention times for 4-oxo-RAs and acitretin were 3 to 4 min and 6.45 min, which corresponded to 61 to 62% and 85% acetonitrile in the mobile phase, respectively. With an increased proportion of acetonitrile from 60 to 90%, the change in the response for acitretin was 5%, indicating that the ionization conditions for acitretin approximated those for 4-oxo-RAs. In addition, acitretin showed similar recovery to 4-oxo-RAs (Table 2), and it was absent from all field sample extracts. Thus, acitretin can be reasonably used as surrogate standard for analyzing 4-oxo-RAs in the environment.

Throughout the entire determination procedure, no contamination of blanks was detected. The overall method recoveries for the target analytes and surrogate standards in various water matrices were between 51 and 81.7%, with a relative standard deviation less than 16% (Table 2). The instrument repeatability was determined by injecting the mix standards three times during the same day ($n = 3$) and different days ($n = 7$) and the instrument intra- and interday precision was generally below 10%. The method detection limit (MDL) was defined as the lowest concentration of analyte in various water matrices that could be analyzed with the described method generating a signal with an S/N ratio of 3. For those substances that were not

Table 2. Recoveries and method detection limits (MDLs) of retinoic acids (RAs) and 4-oxo-RAs in various types of aqueous matrices[a]

	Recovery % ± RSD[b]				MDL (ng/L)			
Analyte	Influent	Effluent	River water	Seawater	Influent	Effluent	River water	Seawater
All-*trans*-RA	69.1 ± 8.8	54.4 ± 8.5	54.1 ± 8.1	58 ± 14.2	0.27	0.04	0.05	0.02
13-*cis*-RA	52.6 ± 7.7	57.7 ± 1.3	53.3 ± 3.9	62.3 ± 10.8	0.37	0.05	0.03	0.03
9-*cis*-RA	56.7 ± 5.8	51.8 ± 3.0	57 ± 7.8	51.5 ± 7.9	0.35	0.05	0.06	0.04
All-*trans*-4-oxo-RA	72.0 ± 15.5	68.4 ± 0.7	56.4 ± 3.2	55.7 ± 7.2	0.17	0.04	0.02	0.02
13-*cis*-4-oxo-RA	73.9 ± 4.8	72.5 ± 2.1	55.1 ± 6.4	73.5 ± 6.3	0.15	0.09	0.06	0.06
9-*cis*-4-oxo-RA	81.7 ± 8.6	62.4 ± 2.4	60.5 ± 10.0	71.6 ± 5.8	0.11	0.04	0.10	0.05
All-*trans*-RA-d_5	53.0 ± 6.4	54.8 ± 9.5	51 ± 7.7	61.8 ± 9.7				
Acitretin	75.9 ± 6.2	66.7 ± 5.8	53 ± 5.6	58.4 ± 12.5				

[a] influent (250 ml) spiked with analytes at 30 ng/L; effluent, river water and seawater (2 L) spiked with analytes at 2 ng/L.
[b] $n = 3$.

detected in some water samples, the MDLs were determined by spiking water samples with a mixture of standard compounds at low concentration. In the current study, the MDLs of the six target analytes were 0.11 to 0.37 ng/L for the influent, 0.04 to 0.09 ng/L for the effluent, 0.02 to 0.10 ng/L for the river water, and 0.02 to 0.06 ng/L for the seawater (Table 2). These values are lower than those reported in our previous paper [20].

Occurrence of RAs and 4-oxo-RAs in rivers and sea water

This new method was applied to the analysis of RAs and 4-oxo-RAs in river and seawater samples from Liaodong Bay and its adjacent rivers. Figure 2 (right panels) shows typical MRM UPLC-MS/MS chromatograms obtained from a river water sample. In the 37 river water samples, two RAs isomers, at-RA and 13c-RA, and two of three 4-oxo-RAs isomers, at-4-oxo-RA and 13c-4-oxo-RA, were detected (Fig. 3 and Table S1). Retinoic acids were detected for the first time in the aquatic environment and were more prevalent than 4-oxo-RAs in the rivers: the detection frequencies of at-RA and 13c-RA were 100% and 92%, whereas those of at-4-oxo-RA and 13c-4-oxo-RA were 48.6 and 21.6%, respectively. The concentrations of at-RA and 13c-RA were 0.05 to 1.23 ng/L and less than 0.03 to 0.41 ng/L, and the concentrations of at-4-oxo-RA (<0.02–1.00 ng/L) and 13c-4-oxo-RA (<0.06–0.81 ng/L) in the current study were comparable to those in the Tonghui and Qing rivers in Beijing [20]. Low total retinoid concentrations (0.09–0.40 ng/L) were observed at the sites near estuaries (sites 2, 5, 10, and 34), and no retinoids were detected in any of the 36 seawater samples from Liaodong Bay. In the rivers such as C_2, C_5, and D_2, which are located in residential areas (Fig. 3), at-4-oxo-RA and 13c-4-oxo-RA were detected simultaneously with a higher detection frequency (60–100%) than in other tributaries. The highest concentration of total retinoids in all the sampling sites was found at site 15 (3.17 ng/L), downstream in the C_2 tributary.

Although retinoids were detected in some tributaries at relatively high concentrations, the concentrations in the mainstreams (A, B, C, and D in Fig. 3) were very low. The decrease in concentrations of retinoids from tributaries to mainstreams may be attributable to dilution by backflow of seawater, sorption, or degradation. In the 16 water samples collected from these four mainstreams, RAs were present as the dominant retinoids, whereas at-4-oxo-RA occurred sporadically and 13c-4-oxo-RA was not detected.

To investigate the potential sources contributing to the presence of retinoids in the environment, we collected and analyzed influent and effluent samples from an STP and a lagoon wastewater sample from a duck farm. The levels of retinoids in the influent were 2.41 ng/L for at-RA, 0.74 ng/L for 13c-RA, 17.01 ng/L for at-4-oxo-RA, 2.79 ng/L for 9c-4-oxo-RA, and 12.45 ng/L for 13c-4-oxo-RA, and the effluent concentrations were much lower (at-RA, 0.06 ng/L; 13c-RA, 0.06 ng/L; 4-oxo-RAs, <MDL) than the corresponding influent and the STP upstream (site 23) and downstream (site 24) river water samples, suggesting that untreated sewage rather than the STP effluent was the probable source of retinoids in this area. For lagoon wastewater from the duck farm, the concentrations were 0.97, 0.36, 1.64, and 1.23 ng/L for at-RA, 13c-RA, at-4-oxo-RA, and 13c-4-oxo-RA, respectively. We found that the proportion of total RAs (at-RA and 13c-RA) in untreated wastewaters only accounted for a small part of the total retinoid concentration (8.9% in STP influent and 31.6% in lagoon wastewater from the duck farm) compared with 4-oxo-RAs, which is very different from treated wastewater (STP effluent), where this proportion increased to almost 100%. This result indicated that the river water samples with relatively low proportions of total RAs, such as site 15 (45.7%), site 22 (45.8%), and site 23 (32.1%), were probably influenced by the discharge of untreated domestic wastewater. In 85% of the 37 river water samples, the proportions of total RAs in the total retinoid concentrations were greater than 80%, which was possibly attributable to the persistence of RAs in the environment.

Preliminary risk assessment

Exposure of *Xenopus laevis* embryos to at-RA causes malformations and mortality [36]. Here a preliminary estimation of the potential effects of retinoids on frog embryos was conducted by calculation of hazard quotients (HQs). As an initial estimation of the overall toxicity of retinoids detected in the current

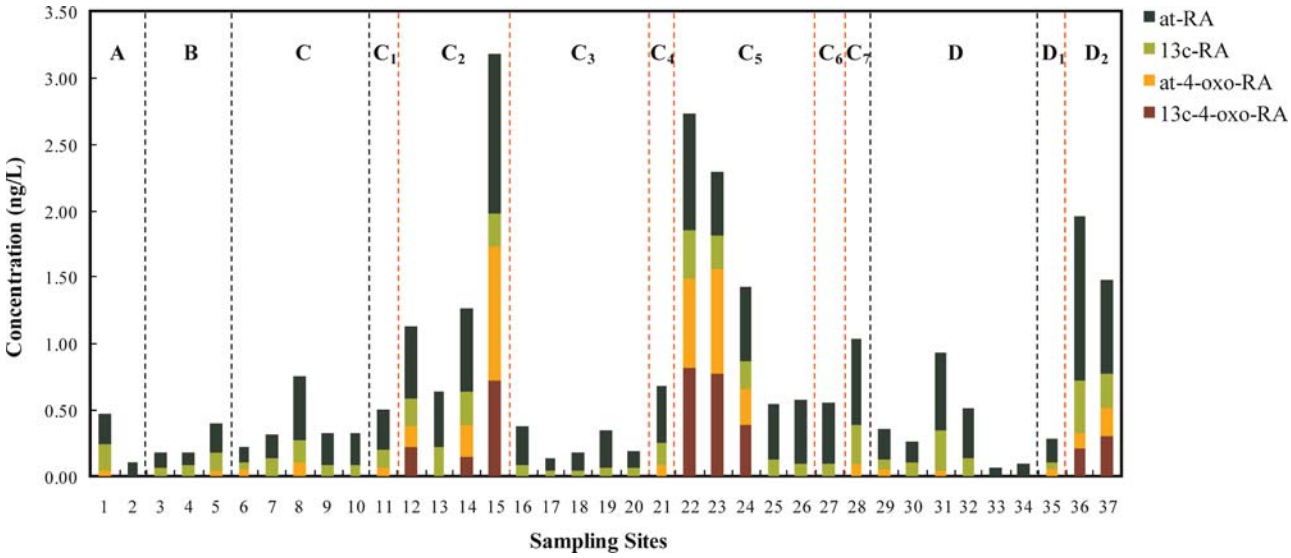

Fig. 3. Concentrations of retinoic acids (RAs) and 4-oxo-RAs in water samples from the adjacent rivers of Liaodong Bay, China. Sampling sites were shown in Fig. 1. A = Xiaoling River; B = Daling River; C = Shuangtaizi River; C_1–C_7 = tributaries of Shuangtaizi River; D = Daliao River; D_1–D_2 = tributaries of Daliao River. (Print version only: White squares = all-*trans*-RA; striped squares = 13-*cis*-RA; black squares = all-*trans*-4-oxo-RA; gray squares = 13-*cis*-4-oxo-RA.) [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

study, an at-RA equivalency factor (RAEF) was developed to normalize the concentration of each retinoid to an equivalent concentration of at-RA. The RAEF value was defined as the median effective concentration (EC50) of a retinoid, which was estimated by determining RARα-mediated activity using a two-hybrid yeast assay, relative to that of at-RA. The EC50 values for 13c-RA, 9c-RA, and 9c-4-oxo-RA were determined according to the dose–response for RARα-mediated activity (Fig. 4), and then their RAEFs were calculated to be 0.04, 0.15, and 0.46, respectively. The RAEFs for at-4-oxo-RA and 13c-4-oxo-RA were 3.87 and 0.46, respectively, as reported in our previous paper [20]. The at-RA equivalents (ATRA-EQs) of water samples from the rivers adjacent to Liaodong Bay were defined as the sum of the product of the concentration of each retinoid detected in the current study multiplied by its respective RAEF, and the calculated ATRA-EQ values were in the range of 0.05 ng/L (site 33) to 5.42 ng/L (site 15) (Table S1). To estimate the risk associated with the protection of 90% of embryos, ATRA-EQs of river water samples were log-transformed to more closely approximate the normal probability distribution (Fig. 5), and then the 90th centile ATRA-EQ concentration was determined to be 2.86 ng/L.

The benchmark dose low (BMDL) of mortality occurrence induced by at-RA was derived from the dose–response curve obtained from an experiment in which *Xenopus* embryos were chronically exposed to at-RA (from stage 8 to stage 65) [36]. The value of BMDL was calculated to be 30.9 ng/L using the U.S. Environmental Protection Agency's benchmark dose software (Ver 2.1).

Finally, a preliminary risk assessment of at-RA using the incidence of mortality as the endpoint was conducted by HQ as the equation

$$\mathrm{HQ} = 90^{\mathrm{th}}\ \text{centile ATRA-EQs concentration}/\mathrm{BMDL}$$

The HQ was calculated to be 0.09, which suggests that current concentrations of RAs and 4-oxo-RAs in the rivers adjacent to Liaodong Bay are less than the threshold for effects (HQ < 1) and are unlikely to cause harm to the survival of frog embryos. Although the current contamination level of retinoids detected in the current study does not appear to be high enough to exert high risk for frog embryos, this does not mean that adverse effects of retinoids on other organisms in the aquatic environment do not occur, and more toxicological data on other aquatic animals will be needed to undertake a more comprehensive risk assessment.

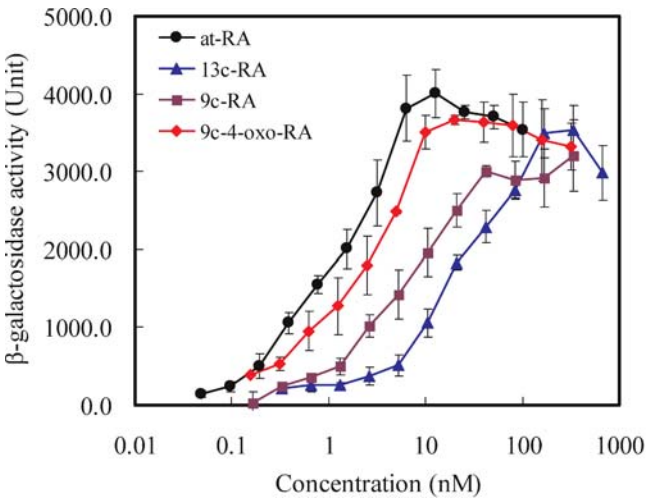

Fig. 4. Dose–response curves of all-*trans*-retinoic acid (at-RA), 13-*cis*-RA (13c-RA), 9-*cis*-RA (9c-RA), and 9-*cis*-4-oxo-RA (9c-4-oxo-RA) in RARα yeast two-hybrid assay. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

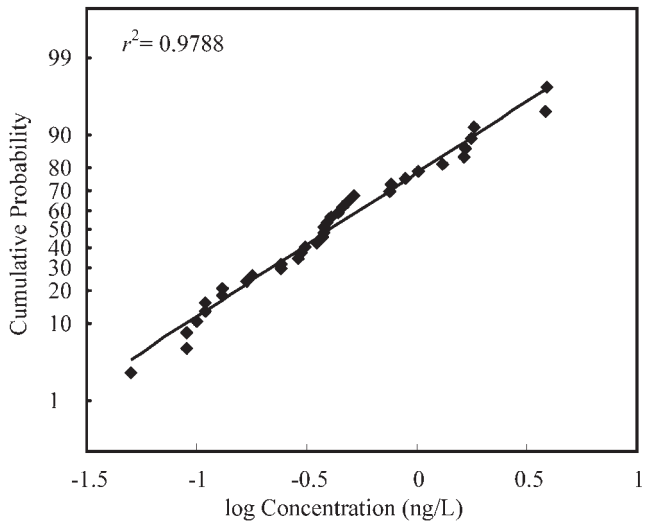

Fig. 5. Cumulative probability curve of all-*trans*-retinoic acid equivalent (ATRA-EQs) of water samples from rivers adjacent to Liaodong Bay, China.

CONCLUSION

In the current study, a sensitive method was developed for simultaneously analyzing the trace levels of RAs and their 4-oxo metabolites in various water matrices using solid-phase extraction and UPLC-MS/MS. The method was then used to investigate RAs and 4-oxo-RAs in Liaodong Bay and its adjacent rivers. Four retinoids (at-RA, 13c-RA, at-4-oxo-RA, 13c-4-oxo-RA) were detected in rivers, with the concentrations between 0.03 and 1.23 ng/L, and this is the first report of the occurrence of RAs in environmental waters. Retinoic acids were detected much more frequently than 4-oxo-RAs in rivers, indicating that RAs were relatively persistent compared with 4-oxo-RAs. The river samples with relatively low proportions of total RAs were probably influenced by untreated domestic wastewater discharge. The estimated hazard quotient for mortality of frog embryos caused by induction by retinoids detected in the current study showed that current concentrations of RAs and 4-oxo-RAs in the rivers adjacent to Liaodong Bay are unlikely to cause harm to the survival of frog embryos.

SUPPLEMENTAL DATA

Table S1. Concentrations of detected retinoic acids (RAs) and 4-oxo-RAs in water samples.

Fig. S1. Structures of the six target compounds and a surrogate standard (acitretin). (107 KB DOC)

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