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Detection, Occurrence and Fate of Indirubin in Municipal Sewage Treatment Plants

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Indirubin which has been isolated from human urine is an extremely potent AhR agonist. This paper first established an analytical method based on liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) for indirubin in complex environmental waters, and then applied this method to investigate its occurrence and fate in sewage treatment plants (STPs). For the various types of aqueous matrices considered, the absolute recoveries were from 64 to 81%, and the limits of quantification were below 0.05 ng/L. Among the seven STPs studied, the average concentrations of indirubin in influents ranged from 8.3 to 29.7 ng/L, and their aqueous-phase removal rates were 72−91%. In the receiving waters, the Tonghui and Qinghe Rivers, the concentrations of indirubin (0.65−3.7 ng/L) in some samples were much higher than those in their corresponding STP effluents, suggesting that there is random discharging of untreated sewage. The fate of indirubin was investigated in mechanical and biological sewage treatment as well as in sewage-sludge treatment at a STP consisting of anoxic, anaerobic, and aerobic tanks. The indirubin was largely removed in the anoxic tank and the secondary clarifier mainly due to the biodegradation and sorption on sludge, respectively. An increase of indirubin was observed in the aerobic tank, which was due to the cleavage of indirubin conjugates.

Introduction

A number of papers have highlighted the potentially detrimental effects on wildlife and humans of industrial compounds and byproducts of industrial and combustion processes such as polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons including polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs) (*1*, *2*). Exposure of vertebrates to dioxin and related chemicals has been shown to cause liver damage, growth retardation, certain types of cancer, birth defects, depression of immunological function, and reproductive problems in several species by binding to activating aryl hydrocarbon receptor (AhR) (*3*−*6*). These AhR agonists have been identified in almost every component of the global ecosystem including fish, wildlife, human adipose tissue, milk, and serum (*7*).

Besides these chemicals, a wide variety of naturally occurring chemicals such as tetrapyroles, indoles, and arachidonic acid metabolites have been also reported to be AhR agonists (*8, 9*). Some of these chemicals were reported to interact with AhR and produce activation or inhibition of signal transduction. Of particular concern is a tryptophan (Trp) metabolite, indirubin. This chemical was reported to activate the AhR in an AhR- aryl hydrocarbon receptor nuclear translocator (Arnt)-containing yeast cell bioassay system and to be an extremely potent AhR agonist 50-fold more potent than 2,3,7,8-tetrachlorinated dibenzo-p-dioxin (TCDD) (*10*). It has been demonstrated that indirubin can induce the microsomal drug-metabolizing enzyme activity by AhR in mammals in vivo, although the potency in vivo is relatively low compared with in vitro results possibly due to its metabolism, distribution, and solubility (*11, 12*). In addition, indirubin is the active component of Danggui Longhui Wan, a traditional Chinese medicine, used for treatment of chronic myclogenous leukemia (*13, 14*). Indirubin elicits antieukaemic activity by inhibiting cyclin-dependent kinases (CDK) (*15*−*17*), and therefore resulting in cell cycle arrest and induction of apoptosis by inhibiting Stat3 signaling in human cancer cells (*18*). Mechanistic research has shown that indirubin can inhibit DNA and protein synthesis in cell-free systems, in cell line and in rats (*19*).

Indirubin has been detected in the roots and leaves of *Isatis indigotica* (Chinese woad) named Ban-lan-Gen" and Da-Qing-Ye" which are used for the treatment of influenza, viral pneumonia, mumps, pharyngitis, and hepatitis in China (*20*) and in *I. tinctoria* (European woad) which is used as a dye and as a medicinal herb in Europe (*21*). In addition to these sources, indirubin was proposed to be formed endogenously in the human body where human cytochrome P450s catalyze the formation from indole of indoxyl and isatin (*22*). Indirubin has been isolated from acid-treated human urine with about 0.2 nM concentration excreted from the urine of Japanese volunteers (*10*). Thus, indirubin could be present in the environment, from domestic sewage discharging and from medical and dye usage, and cause risk for humans and aquatic life. At present, however, no reports about its occurrence and fate in the environment have been found. It can be expected that indirubin will be present in the environmental waters at ng/L level based on its daily excretion by humans and dilution factor; therefore, there is a need to establish a sensitive and specific analytical method to determine such low indirubin concentrations in complex aqueous matrices. Gas chromatography mass spectrometry (GC/MS) is a potential method to analyze indirubin (*23*), whereas the necessary derivatization procedure for detection is very tedious and complicated. Liquid chromatography mass or tandem mass spectrometry (LC-MS (/MS)) is an alternative method due to its sensitivity and specificity without any need for derivatization, and has been used to analyze indirubin in the roots and leaves of *Isatis indigotica* (*24*). However, the matrix interference in complex environmental samples can reduce the detection sensitivity and accuracy, and has proven to be a general problem even for the LC-MS/MS system. Thus, there is a need to develop an effective method for analyzing indirubin in various water matrices.

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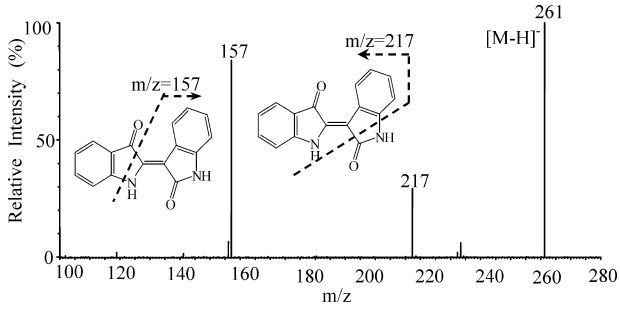

FIGURE 1. LC-MS-MS mass spectrum of indirubin standard (10 μg/L) with its deprotonated molecule ion as precursor ion.

In this study, we developed a valid sample preparation method to extract and clean up trace indirubin in sewage water and sludge, and the detection method was based on LC-ESI-MS/MS. Then, we applied the developed method to investigate the occurrence of indirubin in seven municipal sewage treatment plants (STPs) in Beijing, China, and to clarify the fate in each treatment step of a municipal STP operated with anoxic, anaerobic, and aerobic treatment units for better management of current STPs to remove indirubin. As far as we are aware, this is the first report about the occurrence and fate of indirubin in STPs.

Materials and Methods

Reagents and Materials. Estrone-d_4 was purchased as powders from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), and indirubin was a gift from Dr. Matsuda of the Department of Environmental Engineering, Kyoto University. The purity of the indirubin standard was more than 95% according to a comparison analysis with a standard (≥95% purity) purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing China) (Table S2, Supporting Information). Stock standard solutions for indirubin and estrone-d_4 were prepared at 1 g/L in methanol. Working solutions of the individual standards and of mixtures of all of them were prepared at various concentrations by appropriate dilution of the stock solutions in methanol. HPLC-grade solvents methanol, ethyl acetate, methylene chloride (DCM), hexane, and acetone were purchased from Fisher Chemical Co. (China). Ultra pure water was prepared using an Easypure UV Compact Ultrapure System (Fisher Chemical, U.S.) under a conductivity of 18.3 $\Omega\mu cm^{-1}$. Sep-Pak C18 (200 mg or 1 g, 6 mL, Waters, U.S.) and Florisil (1 g, 6 mL) solid phase extraction cartridges were purchased from Waters (U.S.). Glass fiber filters (grade GF/C, 1.2 μm pore size) were purchased from Whatman (Maidstone, UK)

Sample Collection. For investigating the occurrence of indirubin in STPs, 24 h composite samples of the influents and effluents were collected by using flow proportional samplers (cooled at 4 °C) each day during the 2-week period studied (June 26 to July 2 and July 17−23, 2006) from seven STPs, the main operating STPs in Beijing, China. These seven STPs are all operated with primary and secondary treatment processes without any post disinfections or additional filtration step. All of the plants mainly receive domestic waters, and detailed information on the STPs and sampling dates are summarized in Supporting Information Table S1. All samples were filtered and extracted within 6 h from the time of collection. For investigating the fate of indirubin in STPs, the influent and effluent of each treatment step was carried out (Supporting Information Figure S1) on March 15, 2006. The primary and secondary effluents were taken as 24 h flow-proportional composite samples (cooled at 4 °C), and the suspended solids were collected by filtering these water samples for the analysis of indirubin in the same way as the sludge samples. All other sludge-liquid and sludge samples were taken randomly between 9 and 11 a.m.

Sample Preparation. After filtration, 70 mL of influents, 200 mL of effluents, and 2 L of river water, respectively spiked with 7, 10, and 50 ng surrogate standard were extracted through an Sep-pack C18 (6 mL, 200 mg (effluent and influent) or 1 g (river water), Waters, U.S.), previously conditioned with 6 mL of dichloromethane, 6 mL of methanol, and 12 mL of distilled water at a flow rate of 5−10 mL/min. The cartridge was washed with 10 mL of distilled water, and then was dried under a flow of nitrogen. Twelve mL of dichloromethane was used to elute the analytes. For the influent and effluent samples, daily 24 h composites were extracted, and then the 7-day elutants were pooled as composite samples for a complete week. The extracts were dried under a gentle nitrogen stream. The dry residues were redissolved in 1 mL dichloromethane/hexane (1:1, v/v). The mixed solutions were applied to Sep-pack Florisil cartridges (3 mL, 1 g, Waters, U.S.), which had been preconditioned with 6 mL hexane/dichloromethane (1:1, v/v). After the cartridges were rinsed with 10 mL hexane/dichloromethane (1:1, v/v), the analytes were eluted with 6 mL acetone/dichloromethane (1:9, v/v). For the river water and effluent samples, the solution was evaporated to dryness under a gentle stream of nitrogen, and reconstituted with acetonitrile (0.5 mL) for LC-ESI-MS/MS analysis. For the influent sample, the solution was dried under a gentle nitrogen stream. The dry residues were redissolved in 1 mL of hexane/ethyl acetate (9:1, v/v), and then were applied to silica cartridges (3 mL, 1 g, Waters, U.S.), which had been preconditioned with 3 mL of water-saturated ethyl acetate. After the cartridges were rinsed with 3 mL of hexane/ ethyl acetate (9:1, v/v), the analyte was eluted with 3 mL of hexane/ethyl acetate (36:62, v/v). The solution was evaporated to dryness under a gentle stream of nitrogen, and reconstituted with acetonitrile (0.5 mL) for LC-ESI-MS/MS analysis.

Sample Preparation for Sludge. The method used for measuring indirubin in sludge was also developed in this study. Approximately 1−5 g of freeze-dried sludge mixed with about 20 g Na_2SO_4 were spiked with standard indirubin (for recovery evaluation) and estrone-d_4 and were Sohxlet extracted for 24 h with acetone. The extracts were rotoevaporated and reconstituted by 1 mL dichloromethane/hexane (1:1, v/v), and a cleanup was carried out by a Florisil cartridge as mentioned above. The extract after cleanup by Florisil cartridge in 5 mL of methanol was diluted by water to 100 mL, and then extracted through a Sep-pack C18 (6 mL, 200 mg, Waters, U.S.) as described above. The extract eluted from C18 SPE by 12 mL of dichloromethane was dried under a gentle nitrogen stream. The dry residues were redissolved in 1 mL hexane/ethyl acetate (9:1, v/v), and then were cleaned by a Silica cartridge (3 mL, 1 g, Waters, U.S.) as described above. The final extract in acetonitrile (0.5 mL) was for LC-ESI-MS/MS analysis.

LC-ESI-MS-MS Analysis. The LC apparatus was an Acquity Ultra Performance LC (Waters, Milford, MA, U.S.). Separation was accomplished with a Waters Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm particle size) (U.S.). The column was maintained at 40 °C at a flow rate of 0.3 mL/min. Solvent A was ultra pure water, and solvent B was acetonitrile. The gradient started at 20% B, was brought to 100% B in 6 min. Finally, the gradient was brought down to 35% B in 0.1 min, and this percentage was kept for 4 min until the next injection. The injection volume was 10 μL. Mass spectrometry was performed using a Quattro Ultima Pt tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Z-Spray ionization (ESI) source that was operated in negative mode. Each of MS parameters was optimized by flow infusion of indirubin. The capillary voltage, cone voltage, and multiplier voltage were

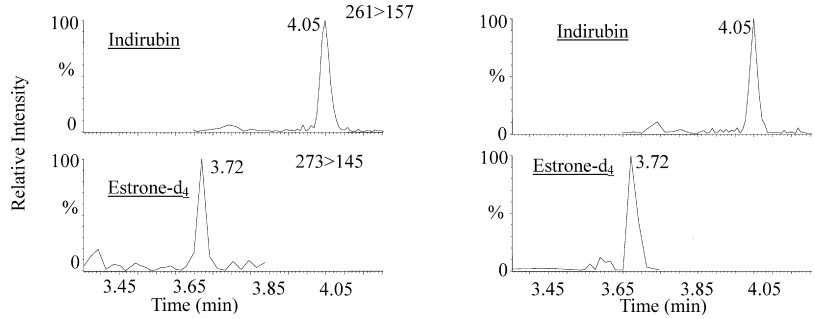

FIGURE 2. LC-MS-MS chromatograms of a composite influent (Qinghe STP, 2006/6/26−7/2) (left panels) and corresponding effluent (right panels) for indirubin as well as a surrogate standard, estrone-d_4.

TABLE 1. Concentrations (ng/L) of Indirubin Entering and Leaving Seven Beijing Sewage Treatment Plants in June and July 2006[a]

	Beixiaohe		Fangzhuang		Gaobeidian		Jiuxianqiao		Qinghe		Wujiacun		Xiaohongmen	
date	in	out	in	out	in	out	in	out	in	out	in	out	in	out
2006/6/26−7/2	16.9	3.7	10.0	1.0	9.4	0.6	12.7	1.8	11.2	1.6	8.3	2.1	10.8	1.1
2006/7/17−7/23	19.3	5.4	29.7	4.2	16.8	1.9	26.8	3.4	11.3	1.9	11.7	1.2	23.5	4.7

[a] Average of duplicate injections. LOQs is 0.05 and 0.02 ng/L for the STP influents and effluents, respectively.

TABLE 2. Concentrations (ng/L) of Indirubin in the Qing and Tonghui Rivers in June and July 2006[a]

Qinghe River date	upstream 4 km	upstream 2 km	Qinghe STP effluent	downstream 2 km	downstream 4 km
2006/6/26−7/2	0.65	0.92		3.11	2.39
2006/7/17−7/23	0.74	0.72		2.97	2.33

Tonghui River date	Upstream 2 km	upstream 0.5 km	Gaobaidian STP effluent	downstream 0.55 km	downstream 2.5km
2006/6/26−7/2	1.74	1.53		1.59	2.74
2006/7/17−7/23	1.46	1.90		3.06	3.72

[a] Average of duplicate injections. LOQ is 0.01 ng/L.

TABLE 3. Concentrations (ng/L) and Mass Flux of Indirubin in a STP on March 15, 2006

	primary influent	primary effluent	anoxic	anaerobic	aerobic	secondary effluent	primary sludge	return sludge	excess sludge	digester
water flow (m^3/d)	1.7×10^5	+600	3.06×10^5	3.06×10^5	3.06×10^5	1.7×10^5	2000	1.36×10^5	6000	4000
TSS (g/m^3)	833	142	2167	2167	2167	23		3200		
mass flux of TSS (kg/d)	1.416×10^4	2.19×10^4	7.8×10^5	7.8×10^5	7.8×10^5	3.91×10^5	1.20×10^{5a}	4.35×10^5	2.17×10^{4b}	1.41×10^{5c}
concentration of dissolved indirubin (ng/L)	17.9	8.2	1.5	0.86	1.5	0.19	218.6	3.4	3.4	141.6
concentration of sorbed indirubin (ng/g TSS)	19.3	46.9	8.9	6.6	9.3	15.7	19.5	15.7	15.7	13.9
mass flux of indirubin (g/d)										
dissolved	3.0	1.4	0.47	0.26	0.45	0.03	0.44	0.47	0.01	0.57
adsorbed	2.7	1.0	7.0	5.1	7.3	0.06	2.3	6.8	0.34	2.0
total	5.7	2.4	7.4	5.4	7.7	0.09	2.7	7.3	0.35	2.5

[a] Subtracting TSS in the influent from that in the effluent of the primary tank. [b] 5% of TSS in the return sludge. [c] Sum of TSS in primary sludge and that in excess sludge.

set at 3.0 kV, 45 V, and 650 V, respectively. The nebulizing, desolvation, and cone gases were supplied with ultra high purity nitrogen. The flow of desolvation gas and cone gas were set to 600 and 0 L/h, respectively. The source temperature and desolvation gas temperatures were held at 100 and 350 °C, respectively. The radio frequency (RF) lens 1 and RF lens 2 were set as 27 and 0 V, respectively. The collision gradient was 2.0 eV. Quantitative analysis of indirubin was performed using LC-ESI-MS/MS in multiselected reaction monitoring (MRM).

Quantitation and Quality Control. All equipment rinses were done with methanol to avoid sample contamination, and one laboratory blank was analyzed every day to assess the potential sample contamination. One field blank made from ultra pure water was also incorporated in the analytical procedure for every day. In this study, the concentrations of

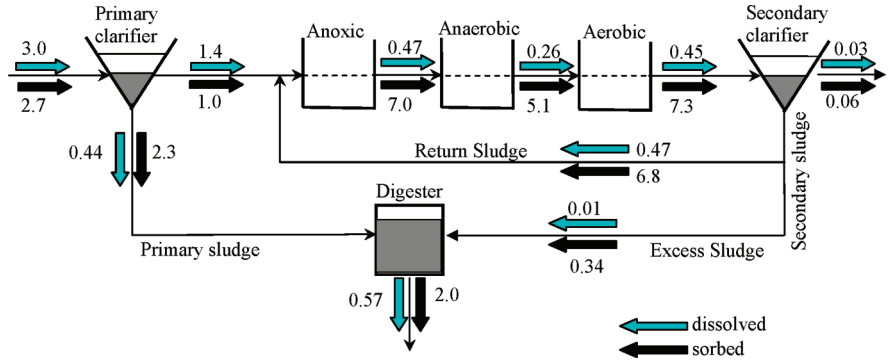

FIGURE 3. Mass flux (g/d) of indirubin.

all field blank and laboratory blank samples are lower than detection limit. Duplicate analyses were carried out for each sample. Recovery experiments were done by spiking standard solutions to an influent, an effluent, a sludge sample from Gaobeidian STP, and a river water sample from the Tonghui River. Analyte addition was made with the criterion of at least three times the original concentration, which was determined prior to the fortification experiment. The overall recovery was used to assess the accuracy of the method and matrix effects, and the RSD was used to evaluate the precision.

Results and Discussion

Optimizing LC-MS/MS Conditions. In order to obtain the best instrumental conditions for unequivocal identification of the very low amounts of indirubin expected in aqueous environmental samples, all instrumental parameters were optimized by infusing 500 μg/L of standard solution at flow rate of 10 μL/min in negative mode. Because the deprotonated molecule $[M-H]^-$ proved to be the most abundant ion, the $[M-H]^-$ ion of indirubin was selected as the precursor ion for collision induced dissociation (CID) fragmentation. With the deprotonated molecule $[M-H]^-$ as precursor ion, the product ions, CH_2NO (m/z 157, a base ion) and C_7H_5O (m/z 217), were observed as the most abundant in the collision-induced dissociation (CID) spectrum of indirubin (Figure 1). Based on the precursor ion and product ions, the MS/MS parameters such as capillary voltage, cone voltage, flow of desolvation gas, cone gas, source temperature, desolvation gas temperature, collision gas pressure, and collision energy were optimized as described in the experimental section. Therefore, the $[M-H]^-$ to $[M-H-CH_2NO]^-$ (m/z 157) transition was selected for quantitation, and the ratio of the quantitation transition and identification transition ($[M-H]^-$ to $[M-C_7H_5O]^-$ (m/z 217) was used for confirmation in the environmental samples.

Quantitation and Quality Control. Throughout the whole determination procedure, no contamination of blanks was detected. To automatically correct the losses of analytes during extraction or sample preparation, and to compensate for variations in instrument response from injection to injection, surrogate standard was used in this study. Deuterated indirubin would be preferable to monitor the analyte in the environment. However, we could not obtain it commercially. In this study, estrone-d_4 was used as the surrogate standard. Since a gradient elution was applied, we evaluated the ionization conditions for indirubin and estrone-d_4. Under the HPLC conditions used in this study, indirubin and estrone-d_4 were eluted at similar retention times (3.72−4.04 min) which corresponds to the range of ca. 70−74% acetonitrile in mobile phase. While the increasing proportion of acetonitrile produces an increasing response, the response for estrone-d_4 at 70% acetonitrile in mobile phase is appropriately approximate to that for estrone-d_4 eluting at ca. 74% acetonitrile in the gradient elution used in this study (the response difference is below 10%). In the recovery experiments, the overall average recoveries of indirubin in STP sludge, influent, effluent, and river water were 65 ± 5.0%, 64 ± 2.6%, 80 ± 1.8%, and 81 ± 1.2%, respectively; those of the surrogate were 82 ± 4.0%, 83 ± 1.0%, 102 ± 1.2%, and 100 ± 0.2%, respectively. Since estrone-d_4 can be recovered from the sample matrix with similar efficiency with those of indirubin in various water matrices, estrone-d_4 can perform a quality control function for analyzing indirubin. Supporting Information Figures S2 and S3 show the cleanup effectiveness for an effluent sample and a sludge sample. It was found that the signal/noise (S/N) ratios for the indirubin and estrone-d_4 were largely improved by the cleanup procedures.

The confirmation ratios for samples were all within 20% of that of the standards, indicating good purification performance of the proposed method. Since indirubin was detected in all sample matrices considered, the estimation of the method detection limit (MDL) was based on the peak-to-peak noise of the baseline near the analyte peak obtained by analyzing field samples and on a minimal value of signal-to-noise of 9. Thus, MDLs for sludge, influent, effluent, and river water were 0.01 ng/g, 0.05, 0.02, and 0.01 ng/L, respectively.

Occurrence of Indirubin in STPs and the Receiving Rivers. Indirubin was analyzed in the influents and effluents collected from seven STPs in 2006. Figure 2 shows the typical MRM LC-MS/MS chromatograms obtained from a composite influent and corresponding effluent sample, and the analytical results are listed in Table 1. Over a period of two weeks, the average influent concentration of indirubin among the seven STPs were in the range of 8.3 ng/L (Wujiachun, 2006/6/26−7/2) to 29.7 ng/L (Fangzhuang, 2006/7/17−7/23), and the effluent concentration were in the range from 0.6 ng/L (Gaobaidian, 2006/6/26−7/2) to 5.4 ng/L (Beixiaohe, 2006/7/17−7/23). The percent aqueous-phase removal rates of indirubin were calculated by comparing the concentrations in the influents and effluents from each plant. It was found that indirubin experienced relatively high and stable aqueous-phase removal rate ranging from 72 to 91% among the seven STPs. Of seven STPs, the aqueous-phase removal rate of indirubin in Beixiaohe STP was lowest, similar with those of natural and synthetic glucocorticoids in the same STP (*25*). This was possibly due to its treatment process only operated with anaerobic and aerobic units (AO), whereas other STPs use an anoxic, anaerobic, and aerobic process (A^2O).

To study the occurrence of indirubin in environmental waters due to the discharge from STPs, in June and July 2006, we analyzed samples taken from the Tonghui and Qinghe Rivers, which receive the effluent from Gaobeidian and Qinghe STPs, respectively. As shown in Table 2, indirubin was frequently detected in the river water samples with the concentrations ranging from 0.65 to 3.1 ng/L in the Qinghe River and from 1.5 to 3.7 ng/L in the Tonghui River. It should

be noted that the concentrations in the downstream-river samples were higher than those in the corresponding STP effluent samples. This was also found in our previous study on the occurrence of natural and synthetic glucocorticoids at the same sampling (*25*), and has been explained by the random discharging of untreated wastewaters into these rivers.

The AhR ligand activity of indirubin (EC_{50}) was determined to be 0.12 nM (31.4 ng/L) using human AhR and ARNT genes coexpressed in yeast, which was about 50 times higher than that of TCDD (*12*). Although Ah receptor-mediated induction of microsomal drug-metabolizing enzyme (hepatic cytochrome P450 1A1/2) activity by indirubin was relatively low compared with TCDD (*11*, *12*), the potential risk due to long-term exposure should be paid attention to.

Mass Flux Analyses of Indirubin in a STP. To clarify its fate in the STP, the mass balance of indirubin in a STP system exemplified by the Gaobeidian STP (Supporting Information, Table S1) was calculated.

In the wastewater stream, the measured indirubin concentrations of each treatment step at the Gaobaidian STP are shown in Table 3. While the indirubin concentration was reduced by 82% in the first anoxic tank, the practical aqueous-phase removal rate in this tank was estimated to be approximately 75% considering the dilution by return sludge which had a lower concentration (3.5 ng/L) than that (8.2 ng/L) of the influent of anoxic treatment. In the following anaerobic tank, the indirubin concentration in aqueous phase was further reduced by 42%, from 1.5 to 0.86 ng/L. The concentration variations of indirubin sorbed on activated sludge in the anoxic tank and the following anaerobic tank (Table 3) were slight considering the potential variations between the two days and the analytical RSD, indicating the occurrence of biodegradation. However it should be noted that both the concentration and the load of indirubin in aerobic treatment increased 72 and 43%, respectively. Although there are no previous reports providing any information about the concentrations of indirubin conjugates in urine or serum, a glucuronidase treatment experiment has suggested that a considerable portion of indirubin in urine exists as glucuronides (*10*). So, the increase of indirubin in aerobic treatment suggested that conjugates such as glucuronides of indirubin not measured in the influent of the aerobic tank would be cleaved as exemplified by the deconjugation occurrence of steroid glucuronidates and sulfates in the STP (*26*). To support this hypothesis, we made an attempt to deconjugate two water samples and two sludge samples which were collected from the anaerobic and aerobic tanks as described in the Enzyme Treatment section of the Supporting Information. The contributions of indirubin conjugates in water and sludge samples to total indirubin loads in aerobic tank were 80.6 and 9%, respectively which were lower than those in anaerobic tank (85.1% in the water sample and 49.5% in the sludge sample), indicating the deconjugation occurred in the aerobic treatment unit. The above fate of indirubin in the A^2O process is obviously different from that of estrogens in a similar STP process. In the case of estrogens, the elimination of estrogens occurred in the aerobic and/or denitrifying (anoxic) reactors (*27*). In the secondary clarifier, the concentration of indirubin was reduced from 1.5 to 0.19 ng/L with 87% aqueous-phase removal rate and its loads of the input (7.7 g/d) and the output (7.7 g/d) in this tank were same, indicating that the aqueous-phase removal was due to the absorption of indirubin on sludge.

In the sludge treatment, a clear increase of indirubin concentration in the water was observed, while the concentration in sludge phases is similar, according to a comparison of the excess and digested sludge. The dissolved and sorbed indirubin in the excess sludge leaving the secondary clarifier was found at 3.5 ng/L and 15.7 ng/g, respectively, and those in the digester liquid from dewatering of digested sludge and sorbed on the sludge were 141.6 ng/L and 13.9 ng/g, respectively. The detected inlet load of the digester (3.8 ng/g) is higher than that of the outlet load (3.0 ng/g) with 17% indirubin reduction possibly by the degradation under the methanogenic conditions in the digester or another reason that the sample taken from the digester do not correspond directly to the analyzed inlet sludge since the retention time of the sludge in the digester is about 20 d. Overall, the sorbed load in the digested sludge was about 43% of the inlet load of the STP (Figure 3 and Table 3) which is largely higher than those of estrogens in a similar STP, indicating indirubin absorbed to sludge.

Thus, we developed a method to analyze trace indirubin in environmental samples, and clarified that indirubin was ubiquitous in STP influents, effluents and river waters, and can be effectively biodegraded in anaerobic and anoxic treatment units. This first report on the occurrence and fate of indirubin in STPs will be useful for its risk assessment in the future. In this study, we found that the deconjugation of indirubin occurred during sewage treatment, and there is a need to further investigate the occurrence and behavior of conjugates.

Acknowledgments

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Supporting Information Available

Additional tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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