

に暴露するような試験になっています。あとで紹介しますが、たとえば妊娠後期だけなど、ある特定の時期に限って暴露すると、その影響が思春期など成育してから後の時期に現れる可能性があります。逆にいうと、暴露をし続けると検出できなくなる可能性があるということも、生物学的に言えば考えられます。

もう一つ、毒性学的に大事なのは影響の非可逆性、その影響が可逆的なのか非可逆的なのか、生体の調節機能を考慮した結果の解釈ですね。それから影響のエンドポイントについては前立腺の重量が増えるなどが指摘されていますが、これまでは指摘されてなかったエンドポイントについても注目する必要があるかもしれないということです。エンドポイントの選び方と暴露時期の選び方、メカニズムでは細胞のシグナル伝達による調節機構のかく乱、こういったことについて注意して標準的な試験法を見直す必要があるのではないかと考えられました。

また野生生物との関係では、野生生物とヒトでは共通する部分と違う部分があります。このへんについても十分検討して、野生生物で見られた結果をヒトに適用していくことができるのか、できないのかを考えることが非常に大事です。

さらに天然物による寄与の考察で、当然ながら天然にヒトや畜産動物が持っているホルモン物質との関係です。すでに存在するこれら物質による影響を超えて、どんな影響があるのかということがありますし、また天然にはヒトや動物が体の中に持っているホルモン以外に、植物中にホルモン様活性を示す物質があり、これがどれだけの問題を提供するだろうかということの検討が必要です。

野生生物への影響という点では、ヒトを含む動物が排泄する天然のホルモン物質や畜産用に用いられたホルモン物質の尿中排泄物が、意味のある濃度で環境に検出されている事実があります。また従来注意されなかった医薬品や健康関連製品中の生理活性物質が一部環境中に放出され、特に病院の廃水や、下水処理場放流水、一般の河川にも検出されるという報告もあります。

最後に本学会に関連して重要なもう一つは、リスクの問題として不確実性の検討ということです。影響の違いといった場合に、どの段階に不確実性が最も大きいのか、例えばヒトの個体間の影響の違いでは、遺伝的な背景によるものなのか、あるいは地域的な違い、また環境影響への感受性を左右

する年齢、生活、摂食パターンの違いがどうなのか、これらがどれだけそのリスクに影響を与えているのか与えてないのか、その幅はどうか、こういったことの検討が要求されます。

私どもの研究のご紹介になりますが、まず低用量影響の再現性があまり見られないという問題について検討いたしました。たとえば、個々の論文では、必ずしも試験条件について詳しく記述していない場合が多く見られます。学術論文としてはそのまま掲載される場合も多くあります。しかし、たとえば飼育条件の微妙な違いや、餌中の植物エストロゲン物質の混入の可能性などにより、また投与群を何群選んだかというようなことで、安全性評価という観点から見ると不十分な論文が数多くあります。特にエストロゲン物質で、植物中のホルモン活性物質については注意されるようになりましたが、ある試験研究機関で注意深く計画された低用量のビスフェノールAの試験が行われました。その際にきわめて低用量の投与群を設定したところ、試験物質を投与しない餌や飲料水中に設定されたと同じくらいの濃度のビスフェノールAが検出され、試験が予定された低濃度での影響について適切な解析ができなくなったということとを正直に報告された例もございました。結果の解析手法についても、動物試験では同じ母親から生まれた子どもを利用しますが、微妙な違いについては、別な母親から生まれた子どもを同じ母親から生まれた子どもと分けて、母親の違いによる影響の寄与を分析する統計解析の方法を検討することが必要になります。

前立腺重量など臓器重量の増減が、報告されていますが、体重比による補正を行うとほとんど違いが見られなくなる、いう場合もありました。私

表1 データ集のフォーマット

文献番号：
著者名：
論文題名：
出典：
チェック項目
1. 対象生物、2. 影響の標的臓器、
3. 影響の種類、4. 曝露方法、
5. 曝露時期、6. 曝露濃度(用量段階)、
7. 観察された影響の種類と濃度
8. 観察時期、9. 論文中に低用量影響への関心、
10. 試験の信頼性 (GLP基準準拠など)
論文の概要：(200~400字)
添付資料： 文献の内容を理解する上で重要な図表
評価者のコメント： 統計的な信頼性など報告の
信頼性についての記述

表2 ヒトとマウス・ラットにおける母親と胎児血中のエストラジオール濃度 (Witorschら,2002)

母親
妊娠後期の血中濃度はヒト(15-20 ng/mL)
ラット・マウス妊娠後期 (30-60 pg/mL)の数百倍
胎児
胎児血中濃度はヒト (5-10 ng/mL)、
マウス (100-150 pg/mL) の50-100倍

私たちはビスフェノールAについて、内分泌かく乱の可能性が指摘された論文が多くあることから、この物質について集中的に論文を深く検討しました。NTPが行った毒性試験で、ラットに体重1キログラムあたり50ミリigramの投与で体重減少が見られました。この値を不確実性係数1000で割って、体重1キログラムあたり50マイクログラムの参照用量が設定され、これが安全基準の目安と看做されてきました。

さてこのビスフェノールAについて、低用量を報告した文献がどのくらいあるかを調べました。先ほどのアメリカNTPの低用量影響ワークショップでは、未公表のデータを含めて2000年までの低用量報告がレビューされました。したがって、私たち厚生労働科学研究班(井上達代表)では、2000年以降に発表された文献を収集して一つ一つについて内容を検討しました。すなわちビスフェノールAについて文献検索で得られた文献の中からヒトの健康への影響ということ、水生生物への影響や分析の文献を省いて168件を得ました。さらに昨年、2005年に追加的に調査いたしました。レビューの結果を表1のフォーマットにまとめたデータ集を作りました(関澤;2005a;2006a)。

その中からいくつかを紹介します。内分泌かく乱のうちエストロゲン活性による影響については、マウスやラットの胎児では母親由来のエストラジオールへの曝露濃度がヒトに比べ、ずっと低いレベルにあります。ヒトの場合の数百分の1というレベルであり、このような場合にいわゆる環境ホルモン、すなわち外から比較的微量のエストロゲン物質を与えると敏感に反応する可能性があると考えられます(表2)。マウスで微量の環境ホルモン物質を与えたときに影響が見られたとしても、ヒトの場合ほとんど胎児がずっと高い濃度のエストラジオールにさらされているため、少々程度の外界からのエストロゲン物質の混入による影響は見られなくなる可能性が高いといえ、種差があると考えられます。いわゆる女性ホルモン作用に

ついては、実験結果の再現性があまり見られなかったのですが、ラットやマウスでようやく検出されるような影響は、必ずしもヒトでは影響が起らない可能性が大きいのではないかと推測されました。

他方、よく引用されるアメリカと日本で行った広範囲の投与レベルでの3世代試験、2世代の長期繁殖試験でも、統計的に有意な影響が見られなかったということについては、先ほど申しましたようにある特定の時期に曝露するということが、ずっと曝露し続けるということの結果が異なる場合があります。専門家にはよく知られたことですが、受容体タンパクの濃度そのものが体の中で変動していて、活性物質に長期に曝露させると反応を示す受容体タンパクの濃度が、低くなるダウンレギュレーションという現象が見られます。この場合には、外部からいくらエストロゲン物質が来ても反応が見られなくなるという可能性があります。ずっと曝露し続けるのではなく、妊娠の後期など、ある特定の時期のみに曝露することにより、多世代の曝露試験で見られなかったような影響が見られる可能性があります。

私どもが目にしたのは、これまでは女性ホルモン作用や男性ホルモン作用、あるいはせいぜい甲状腺ホルモンへの作用に着目した試験が行われてきましたが、最近になってこれまで検討されなかったエンドポイントである神経行動毒性の影響が比較的少量の体重1キログラムあたり10マイクログラムという曝露により見られた報告が出つつある点です。このような濃度で脳内ドーパミン系の異常とか、行動における雌雄の性差の解消や変動が見られるという報告が増えています。

ここであえて指摘しておいたほうが良いと思いますことに、ビスフェノールAについては昨年国内で詳細なリスク評価書というものが独立行政法人の産業技術総合研究所の化学物質リスク管理研究センターから出されました(NEDO/産総研,2005)。私たち自身のデータベース作成にはこの詳細リスク評価書は利用していませんが、この図書は出版物として販売され日本におけるビスフェノールAについての詳細なリスク評価を行った資料として重視される位置にあると思います。ほとんど同じ時期に私たちは厚生労働研究班で詳細な文献調査を行った訳ですが、どういうわけか化学物質リスク管理研究センターの詳細リスク評価書ではこのような多くの神経行動毒性の報告について指摘が

一例もなかったということです。ですからこの詳細リスク評価においてはそもそも文献調査が本当に詳細になされた上でリスク評価が、十分なされたのかどうかということの疑問が生ずる次第です。

神経行動毒性の一例ですが、九州工業大学の粟生先生たちが発表された報告では、飲料水中からビスフェノールAを体重1キログラムあたり15マイクログラムの濃度で妊娠の13日目から出産までの間摂取させます。マウスやラットでは21日目ぐらいで出産しますが、その期間に限って投与します。出生後6ないし9週にかけて神経行動毒性試験として幾つかの試験を組み合わせ実施しますと、探索行動やストレス対処行動の性分化に障害や、うつ・不安の増強が見られました。

神経行動毒性試験は標準化されにくかったので、現在安全性評価における標準的な試験の中にはまだ組み込まれていません。形態的な催奇形性はどちらかという客観的に評価しやすかったと思いますが、神経行動毒性については必ずしもそうではないので、あまり観察されてこなかったですが注目する必要があると私は思います。

結論として、ビスフェノールAに関して胎児期と周産期に低用量暴露した場合に、個体レベルで神経系への影響が検出されているということです。そのためにNTPの長期毒性試験を基にした体重1キログラムあたり50マイクログラムという参照用量（一日許容摂取量にあたる）を見直す必要があるかもしれません（文末注）。今後このようなデータの再現性と毒性学的な意義、ヒトへの適用可能性を十分検討する必要があります。もうひとつの課題としては生体のそういう生理作用の制御、それからホメオスタシスにかかわる内分泌系、免疫系、神経系の共同作用、こういったものに着目した毒性試験の確立とリスク評価方法を今後検討していくということが要求されます。私は日本リスク研究学会の一員として、このような新しい毒性学的問題について対応できるリスク評価の手法について考え、またこのような場合の不確実性について十分検討していく必要があると思います。

特に毒性試験における曝露期間の設定については今まで見られていなかった、曝露時期とエンドポイントの関係との組み合わせ、最終的には動物実験からヒトへの適用の可能性について、またヒトでは個体間の差というのも大きかったり、それぞれ環境が違いますので環境がどのように影響の発現を左右し、それが結果の変動要因となるかを

検討する必要があると思います。

私どもは別途、実際のな応用例の研究の一つとして直接ビスフェノールAが、比較的高濃度で直接体に取り込まれる可能性の検討を、2000年に日本リスク研究学会で発表しています。ビスフェノールA重合樹脂で成型された血液透析装置から重合反応で残存し溶け出すビスフェノールAモノマーに着目し、当時在職していた国立医薬品食品衛生研究所の方たちと調査し、平均的にどの位が血液透析器から体の血液に直接入ってくる可能性があるかを調べました（関澤等，2001）。これと当時設定されていた許容量と比べてみたところ、十分余裕があるだろうと考えわけですが最近の文献調査によると必ずしもそうではなさそうだというようなところになってきます。それから実際にエストロゲン活性を持った物質のヒトへの可能性ということでは、ご存じの植物ホルモンについても私は日本リスク研究学会誌に論文発表しています（関澤，大屋，1999）。最近内閣府の食品安全委員会は、植物ホルモン物質のイソフラボノイドは体に良いが、これをサプリメントとして過剰に摂ると問題が起こりうる、つまり私は先ほどの表でもお見せしましたが、大豆製品を平均65グラム（当時）を一日取っているということが国民栄養調査から分かりました。その大豆製品の中に含まれる植物ホルモン物質であるダイゼインとか、ゲニステインの量というのは分析で分かります。その結果、平均20数ミリグラムぐらいのこういった植物ホルモン物質をとっているということが分かり、それら物質の活性との計算からおよその影響の可能性を推計できます。併せて当時私たちは女子大生の方を対象にして、小規模でしたが340人規模のアンケート調査をしました。その結果、大豆製品の摂取と不正出血との関係では一見関連があるようなデータが得られましたが、サンプルサイズが小さかったので統計的な有意差は見られませんでした。食生活のほかには睡眠時間や運動量についても聞いたところ、貧血については、睡眠時間が5時間以下の方と5時間以上の方では、明らかに有意差が見られました。そのことは、ヒトにおける影響を考察するとき、私たちの生活と環境、遺伝的な背景、性別、年齢といったさまざまな要因が、私たちのリスクやベネフィットに関係してきているということです。こういったことをトータルとして考えていくということが、問題の解明にどうしても必要だと思います。日本リスク研究学会は、社

会科学や医学の専門家といわゆる自然科学の専門家などが会員となっており、ともにリスクの不確実性要因や、リスクコミュニケーションについても考えながら、これから研究を進めていく必要があります。今回の講演は口頭なので、厳密な議論と本主題に関するより詳しい内容については、別に記しましたので、そちらを参照してください（関澤, 2005b; 2006b）。

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(文末注)

欧州連合食品科学諮問委員会(2002)は, ラットの3世代試験における無毒性量5 mg/kg体重/日に種差, 個体差それぞれ10づつと, 低用量影響の不確実性について5の合計500の不確実性係数をあてはめて0.01 mg/kg体重/日を暫定耐容摂取量とした。

## The effects of pH on fluoxetine in Japanese medaka (*Oryzias latipes*): Acute toxicity in fish larvae and bioaccumulation in juvenile fish

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### Abstract

Recent detection of fluoxetine in the aquatic environment and fish suggests a possibly high accumulation of fluoxetine; however, no report is available on the bioaccumulation of fluoxetine in aquatic organisms. Since bioaccumulation of fluoxetine was probably dependent on pH near the  $pK_a$  value of 10.1, experiments were conducted approximately at pH 7, 8, and 9. Distribution coefficients between 1-octanol and water ( $D_{ow}$ ), and those between synthetic membrane vesicles (liposomes) and water ( $D_{lip-wat}$ ) were determined at pH 7, 8, and 9. The  $D_{ow}$  and  $D_{lip-wat}$  values increased significantly with increasing pH. Acute toxicity tests were performed using Japanese medaka (*Oryzias latipes*) prior to the bioaccumulation test, and 96-h  $LC_{50}$  values were 5.5, 1.3, and 0.20  $mg\ l^{-1}$  at pH 7, 8, and 9, respectively. In the bioaccumulation test, concentrations of fluoxetine and its major metabolite, norfluoxetine, in the fish body and liver were measured. The bioconcentration factors (BCF) of fluoxetine for Japanese medaka were 8.8,  $3.0 \times 10$ , and  $2.6 \times 10^2$  in the body and  $3.3 \times 10^2$ ,  $5.8 \times 10^2$ , and  $3.1 \times 10^3$  in the liver at pH 7, 8, and 9, respectively. The BCF values were lower at pH 7 and higher at pH 9 mainly because of the increase in nonionized species with significantly higher hydrophobicity than the ionized species at pH values closer to  $pK_a$ . A similar trend was obtained for the concentration of norfluoxetine in the fish but the pseudo-BCF values (the ratio of the norfluoxetine concentration in the fish and the fluoxetine concentration in test water) were higher than the BCF value of fluoxetine at all pH conditions. © 2007 Elsevier Ltd. All rights reserved.

**Keywords:** Fluoxetine; Bioconcentration factor; Ecotoxicity; Ionization

### 1. Introduction

Fluoxetine and its major metabolite, norfluoxetine, have recently been detected in fish tissues (Brooks et al., 2005) and have become a topic of growing public concern. Fluoxetine was detected in the fish caught from Pecan Creek (Denton, TX, USA) at  $0.11 \pm 0.03\ ng\ g^{-1}$  in the muscle and  $1.34 \pm 0.65\ ng\ g^{-1}$  in the liver (Brooks et al., 2005). The maximum concentration of fluoxetine found in surface water was  $0.012\ \mu g\ l^{-1}$  in the US streams (Kolpin et al.,

2002), and that detected in the effluents of sewage treatment plants (STPs) was  $0.099\ \mu g\ l^{-1}$  (Metcalf et al., 2003). The predicted environmental concentrations calculated by the US and EU guidelines with the estimated removal rate in STPs using STPWIN (USEPA v3.11) of 22.6% are  $0.030\ \mu g\ l^{-1}$  and  $0.841\ \mu g\ l^{-1}$ , respectively (Johnson et al., 2005). However, since the experimentally measured removal rate was 93.1% mainly by sorption onto sludge (Yamamoto et al., 2005), the aquatic environmental concentration is possibly lower than the prediction. Although the predicted bioconcentration factor (BCF) of fluoxetine is only 2.0 at pH 7 (Brooks et al., 2003a), the actual BCF is considered to be much higher because of the detection in fish.

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Several investigators have initiated research into the fate and transport of fluoxetine in the natural aquatic environment. The reported pseudo first-order photodegradation constant by sunlight was  $0.0126 \text{ h}^{-1}$  and the half-life was 55.2 h (Lam et al., 2005). The sorption coefficients ( $\log K_{oc}$ ) of fluoxetine to dissolved organic matter and a model sediment, Elliott Silt Loam Soil, were 4.72 and 4.87, respectively, and were comparable to a four-ringed hydrophobic polycyclic hydrocarbon, pyrene (Yamamoto et al., 2005). Thus, fluoxetine was predicted to be highly accumulative in soil/sediment but the transport could be accelerated by dissolved organic matter.

Several toxicity tests have been extensively performed using various aquatic organisms. While the predicted  $LC_{50}$  for fish was  $2 \text{ mg l}^{-1}$  (Sanderson et al., 2003), the measured 48-h  $LC_{50}$  for *Pimephales promelas* was  $2.28 \text{ }\mu\text{M}$  ( $705 \text{ }\mu\text{g l}^{-1}$ ) (Brooks et al., 2003b). The aggression of *Thalassoma bifasciatum* was decreased after 14 d of oral exposure to fluoxetine at  $6 \text{ }\mu\text{g g}^{-1} \text{ d}^{-1}$  (Perreault et al., 2003). As far as cladocera are concerned, the predicted  $EC_{50}$  was  $150 \text{ }\mu\text{g l}^{-1}$  (Sanderson et al., 2003) and the measured 48 h  $EC_{50}$  for *Daphnia magna* was  $2.65 \text{ }\mu\text{M}$  ( $820 \text{ }\mu\text{g l}^{-1}$ ) (Brooks et al., 2003b). Brooks et al. (2003b) found 48 h  $EC_{50}$  for immobilization in *Ceriodaphnia dubia*, and no observed effect concentration (NOEC) for fecundity decrease to be  $0.756 \text{ }\mu\text{M}$  ( $234 \text{ }\mu\text{g l}^{-1}$ ) and  $0.180 \text{ }\mu\text{M}$  ( $56 \text{ }\mu\text{g l}^{-1}$ ), respectively, whereas Henry et al. (2004) found acute 48-h  $EC_{50}$  and chronic NOEC (number of broods per female) to be 510 and  $447 \text{ }\mu\text{g l}^{-1}$ , respectively. For algae, the predicted  $EC_{50}$  was  $900 \text{ }\mu\text{g l}^{-1}$  (Sanderson et al., 2003), and the measured 50% growth inhibition at 120 h was  $0.077 \text{ }\mu\text{M}$  ( $24 \text{ }\mu\text{g l}^{-1}$ ) and NOEC was  $<0.0436 \text{ }\mu\text{M}$  ( $<13 \text{ }\mu\text{g l}^{-1}$ ) (Brooks et al., 2003b). These aquatic toxicities of fluoxetine were reviewed and compared with those of other human pharmaceuticals by Fent et al. (2006). They found that fluoxetine is one of the most toxic human pharmaceuticals. However, these toxicity tests were performed at a single pH or with no details about pH, and the effects of pH have never been examined. For pentachlorophenol, bioconcentration factors in goldfish (*Carassius auratus*) were strongly affected by pH near the  $pK_a$  value (Stehly and Hayton, 1990). Fluoxetine is a secondary amine, so that it may also be strongly affected by changing pH near the  $pK_a$  value of 10.1 (Fig. 1). An ionized species ( $AH^+$ ) is presumably more easily dissolved in water (i.e., the

octanol/water partition coefficient is lower) than a nonionized species (A), which increases as decreasing pH becomes lower than  $pK_a$ . Thus, the octanol/water distribution coefficient ( $D_{ow}$ ) decreases with the reduction in pH, because of the decrease in hydrophobic nonionized species (A). Hence the accumulation and toxicity of amines, such as that of fluoxetine, are considered to vary significantly at pH values slightly lower than  $pK_a$ .

Consequently, the objectives of this study were (1) to measure the octanol/water and liposome/water distribution coefficients at different pH values, and (2) to determine the toxicity and BCF of fluoxetine using Japanese medaka (*Oryzias latipes*) at different pH values. The pH values of the test water were set at approximately 7, 8, and 9 mainly because the effects of pH near the  $pK_a$  value of 10.1 are apparently more significant than those of the acidic side. Additionally, Brooks and co-workers sampled fish at a northern Texas stream (Brooks et al., 2005) where limestone is prevalent and the pH value could be as high as 8. In fact, the pH value of the upper Trinity River basin was reported by the USGS to range from 7.3 to 8.3 (USGS, 2007).

## 2. Materials and methods

### 2.1. Materials

Japanese medaka (*Oryzias latipes*) acquired from the National Institute for Environmental Studies (Ibaraki, Japan) were used in this study. The fish were acclimated in the laboratory at the University of Tokushima for at least two months. Approximately 15-d-old larvae were used for the acute test after five days of acclimation period, and approximately 2-month-old fish were used for the BCF test.

Fluoxetine (hydrochloride), its major metabolite norfluoxetine (hydrochloride), an internal standard fluvoxamine, and 1-octanol were purchased from Sigma Chemical Co. (St Louis, MO, USA). Biochemical grade bis-tris (bis(2-hydroxyethyl) iminotris(hydroxymethyl)methane), tris (tris(hydroxymethyl)aminomethane), HPLC grade acetonitrile, hexane 5000, diethylether 5000, dichloromethane 300, and iso-amyl alcohol (3-methyl-1-butanol) for special grades were obtained from Wako Pure Chemicals Co. (Osaka, Japan). Palmitoyl-oleoyl phosphatidylcholine

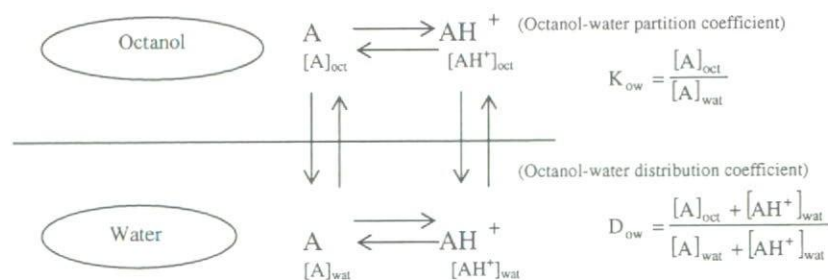


Fig. 1. Schematic diagram of the partition/distribution of the nonionized ( $AH^+$ ) and ionized (A) species of amines between octanol and water.

(POPC) was obtained from Nippon Fine Chemical Co. (Osaka, Japan) to synthesize liposomes. *S*-(–)-*N*-(trifluoroacetyl)-prolyl chloride in dichloromethane (0.1 M), which was used to analyze the derivative reagent with gas chromatograph mass spectrometry (GC–MS), was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

## 2.2. Analysis

Concentrations of fluoxetine in the aqueous phase were measured using high-performance liquid chromatography (HPLC) equipped with UV/vis absorbance and fluorescence detectors. The system consisted of an SLC-10AD controller (Shimadzu, Kyoto, Japan), two LC-10AD pumps (Shimadzu, Kyoto, Japan), a CTO-10AS oven (Shimadzu, Kyoto, Japan), an SPD-10A UV/vis absorbance detector (Shimadzu, Kyoto, Japan), and an RF-10A fluorescence detector (Shimadzu, Kyoto, Japan). A 150 mm × 4.6 mm i.d. packed with 5 μm of end-capped ODS main column (VP-ODS, Shimadzu, Kyoto, Japan) was used with a 10 mm × 4.6 mm i.d. of similar ODS pre-column (GV-ODS, Shimadzu, Kyoto, Japan). The mobile phase was isocratic and consisted of acetonitrile and 10 mM potassium phosphate buffer (pH 3.0, 62:38 v/v%) and the flow rate was set at 1.0 ml min<sup>-1</sup>. The UV/vis absorbance detector was used for quantification at a wavelength of 228 nm, and the fluorescence detector was used for rough identification at excitation and emission wavelengths of 230 and 293 nm, respectively. The retention time was approximately 9.0 min and the lower detection limit was 0.75 μg l<sup>-1</sup> for 500 μl of injection volume.

The concentration of fluoxetine in the fish was measured using a GC–MS after the extraction (Eap et al., 1996) described in detail below. The QP-2010 (Shimadzu, Kyoto, Japan) was used with splitless injection and electron impact (EI) modes. A column of 30 m × 0.25 mm i.d. and 0.25 μm film thickness (DB-5ms, Agilent Technology, CA, USA) was used, and high-quality helium was used as the carrier gas at 1.11 ml min<sup>-1</sup>. The GC oven, injection temperature, and interface temperature were set at 145 (initial temperature), 250, and 280 °C, respectively. The temperature graduation was held for 0.5 min at initial temperature (145 °C), heated at 10 °C min<sup>-1</sup> to 290 °C, and held for 10 min at 290 °C. For the MS, the ion source temperature and ionization potential were set at 200 °C and 70 eV, respectively. Five microliters of solution was injected, and analysis was performed in SIM mode with collected peaks of *m/z* = 117, 166, 237, 253, 341, and 327. Quantification was conducted at *m/z* = 117 for fluoxetine and norfluoxetine, and *m/z* = 166 for fluvoxamine. Chromatograms of a spiked blank fish and an example of exposed fish are shown in Fig. 2. Two separate peaks were found for fluoxetine and norfluoxetine, respectively, and these could be enantiomers as reported by Eap et al. (1996) because their analytical conditions/columns are similar to ours. However, no separated enantiomer standard was available and we could not identify them. Fluoxetine and norfluoxetine were quanti-

fied by adding the two peaks. The detection limits for fluoxetine and norfluoxetine were 63 and 11 μg l<sup>-1</sup>, respectively.

## 2.3. Distribution coefficients in 1-octanol/water and liposome/water systems

The *D*<sub>ow</sub> values were measured at pH 7.0, 8.0, and 9.0 with 10 mM buffers according to the OECD test guideline No. 107 (OECD, 1995). Bis-tris and HCl with 75:25 (v/v%) were used to prepare the pH 7.0 buffer while a mixture of tris and HCl was used for the pH 8.0 and 9.0 buffers with 67:33 and 90:10 (v/v%), respectively. The total amount of 10 ml of 10 mM buffer-saturated 1-octanol with fluoxetine and 1-octanol-saturated 10 mM buffer solution were added into 10 ml dry glass tubes and mixed in the dark using a tumbler for 24 h. The final concentration of fluoxetine in the aqueous phase was determined using a fluorescence spectrophotometer (F-2500, Hitachi, Tokyo, Japan).

Distribution coefficients between synthetic membrane vesicles (liposomes) and water (*D*<sub>lip-wat</sub>) were determined using the equilibrium dialysis method developed by Escher and Schwarzenbach, (1996) and later modified by Yamamoto and Liljestrand, (2004). Liposomes were prepared from POPC dissolved in chloroform using the thin film hydration technique (Mueller et al., 1983). Ten milliliter of 20 g l<sup>-1</sup> POPC in dichloromethane was evaporated to dryness and re-suspended in 100 ml Milli Q water in a sonicator. The POPC suspension was extruded with 1.2 μm Millipore polycarbonate membrane to obtain vesicles with 0.6–0.8 μm diameters (Yamamoto and Liljestrand, 2004). The total organic carbon (TOC) concentration of the liposomes was determined using a TOC analyzer (TOC-5000, Shimadzu, Kyoto, Japan). The buffer solutions described in the previous section were added to the liposome to set the concentration at 122 mg C l<sup>-1</sup> and aqueous solution of fluoxetine to set the pH at 6.9, 7.9, and 8.9. Sodium azide (10 mM) was also added to minimize microbial activity. The apparatus of the combination of two glass vials, a sheet of dialysis membrane (Spectram/Por), and a PTFE connector had been developed by Escher and Schwarzenbach, (1996) and was later modified by Yamamoto and Liljestrand, (2004). The initial concentration of fluoxetine in the aqueous phase was set at 100 μg l<sup>-1</sup> and was connected with the liposome phase by the PTFE connector and a dialysis membrane. After a week of rotary shaking using a tumbler in the dark, the concentrations of fluoxetine in both the liposome and the aqueous phase were measured using HPLC. One week is considered enough to attain equilibrium for this system (Yamamoto and Liljestrand, 2004). Blank samples were prepared with 10 mM buffer solution instead of liposome suspension. All tests and blanks were performed with four replicates, as were the *D*<sub>ow</sub> tests. The values of *D*<sub>lip-wat</sub> (l kg<sup>-1</sup>) were calculated as follows:

$$D_{\text{lip-wat}} = (C_{\text{blank}} - C_{\text{w}}) / (C_{\text{w}} \times [\text{lip}]) \quad (1)$$

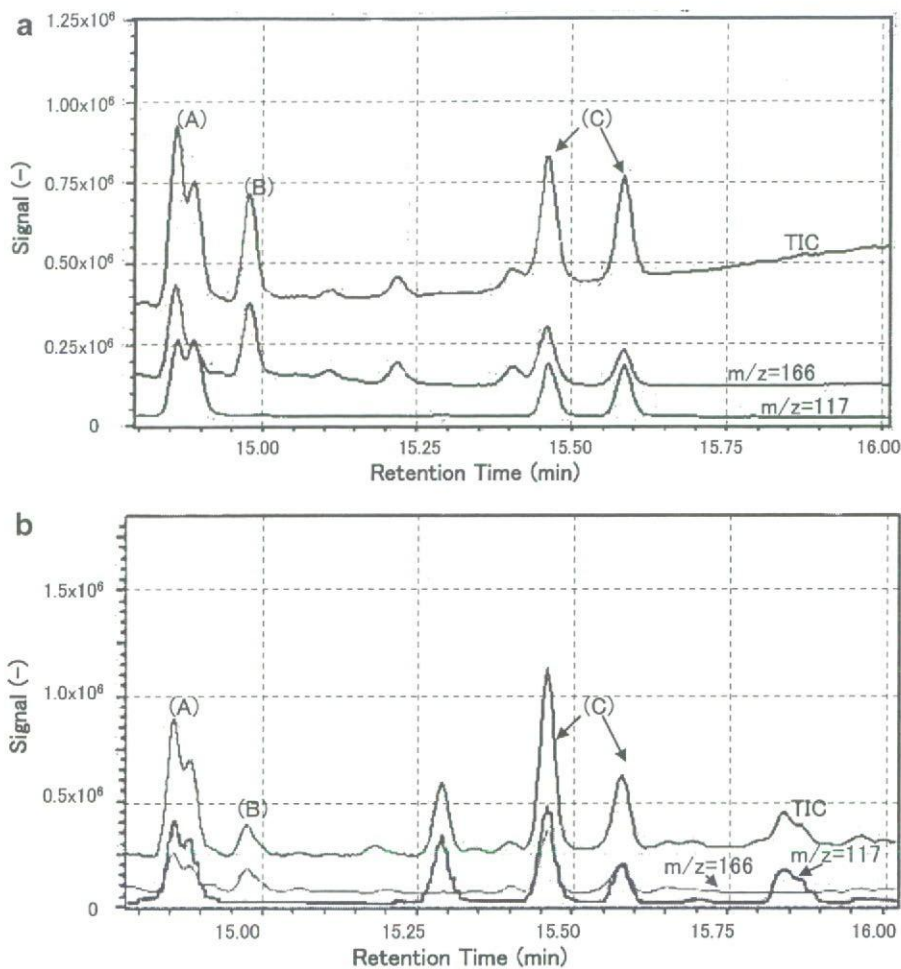


Fig. 2. Examples of selected ion chromatograms of (A) norfluoxetine, (B) fluvoxamine (internal standard), and (C) fluoxetine, of the (a) spiked blank and (b) BCF test.

where  $C_{\text{blank}}$  ( $\text{mg l}^{-1}$ ) is the concentration of fluoxetine in the blank solution,  $C_w$  ( $\text{mg l}^{-1}$ ) is that in the aqueous phase, and  $[\text{lip}]$  ( $\text{kg l}^{-1}$ ) is the TOC of the liposome suspension.

#### 2.4. Acute tests

Acute tests were performed in accordance with the OECD test guideline No. 203 (OECD, 1992) at pH 7.1, 7.9, and 8.8 with 10 mM buffers as presented above. Ten 15-d-old fish were exposed to 100 ml of test water in a 100 ml glass beaker. No significant toxic effects were found for the concentration of buffers (10 mM) for Japanese medaka in blank solutions during the 96-h test period and this concentration was enough to control pH. Ten fish were exposed to fluoxetine solution of each concentration for 96 h. The test water was replaced once in 48 h from the beginning of exposure, and the concentration in the tanks was monitored using HPLC at the beginning and end of the exposure period. The mean measured concentration was used for the data analysis. The maximum relative deviation of the aqueous concentration was 19%. The pH values of the test solutions were also monitored using a

pH meter (D-52, HORIBA, Kyoto, Japan).  $\text{LC}_{50}$  values for 96 h were determined with probit conversion from the number of deaths in 96 h for each pH.

#### 2.5. Bioconcentration factors

##### 2.5.1. Exposure

A preliminary test with a single pH condition was performed before the test with three different pH conditions. Fluoxetine concentrations in the aqueous phase were set at approximately 30 and 300  $\mu\text{g l}^{-1}$ , and Japanese medaka were exposed for 30 d in the preliminary test. For the test with three different pH values, test waters were adjusted at pH 7.2, 8.1, and 8.9 with 10 mM buffers as described above, and eight fish were exposed to 10  $\mu\text{g l}^{-1}$  of fluoxetine, which was determined from 96-h  $\text{LC}_{50}$  and the detection limits for GC-MS (i.e., 63  $\mu\text{g l}^{-1}$  or 12.6 ng per sample for fluoxetine and 11  $\mu\text{g l}^{-1}$  or 2.2 ng per sample for norfluoxetine). For each pH test, blank samples with 10 mM buffer solutions with no fluoxetine addition were prepared to confirm absence of any significant effect by the buffer agent or pH on fish. The exposure period was 30 d as with the preliminary test.



The custom-made exposure system consists of glass tanks, Vivaria R 1815, purchased from Torio Co. (Osaka, Japan) filled with 2,300 ml of test solution. The fluoxetine solution was continuously fed into the tank at a flow rate of  $1.7 \text{ ml min}^{-1}$ . Aqueous concentrations of fluoxetine and pH in these tanks were measured every 10 d using HPLC. After the 30-d exposure period, the body and liver samples were collected from the fish and stored at  $-20^\circ\text{C}$  until the extraction process.

### 2.5.2. Extraction

The extraction of fluoxetine and norfluoxetine from the fish was performed using the method developed by Eap et al. (1996) and Lefebvre et al. (1999) with slight modifications. Two fish bodies (approximately 50 mg) were placed in a 2 ml plastic tube, and  $30 \mu\text{l}$  of  $2 \text{ mg l}^{-1}$  fluvoxamine, an internal standard,  $250 \mu\text{l}$  of  $\text{Na}_2\text{CO}_3$ ,  $200 \mu\text{l}$  of Milli Q water, and  $500 \mu\text{l}$  of *n*-hexane-diethylether (50:50 v/v%) were added. Fluvoxamine was selected as the internal standard because of its similarity in chemical structure to clovoxamine, which is used as an internal standard for the quantification of fluoxetine and fluvoxamine by Eap et al. (1996). For the liver, the pairs identical to the body samples (approximately 1.5 mg) were similarly paired and extracted. After the homogenization, the solution was centrifuged at  $4^\circ\text{C}$  and 10 000 rpm for 30 min. The organic layer was transferred into a 10 ml glass tube and  $500 \mu\text{l}$  of 0.1 M HCl was added. The aqueous layer was collected followed by 1 min of shaking and centrifugation at 2500 rpm for 15 min, and then 1 ml of 1 M  $\text{Na}_2\text{CO}_3$  and  $500 \mu\text{l}$  of dichloromethane-iso-amyl alcohol (85:15 v/v%) were added. After shaking and centrifugation, the aqueous layer was removed and anhydrous sodium sulfate was added to remove the water completely. After shaking and centrifugation at 2500 rpm for 10 min again, the supernatant was transferred to another 10 ml dry glass tube to evaporate until dryness under a stream of nitrogen at  $40^\circ\text{C}$ . A derivatization reagent of  $500 \mu\text{l}$  was added to the glass tube and extracted in a sonicator. The derivatization reagent was prepared every day by adding  $360 \mu\text{l}$  of *S*-(-)-*N*-(trifluoroacetyl)-prolyl chloride solution into 4.5 ml of dichloromethane. The glass tube was topped and sealed with PTFE tape, and the derivatization was performed in a water bath at  $60^\circ\text{C}$  for 1 h. The reagent was evaporated to dryness under a stream of nitrogen at  $40^\circ\text{C}$ ,  $500 \mu\text{l}$  of dichloromethane was added, and the derivatized fluoxetine was extracted in a sonicator. Analysis was performed using GC-MS as presented above after the solution was concentrated to  $200 \mu\text{l}$  under a stream of nitrogen. The BCF value for each pH was calculated as follows:

$$\text{BCF} = C_{\text{fish}}/C_{\text{water}} \quad (2)$$

where  $C_{\text{fish}}$  ( $\mu\text{g kg}^{-1}$ ) is the concentration of fluoxetine in fish and  $C_{\text{water}}$  ( $\mu\text{g kg}^{-1}$ ) is the mean of the aqueous concentrations. The standard solution was prepared by following the procedure shown above after transferring from the

aqueous layer to the dichloromethane-iso-amyl alcohol layer.

## 3. Results

### 3.1. Distribution coefficients between 1-octanol/water and liposome/water

The  $D_{\text{ow}}$  and  $D_{\text{lip-wat}}$  values for fluoxetine are summarized in Table 1. While both coefficients increased with increasing pH, the difference in the  $D_{\text{ow}}$  value was significantly larger than that of the  $D_{\text{lip-wat}}$  value.

### 3.2. Acute test

Table 2 shows 96-h  $\text{LC}_{50}$  values of fluoxetine for approximately 15-d-old *Oryzias latipes*. The number of dead fish in the blank solution of 10 mM buffer at any pH was at most one (i.e., 10%), which was less than the maximum lethal percentage indicated in the OECD test guideline No. 203. The higher pH obviously caused higher toxicity. The difference between  $\text{LC}_{50}$  at pH 9 and 7 was statistically significant ( $p < 0.01$ ) as a result of *t*-tests. NOEC at pH 7.1 was  $3.8 \text{ mg l}^{-1}$  in this study. At pH 8.8, three-tenth of the fish died at  $100 \mu\text{g l}^{-1}$ , the lowest concentration in the study. Therefore, the exposure concentration of the BCF test was set at  $10 \mu\text{g l}^{-1}$ , one order of magnitude lower than the concentration.

### 3.3. Bioconcentration factors

The recovery of fluoxetine from the fish body and liver was  $81.3 \pm 10.8\%$  and  $92.6 \pm 5.3\%$ , respectively, and those of norfluoxetine were  $71.1 \pm 11.0\%$  and  $95.7 \pm 2.6\%$ , respectively. The measured pH values in the test solution during the exposure period were  $7.2 \pm 0.1$ ,  $8.1 \pm 0.1$ , and  $8.9 \pm 0.1$ , and concentrations were  $13.8 \pm 3.8$ ,  $15.0 \pm 3.2$ ,

Table 1  
 $D_{\text{ow}}$  and  $D_{\text{lip-wat}}$  of fluoxetine at each pH (mean  $\pm$  S.D.)

	Ratio of nonionized species [A] (%)	$D_{\text{ow}}$	$D_{\text{lip-wat}}$
pH7	0.079	$3.6(\pm 1.6) \times 10$	$1.7(\pm 0.6) \times 10^4$
pH8	0.79	$4.6(\pm 0.6) \times 10^2$	$2.4(\pm 0.8) \times 10^4$
pH9	7.4	$4.6(\pm 0.5) \times 10^3$	$4.0(\pm 1.6) \times 10^4$

Table 2  
96-h  $\text{LC}_{50}$  Values of fluoxetine for *Oryzias latipes* at pH 7, 8, and 9 (mean  $\pm$  95% confidence interval)

	96 h- $\text{LC}_{50}$ ( $\text{mg l}^{-1}$ )
pH 7 ( $7.1 \pm 0.1$ ) <sup>a</sup>	$5.5 \pm 1.3$
pH 8 ( $7.9 \pm 0.1$ ) <sup>a</sup>	$1.3 \pm 0.2$
pH 9 ( $8.8 \pm 0.2$ ) <sup>a</sup>	$0.20 \pm 0.02^*$

<sup>a</sup> Measured pH values (pH monitoring was performed at the first and last days of the test).

\*  $p < 0.01$  compared with  $\text{LC}_{50}$  value of pH 7 as a result of *t*-test.

Table 3

Bioconcentration factor of fluoxetine and its major metabolite norfluoxetine in the body and liver ( $n = 4$ ; mean  $\pm$  S.D.)

		Body	Liver	Body + liver
Fluoxetine	pH 7	8.8 $\pm$ 5.2	3.3( $\pm$ 0.9) $\times 10^2$	1.3( $\pm$ 0.6) $\times 10$
	pH 8	3.0( $\pm$ 1.3) $\times 10$	5.8( $\pm$ 1.1) $\times 10^2$	3.7( $\pm$ 1.3) $\times 10$
	pH 9	2.6( $\pm$ 1.5) $\times 10^2$	3.1( $\pm$ 0.4) $\times 10^3$	3.3( $\pm$ 1.5) $\times 10^2$
Norfluoxetine	pH 7	8.4( $\pm$ 0.8) $\times 10$	1.5( $\pm$ 0.2) $\times 10^3$	1.0( $\pm$ 0.1) $\times 10^2$
	pH 8	1.3( $\pm$ 0.7) $\times 10^2$	3.3( $\pm$ 0.6) $\times 10^3$	1.7( $\pm$ 0.6) $\times 10^2$
	pH 9	6.5( $\pm$ 1.8) $\times 10^2$	3.7( $\pm$ 2.3) $\times 10^3$	7.2( $\pm$ 1.7) $\times 10^2$
Fluoxetine + norfluoxetine	pH 7	9.3( $\pm$ 1.3) $\times 10$	1.8( $\pm$ 0.3) $\times 10^3$	1.1( $\pm$ 0.2) $\times 10^2$
	pH 8	1.6( $\pm$ 0.6) $\times 10^2$	3.9( $\pm$ 0.6) $\times 10^3$	2.1( $\pm$ 0.6) $\times 10^2$
	pH 9	9.1( $\pm$ 2.1) $\times 10^2$	6.8( $\pm$ 0.2) $\times 10^3$	1.0( $\pm$ 0.2) $\times 10^3$

and  $14.5 \pm 3.3 \mu\text{g/l}$ , respectively. Table 3 shows the BCF values of fluoxetine in the body and liver of approximately 2-month-old *Oryzias latipes* at each pH. No fish died or

were apparently immobilized in either test or blank solutions. Neither fluoxetine nor norfluoxetine was detected in blank fish. The measured BCF value of fluoxetine for the summation of the body and liver was 11 at pH 7.2 in this study, which was slightly higher than the predicted value of 2.0 at pH 7 (Brooks et al., 2003a). Since the major metabolite norfluoxetine was also detected from both samples, the pseudo-BCF value for norfluoxetine (i.e., the denominator is the concentration of fluoxetine in the aqueous phase and not the concentration of norfluoxetine) is also added in Table 3.

In our preliminary tests with two different aqueous concentrations, the BCF value for fluoxetine in the whole body was  $4.5(\pm 1.2) \times 10$  and the pseudo-BCF value for norfluoxetine was  $2.4(\pm 0.3) \times 10^2$  in exposing Japanese medaka to  $30 \mu\text{g l}^{-1}$  of fluoxetine for 30 days, and  $1.0(\pm 0.3) \times 10^2$  for fluoxetine and  $1.1(\pm 0.2) \times 10^2$  for norfluoxetine at  $300 \mu\text{g l}^{-1}$ . The ratios of norfluoxetine and fluoxetine were 5.3 and 1.1 at  $30 \mu\text{g l}^{-1}$  and  $300 \mu\text{g l}^{-1}$ , respectively.

Fig. 3 shows the relationships between pH and distribution coefficient in the octanol/water, liposome/water, and fish/water systems (i.e., the BCF value) in the body and liver. The BCF values of fluoxetine in the body and liver were both lower at pH 7.2 and higher at pH 8.9. The trend of the increase in the BCF values was apparently similar to that of the  $\log D_{\text{ow}}$  value. Contrarily, the pseudo-BCF values for norfluoxetine were neither so increased as the increase in pH nor similar to the trend of  $\log D_{\text{ow}}$  value, but were similar to that of  $\log D_{\text{lip-wat}}$ . The sum of the BCF and pseudo-BCF values for fluoxetine and norfluoxetine increased similar to the  $\log D_{\text{lip-wat}}$  value. The ratios of pseudo-BCF and BCF (norfluoxetine per fluoxetine) were 8.5, 3.9, and 2.2 in the body, and 4.5, 5.9, and 1.2 in the liver at pH 7.2, 8.1, and 8.9, respectively. The ratios were relatively high at pH 7.2 and low at pH 8.9 except for the liver at pH 8.

#### 4. Discussion

The large difference of the  $D_{\text{ow}}$  value at different pH was mainly attributed to the difference in the ratio of the concentration of ionized species ( $[\text{AH}^+]$ ) and nonionized

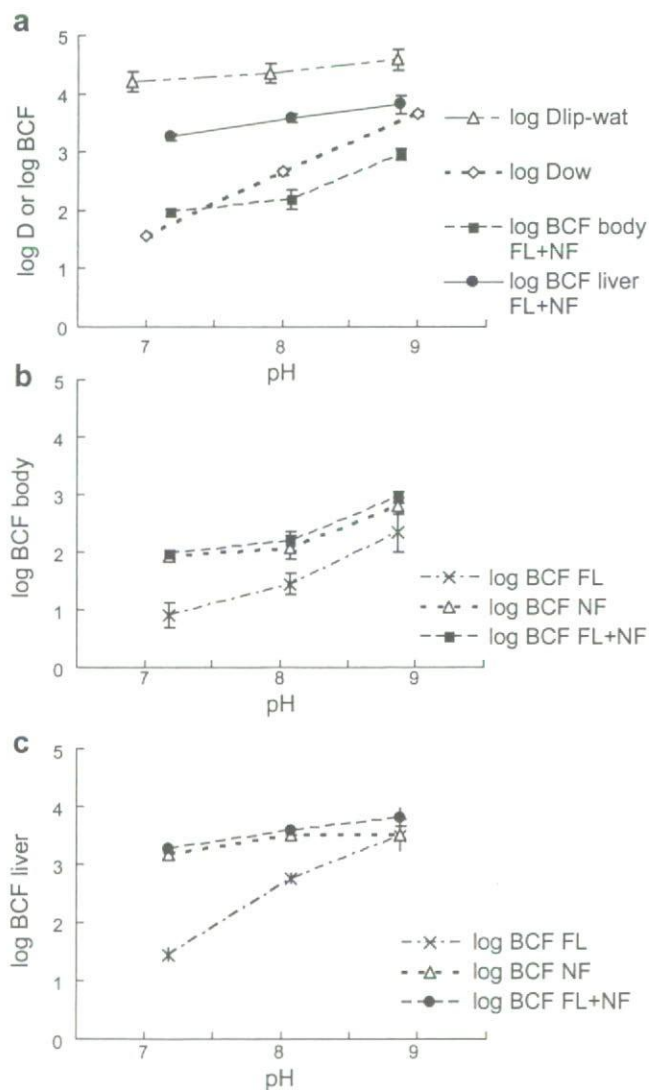


Fig. 3. Relationship between pH and  $D_{\text{ow}}$ ,  $D_{\text{lip-wat}}$ , and BCF. (a) shows  $\log D_{\text{ow}}$ ,  $D_{\text{lip-wat}}$ , and BCF of sum of fluoxetine and norfluoxetine in body and liver, (b) shows  $\log \text{BCF}$  in the body, and (c) shows these in the liver. (FL: fluoxetine, NF: norfluoxetine. Error bars show standard deviations.)

species ( $[A]$ ) as can be seen in the increasing ratio of  $[A]$  in Table 1. The estimated coefficient of octanol and water ( $K_{ow}$ ) values of ionization ( $AH^+$ ) and nonionization ( $A$ ) are less than 1 and  $5.2 \times 10^3$ , respectively. The  $D_{lip-wat}$  value, however, did not vary significantly as the  $D_{ow}$  value; the estimated partition coefficient of liposome and water ( $K_{lip-wat}$ ) of these two are  $1.7 \times 10^4$  and  $3.3 \times 10^4$ , respectively.

The acute toxicity of fluoxetine found in this study for Japanese medaka at pH 7.1 (i.e.,  $LC_{50} = 5.5 \pm 1.2 \text{ mg l}^{-1}$ ) was greater than that in other reports, such as Brooks et al. (2003 b), where no effect was found at  $28.9 \mu\text{M}$  ( $8.9 \text{ mg l}^{-1}$ ) in 48 h. Moreover, the toxicity was found to be as high as  $1.7 \text{ mg l}^{-1}$ , which is the 96-h  $LC_{50}$  value for fish predicted by ECOSAR (US EPA v3.12). Approximately 15-d-old fish larvae, which are apparently more sensitive than juvenile or sexually matured fish, were used in this study. Toxicity was reported slightly stronger for other species including algae, daphnids, and other species of fish as described above. The results of the toxicity tests were obviously different for various ages and species.

The strong dependence of toxicity on pH is partly because of the higher BCF at increased pH due to the higher fraction of the lipophilic nonionized species (Table 1). The toxicity shows positive correlation with the lipophilicity, so that the increasing fraction of lipophilic nonionized species caused the greater toxicity. Despite no apparent effect on blank fish at higher pH, the toxic effects of the buffer reagent and the higher pH on Japanese medaka are unclear and further investigation is necessary to reveal the complete mechanism. The slight difference in ionic strength in the test water (i.e., the estimated ionic strength at pH 7.9 was twice as high as the other two) also possibly causes the difference in the toxicity.

$D_{ow}$  has long been used to estimate the hydrophobicity or bioaccumulation of chemical compounds. Lopes et al. (2006) calculated the BCF value of an acidic pesticide, trichlofon, referring to Kleier, (1994) and Isnard and Lambert, (1988) at different pH values, and the estimated values agreed to the measured ones. In these reports, the BCF values of the ionized ( $AH^+$ ) and nonionized species ( $A$ ) were calculated as per the following equation:

$$BCF = P_{ion} BCF_{ion} + (1 - P_{ion}) BCF_{nonion} \quad (3)$$

where  $P_{ion}$  is the ratio of ionization calculated by following the equation below.

$$P_{ion} = \frac{1}{1 + 10^{pH - pK_a}} \quad (4)$$

$BCF_{ion}$  and  $BCF_{nonion}$  are the bioconcentration factors of the ionized and nonionized species, respectively. These BCF values were estimated using the equation for nonionized compounds suggested by Isnard and Lambert (1988).

$$\log BCF = 0.76 = \log K_{ow} - 0.52 \quad (5)$$

where  $\log K_{ow}$  was used for both the ionized and nonionized species for estimating  $BCF_{ion}$  and  $BCF_{nonion}$ . The

$\log K_{ow}$  value for the ionized species was estimated using the following equation:

$$\log K_{ow ion} = \log K_{ow nonion} - 3.4 \quad (6)$$

This equation moderately agrees to our results;  $\log K_{ow}$  for the ionized species based on Eq. (3) is 0.4 and that for the nonionized species is 3.0. Those of our results were 0.5 and 3.4, respectively. The formula derived from our results is

$$BCF = 0.50 P_{ion} + 3.4(1 - P_{ion}) \quad (7)$$

The estimated log BCF values of fluoxetine based on Eq. (3) and those estimated by Eq. (7) are shown in Fig. 4. The estimated log BCF using Eq. (3) at pH 7.2, 8.1, and 8.9 were 0.60, 1.1, and 1.8, respectively, and the measured log BCF values of fluoxetine in the body were 0.94, 1.5, and 2.4, respectively, and slightly higher than the estimated log BCF values at each pH.

The smaller difference in  $D_{lip-wat}$  value than  $D_{ow}$  value at pH range 7–9 agrees with those obtained by Escher and Schwarzenbach, (1996) for substituted phenols with ionizable groups. Liposome has also been used as a model of biomembrane and found to be better than 1-octanol especially for ionizable compounds, because liposomes are made of phospholipid, a main constituent of biomembrane, and closer to the real biomembrane (Escher and Schwarzenbach, 1996). The result of  $D_{lip-wat}$  suggests the smaller influence of the ratio of  $[AH^+]$  and  $[A]$  on the accumulation of the sum of  $AH^+$  and  $A$  into biomembranes. In Fig. 3, however, the trend of the increase in the log BCF values was apparently similar to that of the  $\log D_{ow}$  value. Contrarily, the trend of the sum of log BCF for fluoxetine and norfluoxetine is similar to that of  $\log D_{lip-wat}$ . Since the pseudo-BCF/BCF ratio (i.e., the norfluoxetine/fluoxetine ratio) was much higher than 1, the compounds originating from fluoxetine in the aqueous phase were as highly accumulated as the metabolite norfluoxetine in the fish body and need to be carefully examined. Moreover, the prediction of BCF values from  $\log D_{ow}$  values is inadequate especially for ionizable species (i.e., weak acids and

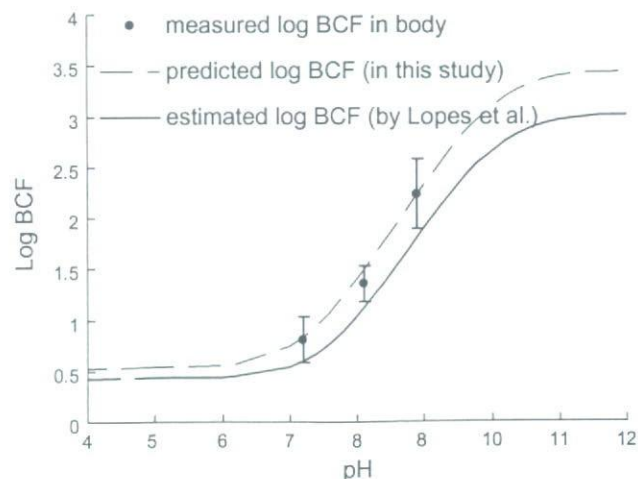


Fig. 4. Measured and predicted log BCF of fluoxetine at different pH.

bases) such as pharmaceuticals. Further investigation is necessary for other polar materials to confirm the relationships between  $\log D_{ow}$ ,  $\log D_{lip-wat}$  and  $\log BCF$ .

The half lives of fluoxetine and norfluoxetine for mammals are 1–4 d and 7–15 d, respectively (Hiemke et al., 2000), and the half-life of norfluoxetine in the fish may also be much longer than that of fluoxetine. In addition, the relatively lower clearance rate of norfluoxetine resulted in a moderate change in the pseudo-BCF of norfluoxetine with increasing pH. Not only the half lives of both compounds but also the higher uptake rate at higher pH caused the higher BCF. As presented above, the fraction of the hydrophobic nonionized species (A) significantly increases with the increase in pH. In this case, the metabolism, the conversion from fluoxetine to norfluoxetine, proceeded relatively faster than the uptake. Conversely, the uptake was relatively faster at high pH because of the higher hydrophobicity of the nonionized species (A), and a part of the fluoxetine absorbed by the fish was possibly accumulated without metabolism. In our preliminary test with two different aqueous concentrations described above, the ratio of norfluoxetine and fluoxetine was higher at lower concentration and lower at higher concentration. Exposure to low concentration might result in the metabolism from fluoxetine to norfluoxetine being relatively faster than the uptake.

Both fluoxetine and norfluoxetine were detected in wild bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), and black crappie (*Pomoxis nigromaculatus*) (Brooks et al., 2005). The concentrations of fluoxetine in the muscle and liver were approximately 0.11 and 1.3 ng g<sup>-1</sup>, respectively, and those of norfluoxetine were approximately 1.1 and 10 ng g<sup>-1</sup>, respectively. The ratios of norfluoxetine and fluoxetine in our results, including the preliminary tests, were 1.1 (whole body), 5.3 (whole body), and 9.5 (body) or 4.5 (liver), at aqueous concentrations of 300, 30, and 10 µg l<sup>-1</sup>, respectively, while those found by Brooks and co-workers, (2005) were approximately 10 (muscle) and 8 (liver). This comparison suggests that the exposure of fish to a low concentration causes a larger ratio, and the aqueous concentration in the river, where fluoxetine was detected in the fish by Brooks and co-workers, (2005), was much less than 10 µg l<sup>-1</sup>. Using the BCF value obtained in this study (i.e., 8.8 at pH 7.2), the aqueous concentration of fluoxetine exposed to the fish can be roughly predicted as 0.011 µg l<sup>-1</sup>, which is similar to the maximum concentration reported by Kolpin et al. (2002). Otherwise, the concentration might be slightly lower and the pH greater than 7, e.g., 0.0033 and 0.00038 µg l<sup>-1</sup> at pH 8 and 9, respectively. As presented above, the pH of the Upper Trinity River Basin in North Texas (e.g., Grand Prairie and Dallas) monitored by USGS ranged between 7.3 and 8.3 (USGS, 2007). If the pH at Brooks and co-workers' sampling point is similar to these values, the fluoxetine concentration was predicted to be a few nanograms per liter. However, further investigation is necessary to clarify the contribution of the other routes of bioaccumulation, such as food.

In this study, the accumulation of fluoxetine was dependent on pH, which agrees to the reports by Fent et al. (1995) and Looser et al. (1998), who had extensively investigated the effects of pH on the bioaccumulation of organotins. However, not much has been revealed about the effects of pH on bioaccumulation, metabolism, or toxicity of other organic micropollutants including fluoxetine, and further investigation is necessary.

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## URINE CONCENTRATIONS OF INDIRUBIN IN RATS AND HUMANS AND ITS POSSIBLE INTERACTION WITH OTHER ARYL HYDROCARBON RECEPTOR LIGANDS

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### Abstract

Urine concentrations of indirubin, a potent aryl hydrocarbon receptor (AhR) ligand, were analyzed in rats and humans using indirubin-specific ELISA assay. Indirubin concentrations were higher in rats (406±54 nM, and 266±36 nM for male and female respectively) as compared to those in humans (76±12 nM, and 68±12 nM for male and female respectively). No significant differences for indirubin concentrations in urine were observed among four estrous-cycle stages in female rats. AhR-specific reporter-gene assay in yeast was used to test xenobiotic-response element (XRE) specific gene-induction activities of AhR ligands. Indirubin showed relatively low EC<sub>25</sub> of 20 nM, whereas other natural AhR ligands, indigo, indole-3-carbinol, and genistein showed EC<sub>25</sub> of from 460 to 4100 nM, and beta-naphthoflavone, a synthetic ligand showed EC<sub>25</sub> of 40 nM. Combinations of indirubin with one of the other ligands showed higher gene induction activities than simple addition of activity of each compound in this assay. Possible physiological role of indirubin as a candidate of endogenous ligands for AhR is discussed.

### Introduction

Aryl hydrocarbon receptor (AhR) was shown to be indispensable for dioxin induced deleterious effects in mice<sup>1</sup>. AhR is ubiquitous from nematodes to humans and is thought to play important functions in organisms, however still not much was known about its physiological roles and its endogenous ligands. Indirubin was found to be a potent ligand of AhR in sewage and was shown to be derived from human urine<sup>2</sup>. Since indirubin is a metabolite of tryptophan, we assumed it as one of potential endogenous ligands for AhR, and to be a clue to elucidate physiological roles of AhR. We developed a sensitive enzyme-linked immunosorbent assay (ELISA)<sup>3</sup> for indirubin and investigated concentrations of indirubin in urines from rats and humans. We also assayed AhR-mediated gene induction activities of several natural AhR ligands including indirubin, using yeast reporter gene assay, to examine whether they can be effective as AhR ligands at the concentrations naturally available to humans and other organisms.

### Materials and Methods

A sensitive ELISA assay specific for indirubin<sup>3</sup> was developed to analyze indirubin concentrations in biological samples. Indirubin (purity 99%) and its antibody were prepared. Other chemicals (pure chemical grade) were purchased from test-chemical suppliers in Japan. Chemicals were dissolved either in distilled water or in dimethylsulfoxide and appropriately diluted by phosphate buffer (pH7.5, 50 mM) before use. Urine samples were appropriately diluted with the phosphate buffer. Urine was collected from 30 male and female rats (BrLHan:WIST@Jcl(GALAS), ages 10 to 11 week) using metabolic cages in a laboratory of the Ina Research Inc. and kept frozen until use. Human urine was collected from healthy young volunteers (10 males and 9 females aged from 21 to 36) with informed consent. Since indirubin can be easily metabolized by CYP1A1 enzyme which it induces, urine was treated with β-D-glucuronidase/sulfatase to hydrolyze conjugated-forms potentially present in urine.

AhR reporter-gene assay was conducted using *Saccharomyces cerevisiae* strain YCM3 carrying human AhR and XRE responsive β-D-galactosidase reporter genes originally developed by Miller<sup>4</sup>, kindly given by Dr. Tomonari Matsuda of Kyoto University. Briefly, yeast cell was incubated with test chemicals overnight for gene induction, collected and assayed for β-D-galactosidase reporter gene induction on the next day. β-Naphthoflavone was

used as the positive control. In addition to indirubin, indigo, indole-3-carbinol, genistein, daidzein, and resveratrol which were reported as agonists or antagonists of AhR were used as samples for yeast gene induction assay.

## Results and Discussion

### (1) Indirubin concentrations in urine

Indirubin concentrations in rat urine are shown in Table 1. Male rats (analysis of remaining samples underway) showed higher indirubin concentrations in urine than female rats. Female rats showed not significant differences in indirubin concentrations during their estrous cycle. Significant increase (166 and 144% for male and female, respectively) of indirubin detection after  $\beta$ -D-glucuronidase/sulfatase treatment suggests part of indirubin was metabolized and conjugated before excretion in urine. The data corresponds to 26 pmole/mg creatinine, and 31 pmole/mg creatinine for male and female respectively

Table 1 Indirubin concentrations in rat urine (nM)

	Average $\pm$ S.D.		Samples (n)
	+ Enzyme treatment	- Enzyme treatment	
Male	406 $\pm$ 54	244 $\pm$ 22	7
Female	266 $\pm$ 36	185 $\pm$ 29	120
Female P-E	259 $\pm$ 45	183 $\pm$ 33	30
Female E-M	266 $\pm$ 42	185 $\pm$ 34	30
Female M-D	272 $\pm$ 30	189 $\pm$ 26	30
Female D-P	265 $\pm$ 28	183 $\pm$ 25	30

P: proestrus, E: estrus, M: metestrus D: diestrus, Enzyme:  $\beta$ -D-glucuronidase/sulfatase

Human urine concentrations of indirubin are shown in Table 2. Although the number of samples was limited, it is apparent that human urine concentrations were much lower than those in rats.  $\beta$ -D-Glucuronidase/sulfatase treatment increased detection of indirubin to threefold (i.e., higher ratio of conjugated form in urine as compared to in rats) suggested that indirubin metabolism/conjugation reactions are more efficient in humans than in rats.

Table 2 Indirubin concentrations in human urine (nM)

	Average $\pm$ S.D.		Samples (n)
	+ Enzyme treatment	- Enzyme treatment	
Male	76 $\pm$ 12	26 $\pm$ 8	10
Female	68 $\pm$ 12	25 $\pm$ 5	9

Enzyme:  $\beta$ -D-glucuronidase/sulfatase

Isatin and oxindole, precursors of indirubin, did not show cross-reactivity, but several hydroxyl-derivatives of indirubin showed various levels (50 to 90% compared to indirubin) of reactions. Currently, we are trying to determine indirubin and its metabolites quantitatively using GC-MS, and LC tandem MS technique.

### (2) Reporter-gene assay of AhR-mediated activity

Fig.1 shows chemical structures of test chemicals for reporter-gene assay. AhR has been known to interact with many polycyclic aromatic hydrocarbons, however other chemicals containing indole or flavone structure are found to react with AhR to express XRE-specific gene in CALUX (chemical activated luciferase gene expression) assay using mouse hepatoma cell line<sup>5</sup>. Resveratrol was shown to be an antagonist to this reaction<sup>6</sup>.  $\beta$ -Naphthoflavone which is a synthetic chemical, was used as a positive control.

In our assay, indirubin showed the highest AhR-ligand activity among test chemicals with lowest 12.5% (of the highest activity in the test) effective concentration ( $EC_{12.5}$ ) of 12 nM (Table 3). Other natural ligands, indigo,

indole-3-carbinol, genistein were much weaker in XRE-specific gene expression activity with EC<sub>12.5</sub> of 210-2300 nM. Daidzein and resveratrol did not show agonist activity. We examined the activity in combination exposure to these chemicals using indirubin as one partner existing endogenously in the body to others which may be

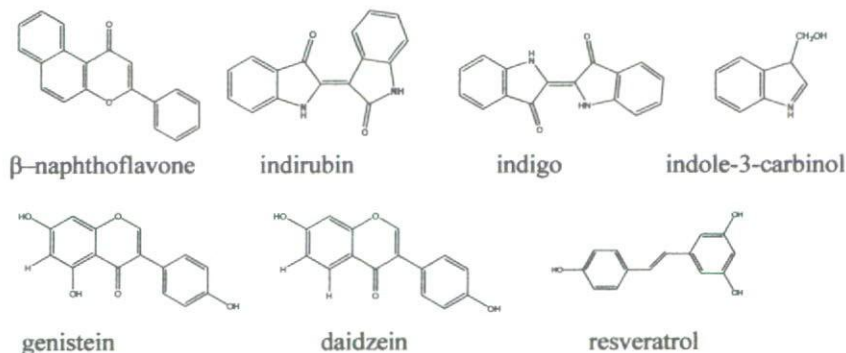


Fig. 1 Chemical structures of the gene induction assay samples

Table 3 Effective concentrations of various chemicals for gene induction assay (nM)

	EC <sub>12.5</sub>	EC <sub>25</sub>	EC <sub>50</sub>
indirubin	12 ± 6	25 ± 9	70 ± 10
$\beta$ -naphthoflavone	19 ± 12	39 ± 22	110 ± 50
indigo	210 ± 40	460 ± 60	1400 ± 100
indole-3-carbinol	330 ± 210	660 ± 320	1900 ± 700
genistein	2300 ± 800	4100 ± 1400	9200 ± 2600

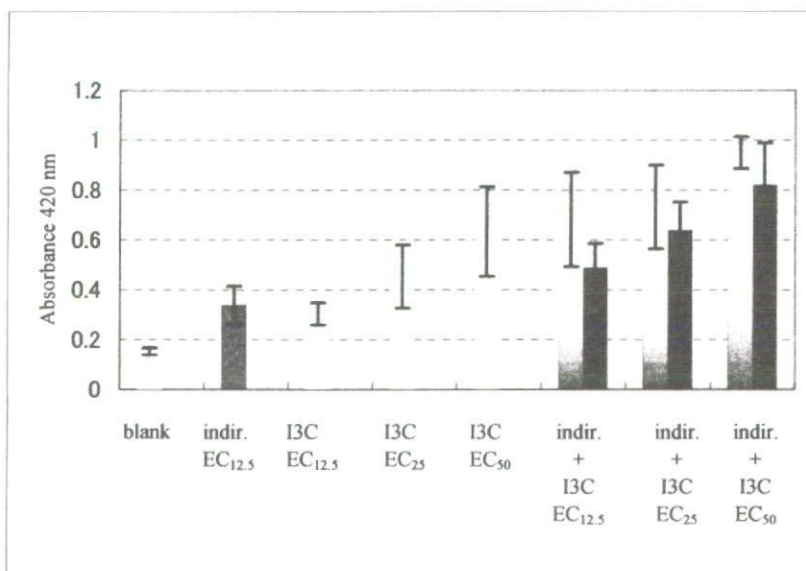


Fig.2 Example of combination assay: blank : buffer only, indir. : Indirubin, I3C: Indole-3-carbinol  
left bar of the two adjacent bars shows mixture activity, right bar of the two shows simple addition value



taken extraneously from foods. Fig.2 showed an example of the results in which combination exposure to indirubin and indole-3-carbinol expressed somewhat higher activity ( $142 \pm 39\%$  for indirubin  $EC_{12.5}$  + indole-3-carbinol  $EC_{12.5}$ ) than the simple addition of the activity of each component. Combination with indigo or genistein also showed higher ( $156 \pm 50\%$  or  $189 \pm 35\%$ , respectively) than simple sum of each component activity.

### (3) Possible role of indirubin in the human body

Relatively low  $EC_{12.5}$  (12 nM) of indirubin in our reporter-gene assay and its concentrations in human urine (76 or 68 nM for male and female, respectively) suggest that indirubin may be present in enough amount to express its activity with AhR in human body. We are now measuring serum concentrations of indirubin in humans and rats.

Since indirubin was reported to be more sensitive in showing AhR activity than 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD)<sup>2</sup>, and only trace amounts of dioxins are present in blood of Japanese (26 pg TEQ/g fat in the blood corresponding to ca 8 pg TEQ/L or 3 fM in blood)<sup>7</sup>, it could be explained that exposure to extraordinarily excess amount of persistent dioxins can only interfere indirubin's physiological function by continuously expressing signals. True activity of indirubin in the human body needs to be examined, since originally yeast cells may not have CYP enzyme activity to metabolize indirubin.

In this regard, the significance of combination assay results needs further investigation. Vegetable constituents, such as flavones, flavonols, anthraquinones, resveratrol and coumestrol were shown to have inhibitory effects on the AhR activity induced by TCDD<sup>6</sup>. Indirubin is known to be a major component of herbal medicine effective to treat leukemia in China<sup>8</sup>. Indirubin is known to interact either directly or indirectly on cell-cycle regulation to arrest cell-cycle at G1-S stage<sup>9</sup>. Since complex cross-talk interactions between AhR and estrogen receptor were reported<sup>10</sup>, it is interesting that some AhR reactive chemicals, such as genistein, daidzein and resveratrol show also estrogen receptor binding and gene induction activities.

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## (16) 下水道未普及地域における河川生物膜 による直鎖アルキルベンゼンスルホン酸 浄化作用の評価

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わが国の下水道人口普及率は現在全国平均で約70%となっているが、地域格差が大きく下水道未普及地域の中小河川では未だに水質汚濁が深刻である。河川の自浄作用についてはT-N・T-Pといった栄養塩全体の濃度やBODのような間接的な有機汚濁指標を用いて検討した例はあるが、具体的な化学物質を用いた研究例は少ない。そこで本研究では陰イオン界面活性剤の直鎖アルキルベンゼンスルホン酸(LAS)に着目し、下水道普及率の低い徳島市近郊6河川を対象に3つの季節で生物膜を回収し、自浄作用を検討した。その結果、LAS汚染度が高い河川ほど浄化作用が大きく、LASの浄化作用とNH<sub>4</sub><sup>+</sup>-Nとの間には弱い相関が見られた。また生物膜の浄化作用に対する寄与率を計算したところ、下水道未普及地域における中小河川のLAS浄化に対して大きな役割を果たすことが示唆された。

**Key Words :** anionic surfactant, biodegradation, biofilm, household effluent, urban streams

### 1. はじめに

河川等の水環境の自浄作用を調べる際はこれまで、間接的な有機汚濁指標であるBODやT-N、T-Pといった栄養塩全体の濃度を用いることがほとんどであった<sup>1)</sup>。しかしながら、家庭用合成洗剤等に含まれる陰イオン界面活性剤の一種である直鎖アルキルベンゼンスルホン酸(Linear Alkylbenzene Sulfonate: LAS) (図-1) は、PRTR(環境汚染物質排出移動登録)法の第一種指定化学物質に分類されており、平成16年度の推計値によれば、水域への排出量が第一位の約19,000 tと非常に多く<sup>2)</sup>、その使用量や水環境中への排出量の多さから様々な調査が行われてきた。最近、わが国でおこなわれた例としては、真名垣らが冬季に全国18の一級河川中のLASの濃度をLCMS/MSを用いて測定し、淀川や多摩川、石狩川などでは最大で6 μg/L程度であるのに対して、鶴見川や菊川などで最大60 μg/Lという比較的高濃度で検出されており<sup>3)</sup>、水棲生物への影響が見られる濃度レベルとも近い<sup>4)</sup>ため、その影響が懸念される。周辺の下水道整備状況や流量等さまざまな要素が関連している。また、河川中のLAS濃度も微生物の活動に伴う自浄作用が盛んである

と考えられる夏季は低く、逆に低温で活動が弱いと考えられる冬季の方が有意に高いという報告もある<sup>5)</sup>。さらに、兵庫県南東部を対象にした調査では、下水道普及率が高いほど河川中の陰イオン界面活性剤濃度が低くなるという調査結果も報告されている<sup>6)</sup>。

一方、徳島県は平成18年3月現在、下水道人口普及率が全国平均の70%を大きく下回る11.5%<sup>7)</sup>、合併浄化槽等普及率を合わせても38.4%とともに全国最下位である<sup>8)</sup>ことから、LASを含む家庭雑排水の一部が河川に未処理のまま放流されている可能性が高い。本研究では、LASの主成分C<sub>12</sub>-LASを対象物質として、河川微生物に

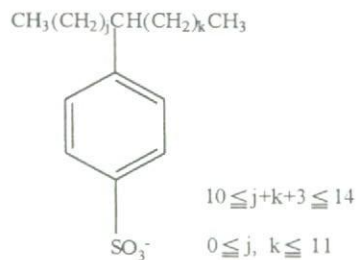


図-1 LASの化学構造

よる浄化能を調べることとした。これまで、行われた河川微生物によるLASの浄化能の定量化に関する検討は、Boeijeら<sup>9)</sup>のポリプロピレン担体を用いた水路実験や、Leeら<sup>10)</sup>の下水処理放流口の上下流での反応速度定数比較が報告されているものの、いずれも季節による違いや河川の汚染度による違いを十分に検討していない。そこで、本研究では2006年8月から2007年1月にかけて徳島市周辺の下水道未普及地域を流域とする6河川を対象に、3度の季節で相対的比較を主な目的とした。それに加えてBOD、TOC、河川中LAS濃度、 $\text{NH}_4^+\text{-N}$ 等の水質項目を生物膜採取中に測定し、LAS浄化能との相関を調べた。また、各河川におけるLAS負荷量をPRTR推計値や流域人口、流量、浄化槽普及率等をもとに推計するとともに、実験室内実験結果をもとに河川での単純なLAS浄化モデルに当てはめ、浄化率を算出した。

## 2. 河川水質調査及び実験方法

### (1) 選定河川

対象は下水道未普及地域を流域とする河川に限定した。また家庭用界面活性剤が直接排出されているおそれの高い流域人口の比較的多い河川と、流域人口が比較的小さい河川の両方を選定することにした。各河川のサンプリングポイントの水深、川幅、流量は冬季に測定しており、それぞれ田宮川が20 cm、2.7 m、0.28 m<sup>3</sup>/s、冷田川が25 cm、6.5 m、0.18 m<sup>3</sup>/s、多々羅川が60 cm、8.3 m、0.18 m<sup>3</sup>/s、打樋川が5 cm、5.3 m、0.027 m<sup>3</sup>/s、芝生川が60 cm、10 m、1.2 m<sup>3</sup>/s、正法寺川が75 cm、14 m、1.8 m<sup>3</sup>/sであった。各河川的位置を図-2に示す。



図-2 対象河川と採取地点の周辺地図

### (2) 生物膜回収装置

試験管立てに5 mm×20 mm×75 mmのスライドガラスを設置し、漂流物や河川に生息する大型の動物に影響を受けないよう防護ネットで覆った。スライドガラスの向きが河川の流れと平行になるように約3週間川岸から約30cm離れた川底に重石を付けて沈設し、生物膜を回収した。

### (3) 添加回収実験

100 mL三角フラスコに試験水を入れ、図-1に示すLASのうちその主成分として知られるC<sub>12</sub>-LAS(東京化成製標準品)を初期濃度1.0 mg/Lになるように添加した。試験水の量は超純水のみ、河川水のみ、河川水をそれぞれスライドガラスが完全に浸るように130 mLとした。超純水のみ、河川水のみ、河川水に採取した生物膜付着スライドガラスを1枚入れたものの3種類を2連ずつ用意し、河川水はOECDテストガイドラインにおける生分解試験<sup>11)</sup>を参考にメンブレンフィルター(孔径=3 μm)でろ過したものをを用いた。夏季25℃、秋季20℃、冬季15℃に設定、90 rpmで往復振とうして0、2、6、24、48、72、120時間に蛍光・吸光検出器付HPLCで溶液中のC<sub>12</sub>-LAS濃度を測定した。また、実際の河川では光分解の影響は生分解に比べて影響は小さいと考えられること<sup>12)</sup>、さらに実験系の単純化のために光分解の影響を避け、暗所で振とうした。なお、付着した生物膜量は各季節、各河川ごとに別のスライドガラスを3枚用意し、1枚当たりの乾燥重量を測定した。

### (4) 水質項目測定

各季節ごとの生物膜回収装置の沈設期間中で、降雨による影響を大きく受けないと考えられる日を選び、2回ずつ水質を測定した。以下にそれぞれの測定方法を示す。

#### a) 河川水中のC<sub>12</sub>-LAS濃度測定<sup>13)</sup>

2006/8/17および8/25(夏季)、10/20および11/8(秋季)、12/18および2007/1/11(冬季)の計6回測定した。河川水は採取後4℃で保存しながら速やかに実験室内に持ち帰った。ガラス繊維ろ紙(孔径=1.0 μm)でろ過後、Sep-Pak Plus C18カートリッジに通水したものをメタノールで溶出し、蛍光・吸光検出器付HPLC(島津製作所製LC-10ADVP)で測定した。

#### b) BOD測定<sup>14)</sup>

2006/8/1および8/28(夏季)、10/20および11/9(秋季)、12/18および2007/1/11(冬季)の計6回測定した。採取後速やかに実験室内に持ち帰った河川水を緩衝液、硫酸マグネシウム溶液、塩化カルシウム溶液、塩化鉄溶液を加え、101 mLふらん瓶3連にわけた。一つは15分後、残り

は、5日後にDO計（堀場製作所製）によってDOを測定し、その差をBODとした。

c) TOC 測定

2006/8/1 および 8/28（夏季）、10/20 および 11/9（秋季）、12/18 および 2007/1/11（冬季）の計6回測定した。採取した河川水にアジ化ナトリウムを入れ、有機物の分解を阻害し、速やかに実験室内に持ち帰った。これをガラス繊維ろ紙（孔径=0.7 μm）でろ過し、島津製作所社製 TOC-5000 で測定した。

d) NH<sub>4</sub>-N測定<sup>19)</sup>

2006/10/20 および 11/9（秋季）、12/18 および 2007/1/11（冬季）の計4回測定した。採取後速やかに実験室内に持ち帰った河川水をガラス繊維ろ紙（孔径=1.0 μm）でろ過し、インドフェノール青法を用いて測定した。

### 3. LAS浄化モデル

(1) 河川水や付着微生物膜によるLAS浄化作用の定量化

添加回収実験における河川水中の浮遊微生物やスライドガラス表面への付着微生物膜の量は、実験期間内は一定であると仮定した。この仮定は理論的には正確性に欠けるものの、採取した微生物膜量が基質であると考えられるC<sub>12</sub>-LASなどの量より過剰であること、実験実施中に生物膜量の時間変化を調べるのが困難であること、この単純化により溶液中のC<sub>12</sub>-LAS減少を擬似的に下式のように一次反応に当てはめることができるなどの理由による。

$$C/C_0 = \exp(-kt) \quad (1)$$

ここで、C<sub>0</sub>はC<sub>12</sub>-LASの初期濃度、Cは時間t(h)後のC<sub>12</sub>-LAS濃度、k(1/h)は一次反応速度定数である。本研究ではこのC<sub>12</sub>-LAS除去の反応速度定数kを算出することで、各河川の各季節でのLAS浄化作用を相対的に比較することとした。本研究では河川水のみk<sub>w</sub>と河川水+生物膜のkの両方を算出した。

(2) 反応速度定数を用いた河川中LAS浄化モデル

まずはじめに、単純に河川水+生物膜の系での反応速度定数kから河川水のみでの反応速度定数k<sub>w</sub>を差し引くことで、生物膜のみによる反応速度定数を求めた。さらに、実験系での河川水量(V<sub>L</sub>=130 mL)と生物膜付着面積(スライドガラスの表面積A<sub>L</sub>≈16 cm<sup>2</sup>)の比率(V<sub>L</sub>/A<sub>L</sub>≈8.1 cm)を計算し、実際の河川での実測した水深Dおよび川幅Wから河床が平面であると仮定して得られた水量(V<sub>R</sub>)と河床面積(A<sub>R</sub>)の比(V<sub>R</sub>/A<sub>R</sub>≈

DW/(2D+W))を計算した。この両者の比によって実験系での付着生物膜のみによる反応速度定数k<sub>0</sub>を実際の河川での付着生物膜のみによる反応速度定数k<sub>br</sub>に換算した。

$$k_{br} = k_0 \frac{V_L A_R}{A_L V_R} \quad (2)$$

そして、河川水のみでの反応速度定数k<sub>w</sub>とこのk<sub>br</sub>をもとに、流量や流速など実測データと合わせて生物膜採取地点から1 km先までの浄化率を試算した。ここでは、追加的LAS負荷や流量・流速の変化、生物膜量（および質）の時間的変化がなく、速度定数k<sub>w</sub>とk<sub>w</sub>+k<sub>br</sub>による分解のみを考慮した簡易な押し出し流れモデル(拡散なし)を仮定した。つまり、完全混合槽と考えられる微小区間でのマスバランスは以下のように仮定した。

$$V \frac{dC_{out}}{dx} = (C_{in} - C_{out})Q - kC_{out}V \quad (3)$$

なお、ここでVは微小区間の体積、C<sub>in</sub>とC<sub>out</sub>はそれぞれC<sub>12</sub>-LAS流入および流出濃度、Qは流量、kはk<sub>w</sub>もしくはk<sub>w</sub>+k<sub>br</sub>である。

### 4. 結果・考察

(1) 各河川・各季節のLAS浄化作用の相対比較

最初に添加回収実験における代表的なC<sub>12</sub>-LAS濃度の時間変化の例を図-3に示す。図に示すように、初期濃度に対する相対濃度C/C<sub>0</sub>は超純水のみでは減少は緩やかで120時間後でも初期濃度の約7割程度が残存していたのに対して、河川水を入れると120時間後には初期濃度の10%未満に、さらに生物膜付スライドガラスを入れると48時間後までに初期濃度の10%未満になった。

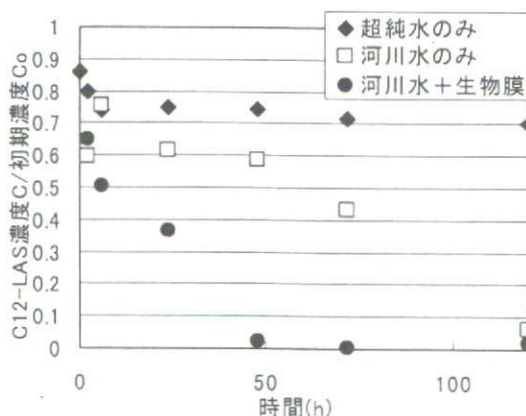


図-3 C<sub>12</sub>-LAS 濃度時間変化(冬季 田宮川)