表2 国内の水道におけるジアルジアの検出状況（平成10～14年度）

年度	地域	水道種別	浄水処理方法	対 応	備 考
10	福井県永平寺町	簡易水道	急速ろ過	浄水処理管理の強化	
11	山形県朝日村	上水道	塩素処理	他施設から受水	クリプトスポリジウムを同時に検出
12	青森県三戸町	簡易水道	塩素処理	膜ろ過施設設置	
12	岩手県平泉町	簡易水道	塩素処理	水源変更急速ろ過施設設置	
13	岩手県釜石市	簡易水道	緩速ろ過	浄水処理管理の強化	
14	山形県新庄市	簡易水道	塩素処理	水源周辺管理の強化	

中に多数のシストがある場合は直接塗沫標本で観察することができる。永久標本を作製して詳細に内部構造を観察するにはコーン染色変法等が勧められる。シストの数が少ない場合は，集シスト法としてFEA法（酢酸エチルを用いたMGL法の変法）を用い，得られた沈渣を鏡検する。

おわりに

ランブル鞭毛虫の分類は流動的で，今後の遺伝子型別等の進展によっては数多くのサブタイプが見出されたり，種の再構築がなされたりする可能性がある。遺伝子型と宿主特異性の関係をさらに詳細に調査し，ヒトと共通して感染する遺伝子型の明確，感染源あるいは汚染源対策を充実させていかなければならない。

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宿主アメーバからみた
レジオネラの水系汚染対策

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はじめに

レジオネラ汚染防止対策の要点は，環境中でのレジオネラの増殖様式と伝播様式の2点を正しく理解することである．レジオネラがいかにして環境を汚染し，どのような経路を経てヒトに感染していくかを知れば自ら対策がみえてくるはずである．対策を論ずることの必要性は重々承知しているが，一歩引いてレジオネラ問題を考えてみると，ところかまわず知に走る人の姿がみえてくる．しかし，所詮は人も自然の中に生かされているにすぎない．自然の恩恵を享受することはできても，その営みを制御することはできないと知るべきである．この観点に立つと，直面するレジオネラ問題に対して臆することなく白旗をあげることが歴とした「人知」にみえてくる．

■環境水中でのレジオネラの増殖様式

レジオネラ属菌の詳細については他書に譲るが，レジオネラ属菌の特徴は細胞寄生性であることで，ヒトに感染した場合にはマクロファージなどに寄生して，その中で増殖する．同様に，環境中にあってはアメーバなど原生動物を宿主として増殖している(図1)[1]．すなわち，環境中のレジオネラは宿主となる原生動物が存在することではじめて増殖が可能となる．これまでにレジオネラの宿主として温水(≦42℃)に棲息する Acanthamoeba や

Naegleria, Hartmannella などといったアメーバ類や Tetrahymena(繊毛虫)などが報告されている[2]．一般の従属栄養細菌類とは異なり，レジオネラ属菌は細菌捕食性の原生動物に取り込まれても消化されることなく寄生・増殖し，やがて宿主を破壊する．例えば，Acanthamoeba を宿主とした場合，感染後2日程度で増殖したレジオネラが宿主の細胞質をほぼ埋め尽くす(図1d，図2)．やがて細胞の中で活発に運動を始め，水に浮遊させた条件で観察すると運動が始まってから10分程度で細胞膜を破り水中に遊出する(図1f，図3)．レジオネラの増殖は盛んでアメーバ1匹当たり優に1,000個を超える数となる．

これまでの感染事例で浴槽水など原因となった水の菌数は $10^{3 \sim 6}$ cfu/100mL の範囲であった．単純計算すると，そのような環境水中には $10^{0 \sim 3}$ 個/100mL の感染したアメーバが存在したことになる．これを 20m³ 程度の営業用の浴槽にあてはめると，浴槽水中には $10^{5 \sim 8}$ 個オーダーの感染したアメーバが必要となる．生態学的な妥当性からすれば，存在するすべてのアメーバが一斉に感染することは考えられず，寄生されているものはせいぜい1%にも満たないと想定される．この単純な仮定が大筋で正しいとすると，事故につながるような浴槽の微生物量は生半なものではないことがうかがえるであろう．
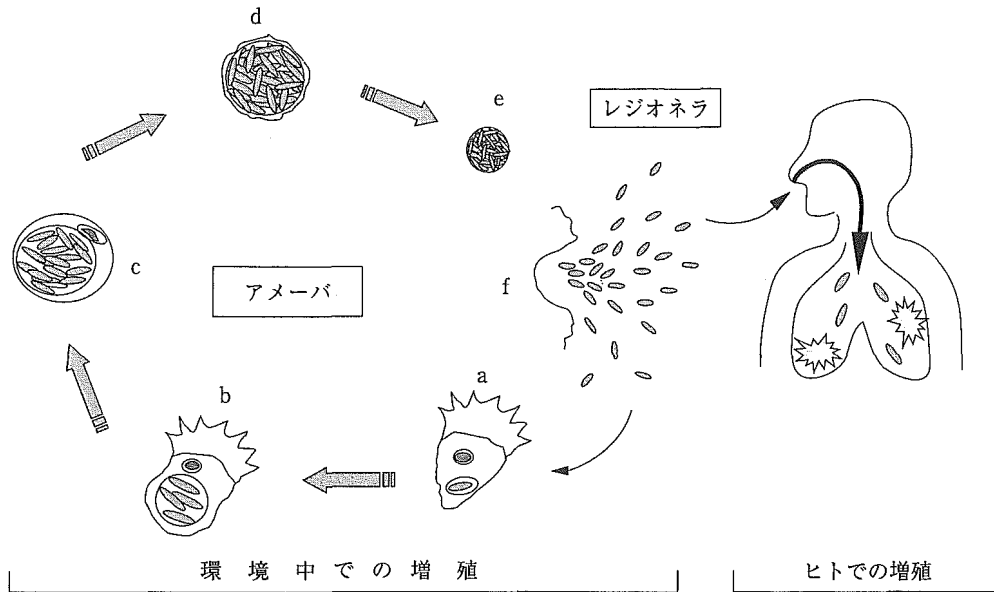
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図1　レジオネラの増殖様式

環境中での増殖　　　ヒトでの増殖

■レジオネラの伝播経路と汚染原因

すでによく知られるところであるが，レジオネラの感染様式は汚染された水から発生したエアロゾルを吸引することによる．一般に，菌を内包したままで肺胞内に到達し得るエアロゾルの粒径は2〜5μm程度とされている[3]．

われわれの生活環境にあって，エアロゾルの発生につながる装置としては空調設備の冷却塔，浴槽の各種付属設備，加湿器や噴霧器，あるいは水を用いる歯科・医療器具などさまざまなものがあげられる．これらの装置では汚染理由がそれぞれ異なるが，根本的な理由は共通する．すなわち，温水などに発生するバイオフィルム対策の欠除がレジオネラ問題を招来させると考えてよい．

以下，わかりやすい例として循環式浴槽を取り上げて説明する．循環式浴槽の基本構造を図4に示した．本装置は浴槽水をろ過循環させて長期にわたり継続使用しようとするものである．このような装置には入浴者が持ち込む汚れ（有機物汚染）を取り除くための装置，ろ過槽が設置され，槽内に積極的に微生物を繁殖させている．いわゆる生物浄化といわれるもので，浴槽水中に持ち込まれた有機物を微生物に捕食・吸収させて除去しよう
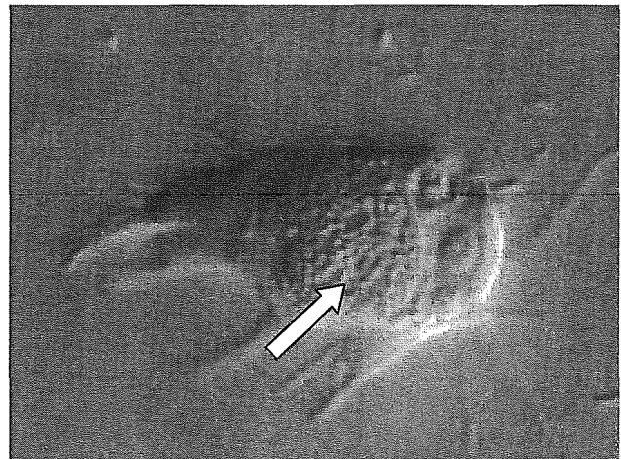

図2　アカンソアメーバに寄生したレジオネラ
細胞質の大半を埋めるレジオネラ（矢印）

とするものである．下水処理などにおける活性汚泥に対応するもので，浴槽水中の有機物除去には効果的であることはよく知られる．図5に示すごとく，浴槽水中の有機物量（過マンガン酸カリ消費量として表現）は細菌数の増加に伴い顕著に減少する．たしかに，浴槽水の過マンガン酸カリ消費量に着目した場合は生物浄化によって見かけの清浄度は回復・保障される．しかしながら，循環式浴槽全体を閉じた系として考えると，全く別のストーリーがみえてくる．すなわち，本システム
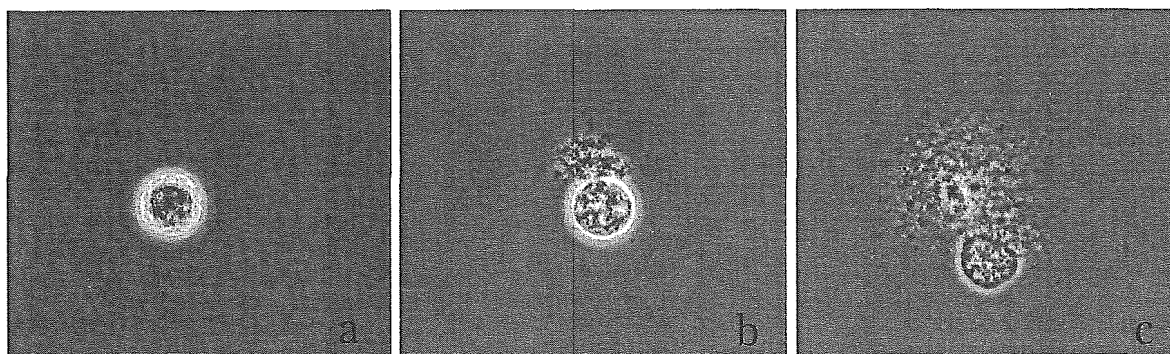
図3　レジオネラによる宿主アメーバの破壊の様子
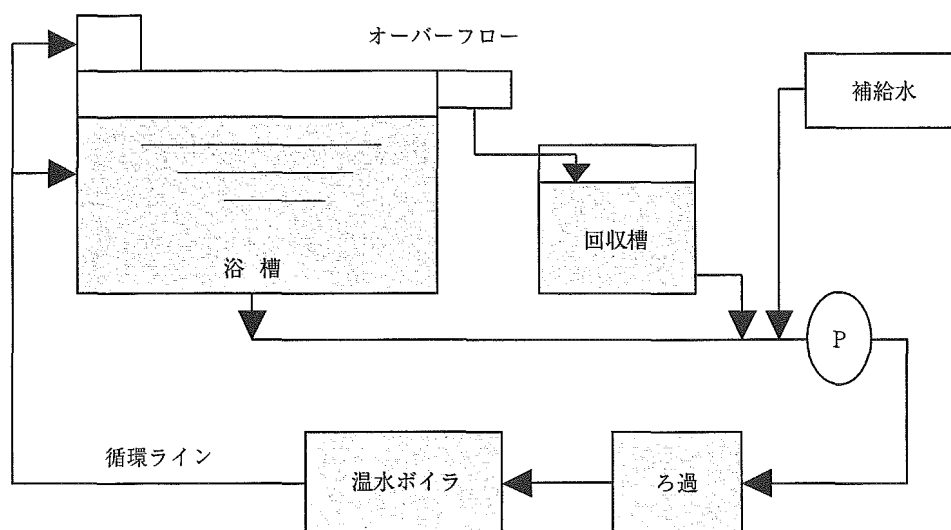a：破壊寸前のアメーバ．管壁当への付着ができなくなり，浮遊状態となる．b：破壊直後．
c：レジオネラが周囲に分散していく

図4　循環式浴槽水のフロー

では入浴者によって持ち込まれる有機物を微生物に変えてはいるが，系外に運び出しているわけではない．そのため，生き物を含めた総有機物量は系内に蓄積され続ける仕組みといえる．さらに，微生物へ形が変わることで厄介なことに系内にレジオネラの増殖の場が形成されてしまう．

厚生労働省は，先の「公衆浴場法第3条第2項並びに旅館業法第4条第2項及び同法施行令第1条に基づく条例等にレジオネラ症発生防止対策を追加する際の指針について（健発第1029004号，平成14年10月）」で遊離残留塩素（1日2時間以上0.2〜0.4mg/Lを保つこと）による浴槽水の管理を指示している．循環式浴槽の設計思想は生物浄化に依拠した有機物除去であり，この装置に遊離残留塩素管理を持ち込むことはシステムの心臓部ともいえる生物浄化機能を破壊することにほかならず，この時点で循環式浴槽という発想は実質的に破綻する．食品の分野では HACCP（Hazard Analysis and Critical Control Point：危害分析重要管理点）という概念が浸透しつつある．この概念の骨子は危害因子の排除と予防であるが，これにならえば，ろ過槽の撤去と残留塩素による管理こそが循環式浴槽の対策といえるのではないかと考える．
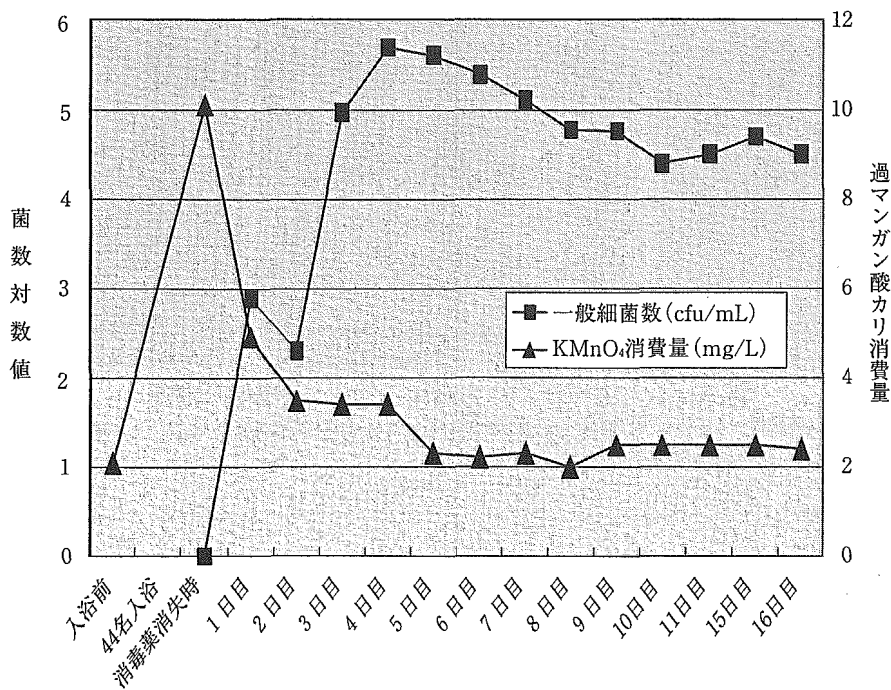
図5　浴槽水中の一般細菌と過マンガン酸カリ消費量の挙動

■汚染防止対策

1．冷却塔水

　レジオネラ症の歴史的背景から冷却塔水のレジオネラ汚染は有名で，かつての集団感染ではほとんどこの装置が汚染源とされていた．事実，冷却塔水が外気に直接触れる構造となっており，微生物の侵入繁殖は防げない．少々古いデータであるが，日本全国の冷却塔水180試料について行ったアメーバとレジオネラの調査によれば，*Acanthamoeba* や *Hartmannella* をはじめとして多種類のアメーバが検出され，検出率はおよそ90％に達していた[4]．その一方で，空調設備の維持のために冷却塔水の水質管理を徹底する必要があること，大気中へのエアロゾルの飛散を抑える構造が考案されていること，さらに，室内への外気取入口や窓，および人が活動する場所から冷却塔を10m以上離すことなどが義務づけられていることが功を奏してか，わが国では冷却塔を介したレジオネラ症の報告は聞かれなくなっている．

2．入浴施設

　循環式の浴用施設が普及するに伴って，入浴施設を介したレジオネラ症の報告が増えている．入浴施設にはジャグジー，ジェットバス，打たせ湯（滝を模した装置），そのほか，エアロゾルの発生しやすい装置が多数存在する．循環式浴槽にこれらの装置を併設しないことが厚生労働省から通達されている．また，すでに述べたように，厚生労働省から浴槽水の遊離残留塩素管理が通達されている．図6は浴槽水の遊離残留塩素濃度と浴槽水pH，および宿主アメーバの出現状況をプロットしたものであるが，0.2mg/L以上の遊離残留塩素濃度が維持されているほとんどの浴槽でアメーバが不検出になっており，安全が確保されていることが示されている[5]．

　なお，循環浴槽系ではシステム内の殺菌・洗浄が伴わない換水は汚染防止にほとんど効果を示さないことが指摘されている．ある調査によると，10^5cfu/100mL程度のレジオネラを含む浴槽水を完全に換水しても，数時間後には 10^4cfu/100mLのレベルに達することが示されている．当該循環
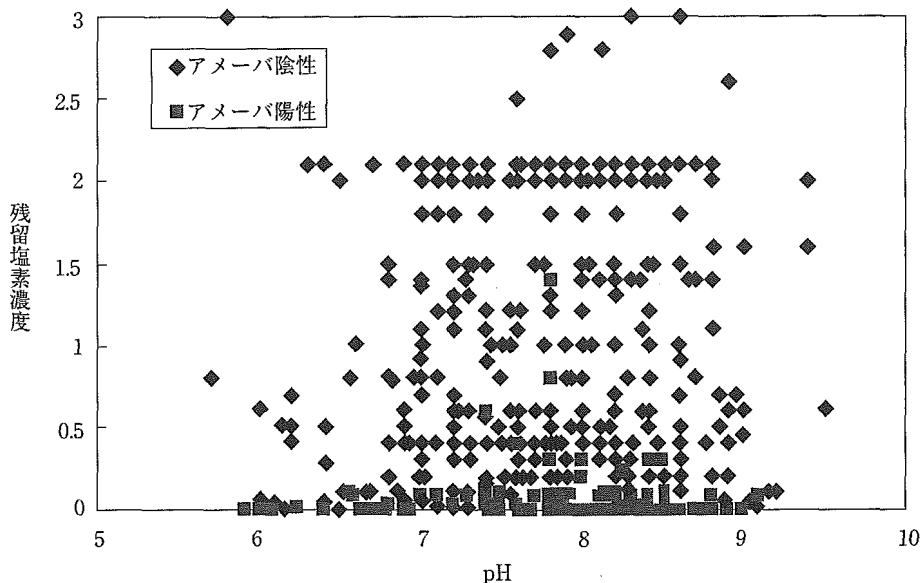
図6　残留塩素濃度と浴槽水pHとアメーバ検出の関係
厚生労働科学研究費補助金（がん予防等健康科学総合研究事業）総合研究報告書「平成13-15年度温泉・公衆浴場，その他の温水環境におけるアメーバ性髄膜脳炎の病原体*Naegleria fowleri*の疫学と病原性発現に関する研究」より転写

装置のろ過槽内の汚泥には 5×10^6cfu/g のレジオネラが存在しており，ろ過槽を含めた系全体の洗浄・消毒の重要性があらためて指摘された．

3．貯湯槽

温泉水，井水，水道水など，いかなる水であっても有機物を全く含まない水はない．そのため，貯湯槽を長期にわたり使い続けると徐々にではあるがバイオフィルム形成が起こり，やがてレジオネラ汚染につながる．そこで，湯水の補給口や貯湯槽底部の温度に至るまで貯湯槽の温度を 60℃以上に保つこと，最大使用時においても 55℃以上を保つよう指導されている．この温度に達しない場合には湯水の消毒が必要となる．また，原水の水質を考慮して，定期的に貯湯槽内のバイオフィルム除去を行うことが望ましい．

4．そのほかの器具など

レジオネラ症は冷却塔や修景用噴水，超音波式ネブライザー，加湿器や温泉浴槽，場合によってはシャワーや呼吸補助装置などを介した発生が知られている[6]．例外的にではあるが，プランターの土や腐葉土で増殖していたレジオネラ（*L. longbeachae*）が原因と考えられる集団感染事例の報告がある[7, 8]．

加湿器や噴霧器，あるいは水を用いる歯科・医療器具などにおいては定期的な高濃度の塩素消毒，水の滞留を防ぐ工夫と，洗浄しやすい単純な構造であることが求められる．あわせて，ホースなどの交換を頻繁に行うことが汚染防止につながる．

■利便性の追求とリスク

レジオネラ問題に関しては利用者（消費者）側の問題も指摘される．例えば昨今の温泉ブームである．そこでは，無批判に大規模な浴槽やジェット噴流式の浴槽などが好まれる傾向がある．浴槽の容量が大きくなれば水量の関係から必然的に循環装置の設置につながる．有機物汚染が進んだ温水に微生物が繁殖しないはずもなく，その中にレジオネラなど病原性を示す微生物が存在しても何の不思議もない．直面しているレジオネラ問題は，便利さや快適さを求める過程で人が作り出した温

水環境を介して起きている．換言すれば，便利さや快適さを求める過程で発生した人災といえる．理論的には，この問題の解決はきわめて容易である．そもそも，循環式浴槽がなければこの問題は起きなかったわけで，設備を使用しなければ本件は解決する．しかしながら，使用されていることを前提とした現実対応はきわめて難しく，その衛生管理に要する労力は計り知れない．ここでは触れなかったが，やむを得ず用いている塩素消毒に問題がないわけではない．塩素を使用する限り副生成物による健康影響は避けられない問題である．本件に関しては原点に立ち返って必要性について是非論を交わすべき時期にきていると考える．

ちなみに，公衆浴場法では浴槽水の有機物汚染に係る水質基準として過マンガン酸カリ消費量が25mg/L 以下であることと規定されている．また，浴槽原水の基準値は過マンガン酸カリ消費量10mg/L 以下とされている．この基準値が何を根拠として定められたのか不勉強にして知らないが，原水の水質を考慮して 15～25mg/L の範囲の持ち込みが上限となる．われわれの試算では，入浴者一人が持ち込む有機物量は過マンガン酸カリ消費量に換算して，およそ0.5 g であった．仮に入浴者の持ち込む汚れ(有機物)を通して入浴者数を規制するものであるとすれば，20m³ の浴槽水に延べ 600～1,000 人が入浴すると公衆浴場法に定める基準値を超える(違反)計算である．200L 程度の家庭用の浴槽にあてはめると延べ 6～10 人の入浴者に相当する．この基準の是非についても合わせて議論していくべきものと考えている．

いずれにせよ，利便性の追求が生活環境の悪化を交換条件としているとするならば，あまりにも知恵のないことである．

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Short communication

Analysis of herbicides in water using temperature-responsive chromatography and an aqueous mobile phase

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Abstract

A simple and rapid method has been developed for herbicides in water using temperature-responsive liquid chromatography (LC) and a column packed with poly(N-isopropylacrylamide) (PNIPAAm), a polymer anchored on the stationary-phase surface of modified silica. PNIPAAm reversibly changes its hydrophilic/hydrophobic properties in water in response to temperature. The method was used to determine five sulfonylurea and three urea herbicides. Separation was achieved with a 10 mM ammonium acetate (pH 3.0) isocratic aqueous mobile phase, and by changing the column temperature. The analytes were extracted from water by off-line solid-phase extraction (SPE) with an N-vinyl-pyrrolidone polymer cartridge. The average recoveries of the eight herbicides from spiked pure water, tap water and river water were 70–130% with relative standard deviations (RSDs) of <10%. The limits of quantitation (LOQ) of the eight herbicides were between 1 and 4 μg l^{-1}.

1. Introduction

Herbicides are used in rice paddies, golf courses, and other types of fields. They are transported by aquifers in groundwater and are widely distributed in the environment. Sulfonylurea herbicides are labile, weakly acidic compounds. Sulfonylurea and urea herbicides are used at lower concentrations, and are more rapidly degraded in soil than older herbicides. Therefore, parts-per-billion concentrations of these herbicides are to be expected in the water supply. These herbicides have been analyzed in water by liquid chromatography (LC) with UV detection [1,2], capillary electrophoresis with UV [3], LC with mass spectrometry (MS) [4,5], immunoassay [6], bioassay [7] and radio immunoassay [1].

Recently, various polymers have been developed which change their structure in response to surrounding conditions, such as the pH, electric field, and temperature. Such polymers have been widely utilized in drug delivery systems [8], cell culture dishes [9], cell sheets [10] and bioconjugates [11]. Poly(N-isopropylacrylamide) (PNIPAAm) is one of these; it exhibits a thermally reversible phase transition in response to temperature changes across a lower critical solution temperature (LCST) of 32 °C in aqueous solution [12]. In water, the polymer chains of PNIPAAm hydrate and expand below this LCST, while they dehydrate to form a compact conformation above it. We previously reported a considerable and reversible change in the hydrophilic/hydrophobic properties of PNIPAAm-grafted surfaces in response to a change in temperature. Taking advantage of this characteristic, we developed an LC column packed with PNIPAAm to selectively separate analytes by controlling the external column temperature [13–17].

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Temperature-responsive chromatography is a method with little load on the environment, because no organic solvent is used in the mobile phase. Urea herbicides in environmental water have been widely studied by Hogenboom and co-workers [2,18,19] and very rapid analyses were made by using a single short column for both SPE and analytical separation. However, there are fewer reports on sulfonylurea herbicides [5]. The aim of this study was to achieve the separation of both groups of herbicides by temperature-responsive LC with an aqueous mobile phase.

2. Experimental

2.1. Chemicals

Analytical-grade standards of bensulfuron-methyl (99.7%), imazosulfuron (99.7%), pyrazosulfuron-ethyl (99.9%), halosulfuron-methyl (100%), siduron (98.9%), daimuron (100.0%) and diuron (100.0%) were purchased from Wako Pure Chemical Industries, Osaka, Japan. Analytical-grade flazasulfuron (99.9%) was purchased from Hayashi Pure Chemical Industries, Osaka, Japan. The structures of these herbicides are shown in Fig. 1. N-isopropylacrylamide (NIPAAm) was kindly provided by KOHJIN, Tokyo, Japan and was purified by recrystallization from n-hexane. 3-mercaptopropionic acid (MPA), 2,2'-azobisisobutyronitrile (AIBN), N,N-dimethylformamide (DMF), ethyl acetate, 1,4-dioxane, N,N'-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide,

HPLC-grade tetrahydrofran (THF) and ammonium acetate were purchased from Wako Pure Chemical Industries. Aminopropyl silica beads (average diameter, 5 μm; pore size, 120 Å) were purchased from Nishio Industries, Tokyo, Japan. The pure water used for sample preparation and the LC mobile phase was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The synthesis of PNIPAAm and a modification of amino-propyl silica with the NIPAAm polymer were carried out by radical polymerization, as previously reported [13,20].

2.2. Temperature-responsive LC

A PNIPAAm-grafted silica beads were packed into a stainless-steel column (150 mm × 4.6 mm i.d.). LC was carried out on an Agilent 1100 series (Agilent, Waldbronn, Germany) instrument equipped with a UV detector and a Rheodyne Model 7750 injector. The column oven was a product of Shodex AO-30C (Showa Denko, Tokyo, Japan). The mobile phase was 10 mM ammonium acetate (pH 3.0). The thermoresponsive elution behavior of the herbicides was monitored at 240 nm at a flow rate of 1.0 ml min^{-1} at various temperatures. The injection volume was 20 μl.

2.3. Preparation of standard solutions

Stock solutions (1000 mg l^{-1}) of each analytical standard were prepared in THF. Next, working standard mixtures were prepared by diluting each herbicide stock solution with THF. These stock solutions were stored at 4 °C. Standard solutions

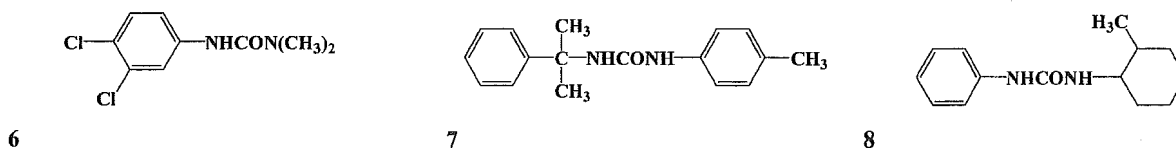## Sulfonylurea herbicides
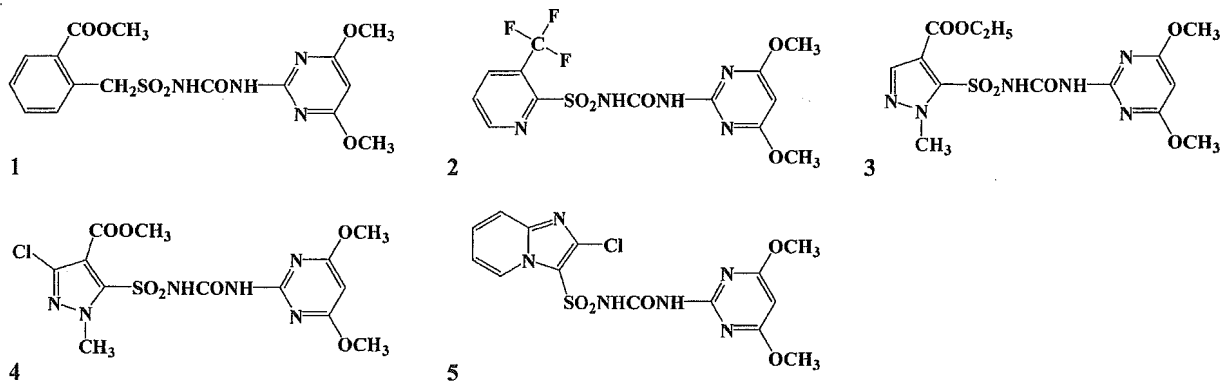
Urea herbicides

Fig. 1. Structures and common names of the eight herbicides. 1, bensulfuron-methyl; 2, flazasulfuron; 3, pyrazosulfuron-ethyl; 4, halosulfuron-methyl; 5, imazosulfuron; 6, diuron; 7, daimuron; and 8, siduron.

were prepared by diluting the stock solution with THF. The standard solutions were used for calibration plots and spiking of the water samples.

2.4. Water samples

Three types of water were analyzed: pure water, tap water and river water. The tap water was from a tap in the laboratory. L(+)-Ascorbic acid sodium salt (Wako Pure Chemical Industries) was added to the tap water at 0.005% (w/v), which eliminated chlorine that could react with and degrade some of the compounds of interest. The river water was collected from the Tama River near Tokyo; it was filtered through a glass-fiber filter before use.

2.5. Analytical methods

For recovery studies, three water samples (0.5 l each) were spiked with 1 ml of 2 mg l^{-1} (except for 0.5 mg l^{-1} diuron and daimuron) of the composite standard. Then, the spiked water samples were passed through a SPE cartridge to extract the analytes [5]. SPE was performed with cartridges prepacked with N-vinyl-pyrrolidone polymer resin (Oasis HLB Plus Extraction Cartridges) from Waters (Milford, MA, USA). The SPE cartridges were equilibrated with 5 ml of methanol and then 5 ml of pure water. The water samples were extracted at a 10 ml min^{-1} flow rate. Then, the cartridges were washed with 10 ml of pure water at a 5 ml min^{-1} flow rate and dried with air passed through the cartridge for 40 min. The herbicides were eluted from the cartridges with 3 ml of methanol at a speed of 1–2 drops s^{-1}. After evaporating the samples to near-dryness under a gentle nitrogen stream, the materials were dissolved to a final volume of 1.0 ml in THF.

3. Results and discussion

3.1. Sulfonylurea herbicides

Sulfonylurea herbicides were separated based on their temperature-controlled hydrophilic/hydrophobic properties by using an LC system connected to a column packed with PNIPAAm-modified silica beads. Fig. 2(a) shows van't Hoff plots for sulfonylurea herbicides separated using a PNIPAAm-modified column in 10 mM ammonium acetate (pH 3.0). The linearity in the van't Hoff plots is commonly observed for commercially available reversed-phase columns under standard chromatographic conditions. On the PNIPAAm-modified column, however, a deviation from linearity was found between ln k values and the reciprocal temperature (1/T). Interestingly, the slope of the van't Hoff plots of each analyte on the PNIPAAm-modified column changed markedly at the LCST boundary (Fig. 2 (a)). This corresponds to a phase transition of the polymer modified on the surface. Typical chromatograms for the standards of the five sulfonylurea herbicides using the PNIPAAm-modified column at 10 and 50 °C are shown in Fig. 3.

The log P values of these herbicides are given in Table 1. log P values were calculated by the CACHe system (Fujitsu, Japan). We reported in previous papers that the order of separation on a temperature-responsive-polymer-modified column depends on the hydrophobicities, corresponding to increasing log P values [13]. In this study, the retention time of the strongly hydrophobic imazosulfuron was remarkably increased, compared with four other sulfonylurea herbicides. When trying to separate the same herbicides on an ODS column using an aqueous/organic solvent, the three peaks of bensulfuron-methyl, flazasulfuron and imazosulfuron overlapped, and the two peaks of pyrazosulfuron-ethyl
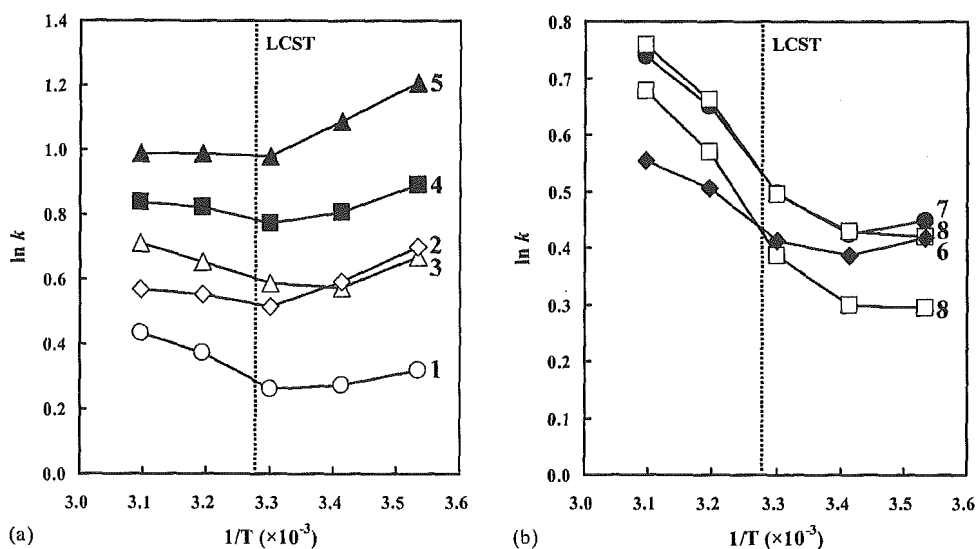

Fig. 2. van't Hoff plots of (a) sulfonylurea and (b) urea herbicides. For LC conditions, see Section 2. For peak numbers, see Fig. 1.
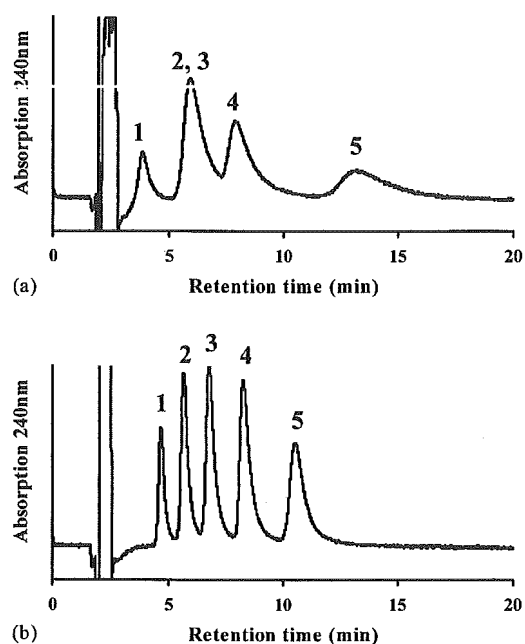
Fig. 3. LC–UV of standards using a PNIPAAm-modified silica column at (a) 10 °C and (b) 50 °C. For LC conditions, see Section 2. For peak numbers, see Fig. 1.
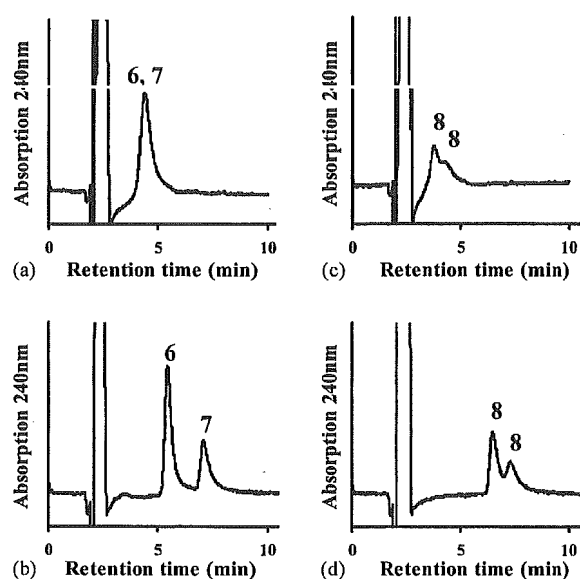

Fig. 4. LC–UV of standards using PNIPAAm-modified silica column at (a) and (c) 10 °C, and (b) and (d) 50 °C. For LC conditions, see Section 2. For peak numbers, see Fig. 1.

3.2. Urea herbicides

The urea herbicides were separated using conditions similar to those for the sulfonyulurea herbicides. Fig. 2(b) shows van't Hoff plots for urea herbicides using a PNIPAAm-modified column. For urea herbicides, the $\ln k$ values increased markedly above the LCST (or lower $1/T$ values), indicating a hydrophobic interaction between the analyte molecules and the hydrophobized stationary phase surface of the column. The difference in retention behavior of the sulfonylurea and urea herbicides reflects differences in their physicochemical properties. Typical chromatograms for the standards of the two urea herbicides, and siduron using the PNIPAAm-modified column at 10 and 50 °C are shown in Fig. 4. Siduron gave two peaks corresponding to its cis/trans isomers. The retention times of urea herbicides also increased with the log P values. An increase in the retention times with increasing temperature was clearly observed.

3.3. Analytical performance

The calibration plots of all eight herbicides using temperature-responsive LC at 50 °C were linear. The concentrations range of the five sulfonylurea herbicides were 0.2–10 mg l^{-1} (six data points in triplicate), those of diuron and daimuron were 0.2–2.0 mg l^{-1} (four data points in triplicate), and those of siduron were 0.5–10.0 mg l^{-1} (five data points in triplicate). In all cases, the R^2 values were at least 0.995 (Table 1). Because siduron has two isomers, the area of the two isomer peaks was calculated and summed to give the total amount of siduron. The LODs of the eight herbicides were 0.2–0.5 mg l^{-1} (Table 1).

and halosulfuron-methyl also overlapped (data not shown). In contrast, upon raising the column temperature of the temperature-responsive system, these five sulfonylurea herbicides could be separated from each other with an aqueous mobile phase.

In this study, the mobile phase was adjusted to pH 3 which was lower than the pK_a values of these herbicides, bensulfuron-methyl (pK_a 5.2), flazasulfuron (pK_a 4.37) and imazosulfuron (pK_a 4.0), in order to suppress their ionization and effect their interaction with the surface of the stationary phase. With increasing temperature, the temperature-responsive surface of the stationary phase changed from hydrophilic to hydrophobic, the retention time increased as a result of hydrophobic interaction, and the separation of the five sulfonylurea herbicides markedly improved.

Table 1
Calibration, LOD and log P data for the eight herbicides

Compound	Calibration equation[a]	R^2	LOD (mg l^{-1})	log P
Bensulfuron-methyl	$y = 12.493x + 0.6557$	1.000	0.5	1.49
Flazasulfuron	$y = 9.8272x - 0.5951$	0.998	0.5	1.93
Pyrazosulfuron-ethyl	$y = 8.976x - 1.1398$	0.997	0.5	0.66
Halosulfuron-methyl	$y = 12.011x - 1.3876$	0.998	0.5	1.21
Imazosulfuron	$y = 16.043x - 0.951$	1.000	0.5	2.15
Diuron	$y = 20.209x + 0.6761$	0.996	0.5	2.15
Daimuron	$y = 11.74x - 0.2518$	0.995	0.2	3.61
Siduron	$y = 13.661x - 0.2925$	0.999	0.2	2.86

[a] y = area; x = concentration (mg l^{-1}).

Table 2
Performance data for extracting five sulfonylureas and three ureas from pure water, tap water and river water

Compound	Pure water			Tap water			River water		
	Recovery[a] (%)	RSD (%)	LOQ (μg l^{-1})	Recovery[a] (%)	RSD (%)	LOQ (μg l^{-1})	Recovery[a] (%)	RSD (%)	LOQ (μg l^{-1})
Bensulfuron-methyl	91	3.6	4	94	2.2	1	88	6.4	4
Flazasulfuron	90	1.9	1	86	1.7	1	72	9.7	4
Pyrazosulfuron-ethyl	93	1.6	1	98	2.5	1	100	5.0	4
Halosulfuron-methyl	90	2.7	1	98	1.1	1	97	4.5	4
Imazosulfuron	86	1.8	1	98	1.8	1	89	6.7	4
Diuron	91	4.5	1	84	6.8	1	97	4.5	1
Daimuron	127	2.8	1	100	5.3	1	94	6.0	1
Siduron	93	2.5	1	87	3.2	4	100	6.0	4

[a] Mean values from three individual samples.

3.4. Application

Water samples were prepared by adding $4\,\mu$g l^{-1} (final concentration) of all herbicides, except for diuron and daimuron, which were added at a final concentration of $1\,\mu$g l^{-1} to pure water, tap water, or river water. Then, 0.5 l of each sample was concentrated 500-fold by SPE. Using temperature-responsive chromatography, these eight herbicides were detected with acceptable recoveries and precisions (70–130% and relative standard deviation, RSD $\leq$10%, respectively) (Table 2).

4. Conclusions

Temperature-responsive LC with an aqueous solution without organic solvents as mobile phase can be used to determine sulfonylurea and urea herbicides. Combined with off-line SPE, trace levels of the herbicide can be quantified in real-life samples.

In temperature-responsive LC, analyte behavior is controlled merely by the temperature, without any changes in the mobile-phase composition.

Acknowledgement

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Use of cholinesterase activity as an indicator for the effects of combinations of organophosphorus pesticides in water from environmental sources

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Abstract

Organophosphorus pesticides (OPs) are commonly detected in agricultural products, animal-derived foodstuffs, and environmental samples. Until now, the focus of research has been to evaluate the adverse effect of a single OP. While each OP may be present at concentrations under recognized as "no observed adverse effect level (NOAEL)", the combined effects of multiple OPs present at these low concentrations have not been sufficiently studied. Therefore, we developed an in vitro testing method to evaluate the toxicity of multiple OPs based on the degree of inhibition of cholinesterase (ChE) activity. This method requires only 10 min to complete and no specialized technology. We examined 15 OPs by this method and categorized them into three groups according to the degree of ChE inhibition. A relationship between the OPs' chemical structures and the degree of ChE inhibition emerged with the moiety –P–O–C=N– showing the strongest action. The degree of ChE inhibition increased with multiple OPs, and the degree of inhibition seemed to be additive. These results demonstrate that the combined toxicity of multiple OPs present in food or environmental samples is an easily determined and toxicologically relevant measure of overall toxicity of complex OPs mixtures. It is possible to apply this testing method as a monitoring technique in water quality management in order to control OPs. As a result, this method can play the role for the potential risk reduction to the ecosystem and may contribute to the preservation of the environment.
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Keywords: Organophosphorus pesticides; Hazard assessment; Cholinesterase activity; In vitro method; Combined toxicity; Additive action

1. Introduction

Recently, various environmental pollutants have become a major source of concern with regards to their potential adverse effects on humans exposed to combinations of these environmental pollutants. Water influences the health of both humans and animals and is greatly affected by water pollutants such as he organic compounds discharged from industrial, agricultural, and domestic drainage. Among the organic compounds, most concern, due to their

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extensive use worldwide and common production (Donaldson et al., 2002), are organophosphorus pesticides (OPs). Present in agricultural runoff, OPs may enter the aquatic environment and reach high concentrations downstream from discharge sites, potentially producing adverse effects in humans and wildlife (Haywood and Karalliedde, 2000; Fleischli et al., 2004).

Acetylcholinesterase (AChE; E.C. 3.1.1.7), the target enzyme of most OPs and their active metabolites, is a key neuroregulatory enzyme that is common to many species from insects to reptiles, birds and mammals. In addition, pseudocholinesterase (PChE), an enzyme present in serum, is also targeted and inhibited by OPs (Ma and Chambers, 1995, 1994; Sultatos, 1987; Sultatos et al., 1985). Toxicity of OPs is a composite effect that depends on concentration, duration of exposure, and organism size. Different organisms display different degrees of sensitivity to cholinesterase (ChE) inhibitors as OPs act as suicide substrates for AChE and PChE, irreversibly phosphorylating critical serine residues in the enzyme active site, leading to irreversible inactivation of the ChE in cholinergic synapses and neuromuscular junctions (Betancourt and Carr, 2004; Liu et al., 2002; Amitai et al., 1998; Mileson et al., 1998). To attain an acceptable daily intake (ADI) of OPs, a single pesticide is administered using a suitable animal model to assess toxicity. Unfortunately, OPs are rarely present in environmental samples as single chemical compounds, but commonly coexist as mixtures of different pesticides; thus, it is important to evaluate the total toxicity of multiple OPs in a sample using an indicator of overall potential toxicity.

While it is expensive and time consuming to evaluate the combined effects of multiple pesticides in laboratory animals, there are few efficient alternative test methods. The limited investigation of acute interactive toxicity of mixtures containing two OPs has previously been done (Karanth et al., 2004, 2001; Hazarika et al., 2003; Richardson et al., 2001). The multiple OPs we chose to evaluate can be grouped into two categories according to their structures: (1) nine chemical compounds containing a P═S moiety: butamifos, chlorpyrifos, diazinon, EPN, fenitrothion (MEP), isofenphos, isoxathion, prothiofos, and tolclofos-methyl; and (2) six chemical compounds containing a P═O moiety: acephate, dichlorvos (DDVP), edifenphos (EDDP), fosetyl, iprobenfos (IBP), and trichlorfon (DEP). In this paper, we establish a simple, inexpensive, and robust in vitro evaluation system suitable for hazard assessment of combinations of multiple OPs. Using 5-methyl -2-thenoylthiocholine-iodide (MTTC) as an indicator of ChE inhibition, the overall toxicity of OP mixtures, representative of those typically encountered in environmental water samples, was examined.

2. Materials and methods

2.1. Chemicals

Butamifos oxon, chlorpyrifos oxon and tolclofos-methyl oxon were obtained from Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan) and chlorpyrifos was obtained from GL Sciences Inc. (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Laboratory water was purified using a Milli-Q gradient A10 Elix with an EDS polisher system (Millipore, Bedford, MA, USA).

2.2. Standard solutions

Stock solutions of PChE (1250 IU/L) and MTTC (2.0 mM) were prepared and stored at 4 °C until further needed. A 0.25 mM chromogen solution of 5, 5'-dithiobisnitrobenzoic acid (DTNB) was prepared using 0.1 mol/L phosphate buffer (pH 7.4).

Individual standard solutions of pesticides were prepared in dimethyl sulfoxide, and each experiment was performed using seven dilutions of each standard solution. Evaluation of a single pesticide was performed using final concentrations of 10, 5, 2, 1, 0.5, 0.2, and 0.1 μg/mL. Combinations of two pesticides were evaluated by fixing the concentration of one pesticide at 0.1 μg/mL and varying the concentration of the second pesticide to 5, 2.5, 1, and 0.5, 0.25, 0.1, and 0.05 μg/mL. The pesticides that showed strong inhibition were diluted 100 fold. Combinations of three pesticides were evaluated by fixing the concentrations of two compounds at 0.05 μg/mL each, and varying the concentration of the third pesticide to 5, 2, 1, 0.5, 0.2, 0.1, and 0.05 μg/mL. The pesticides that showed strong inhibition were diluted 5000 fold.

2.3. The evaluation of ChE activity

The active ChE enzymatically cleaves the substrate MTTC to release thiocholine. The released thiocholine reacts with the chromogen DTNB to generate a yellow product (Fig. 1), quantifiable at 405 nm by UV absorption that is impeded when ChE activity is inhibited by pesticide (Karahasanoglu and Ozand, 1967).

All experiments were done in triplicate. A 7 mIU solution of ChE and each appropriate pesticide sample were uniformly mixed in a ratio of 4:1. MTTC substrate solution (63 μL) was added to 7 μL of the mixed solution of ChE and OP in a 96 microwell plate and then 280 μL of the DTNB chromogen solution was added. The plate was incubated at 37 °C for 7 min, and the absorbance at 405 nm was measured using an
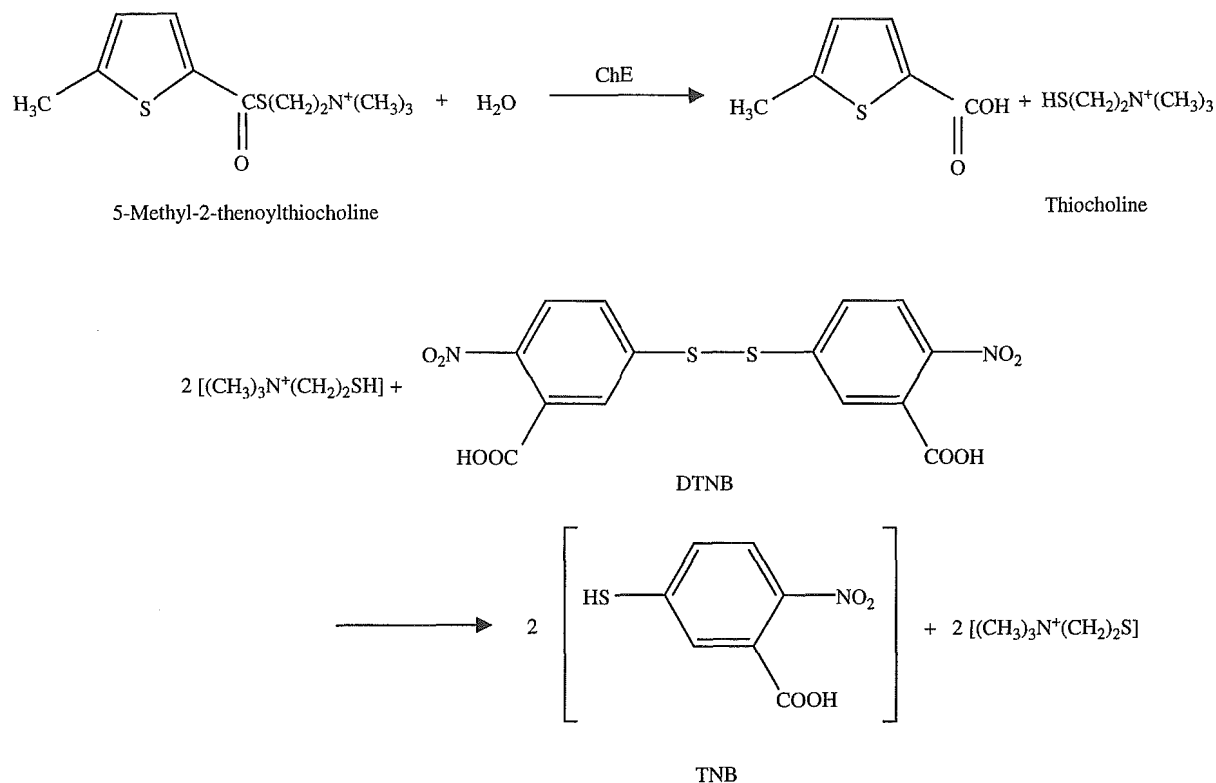
Fig. 1. The chemical reaction for generation of thiocholine and subsequent measurement of ChE activity.

Table 1
Intended use and concentration of 20% inhibition for the oxon form of nine pesticides

Group	Pesticides	Intended use	IC_{20} (µg/mL)
A	Chlorpyrifos oxon	I	0.0011
	Isoxathion oxon	I	0.0013
	Diazinon oxon	I	0.0089
B	Prothiofos oxon	I	0.13
	EPN oxon	I	0.14
	MEP oxon	I	0.33
C	Butamifos oxon	H	1.04
	Tolclofos-methyl oxon	G	5.09
	Isofenphos oxon	I	—

I: insecticide, G: germicide, H: herbicide.

Table 2
Intended use and concentration of 20% inhibition for six pesticides

Group	Pesticides	Intended use	IC_{20} (µg/mL)
A	DDVP	I	0.048
	EDDP	G	0.08
B	DEP	I	0.49
C	IBP	G	2.81
	Fosetyl	G	0.3
	Acephate	I	—

I: insecticide, G: germicide.

Ultrospec Visible Plate Reader II 96 (Amersham Biosciences, Tokyo, Japan).

The method employed typically yields a 40% background at 405 nm and IC_{20} results (Tables 1 and 2) are calculated and corrected by setting the maximal inhibition (Figs. 3 and 4) to 100%.

3. Results and discussion

3.1. Inhibition of ChE activity by 15 kinds of OPs

Parent compounds containing a P=S moiety exhibited limited inhibition (Fig. 2); however, these species undergo desulfuration to form active oxon metabolites, containing a P=O moiety. This desulfuration can occur
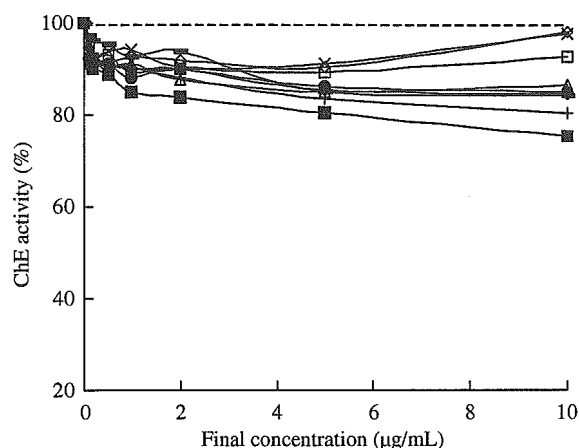
Fig. 2. ChE activity for the parent compounds of nine pesticides: —■—; isofenphos, —●—; tolclofos-methyl, —▲—; butamifos, —□—; MEP, —◊—; EPN, —■—; prothiofos, —+—; diazinon, —×—; isoxathion, —♦—; chlorpyrifos.
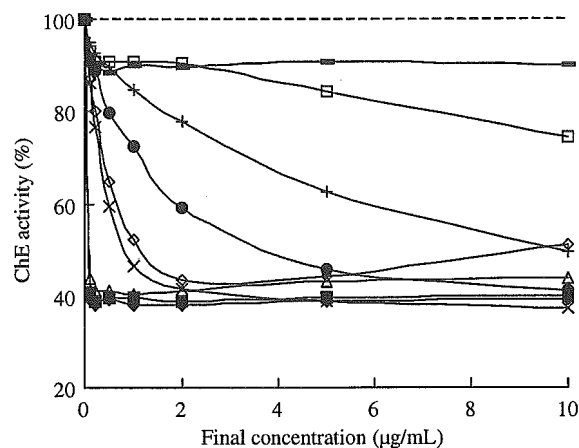

Fig. 3. ChE activity for the oxon form of nine pesticides: —■—; isofenphos oxon, —●—; tolclofos-methyl oxon, —▲—; butamifos oxon, —□—; MEP oxon, —◊—; EPN oxon, —■—; prothiofos oxon, —+—; diazinon oxon, —×—; isoxathion oxon, —♦—; chlorpyrifos oxon.

through metabolic activation by organisms or by nonbiological oxidation in the environment (Ma and Chambers, 1995, 1994; Sultatos, 1987; Sultatos et al., 1985; Butler and Murray, 1997). Evaluation of the inhibitory activity of the oxon metabolites of these nine pesticides confirmed that the degree of inhibition was stronger than that of the parent compounds (Fig. 3). OPs were classified into three groups according to the level of ChE inhibition (Table 1). Group A showed strong inhibition of ChE with 20% inhibition (IC_{20}) at 100 ng/mL or less. Group B showed slight inhibition with IC_{20} between 0.1 and 1 μg/mL. Group C showed limited inhibition with IC_{20} exceeding 0.1 μg/mL. The degree of ChE inhibition for six pesticides containing an original P=O moiety is shown in Fig. 4, and these pesticides were similarly classified into three groups (Table 2). Of the pesticides, fosetyl inhibited ChE activity at low concentrations but the degree of inhibition did not increase with increasing concentrations (Fig. 4). Accordingly, we classified fosetyl into group C even though the IC_{20} was calculated to be 0.3 μg/mL. Consequently, the results suggest that the toxicity of OPs is due to the presence of the P=O moiety that makes the P=O pesticide more similar in structure to acetylcholine, the substrate of ChE, than the comparable P=S pesticide. However, there was no obvious relationship found between the degree of inhibition and intended use (i.e. insecticide, germicide, herbicide) of the pesticide (Tables 1 and 2).

3.2. The relation between ChE activity inhibition and the chemical structure

Strong inhibitors such as chlorpyrifos oxon, isoxathion oxon and diazinon oxon (Fig. 5) in group A
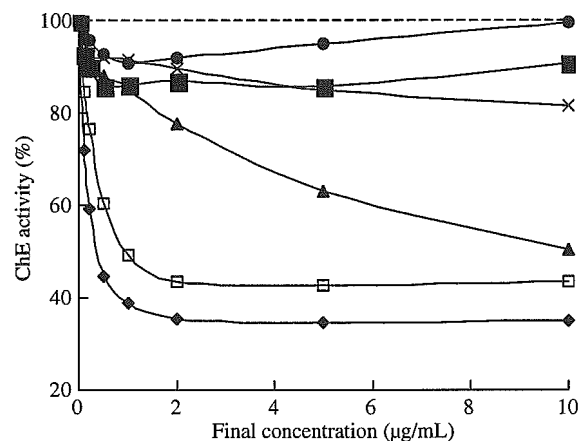

Fig. 4. The degree of ChE activity of six pesticides: —□—; acephate, —×—; fosetyl, —■—; IBP, —▲—; DEP, —●—; EDDP, —♦—; DDVP.

are classified as nitrogenous heterocyclic ester thionophosphate insecticides, and this –P–O–C=N– structure may be critical to the pesticide's inhibitory activity. ChE inhibition produced by isofenphos oxon, acephate and butamifos oxon was weak; thus these compounds belong to group C (Fig. 6). They are classified as ester amidophosphates, and this structure provides selective toxicity against insects compared to mammals. Many OPs developed after the 1960s typically are of this class and present relatively low risks of toxicity for mammals. While there seems to be a relation between the structure and the degree of ChE inhibition, the precise role of the
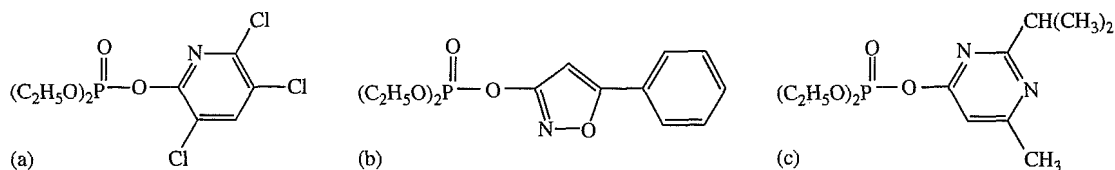
Fig. 5. Chemical structures of (a) chlorpyrifos oxon, (b) isoxathion oxon and (c) diazinon oxon.
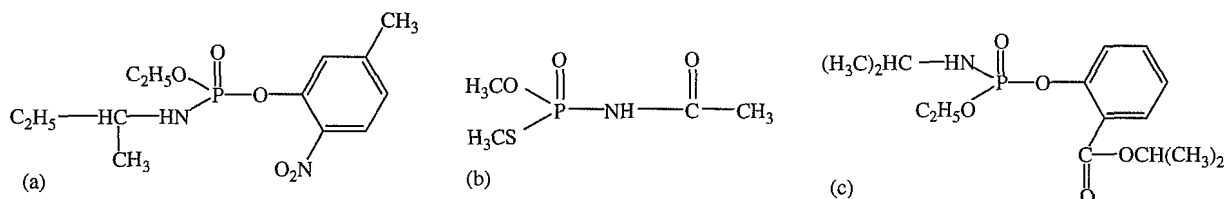

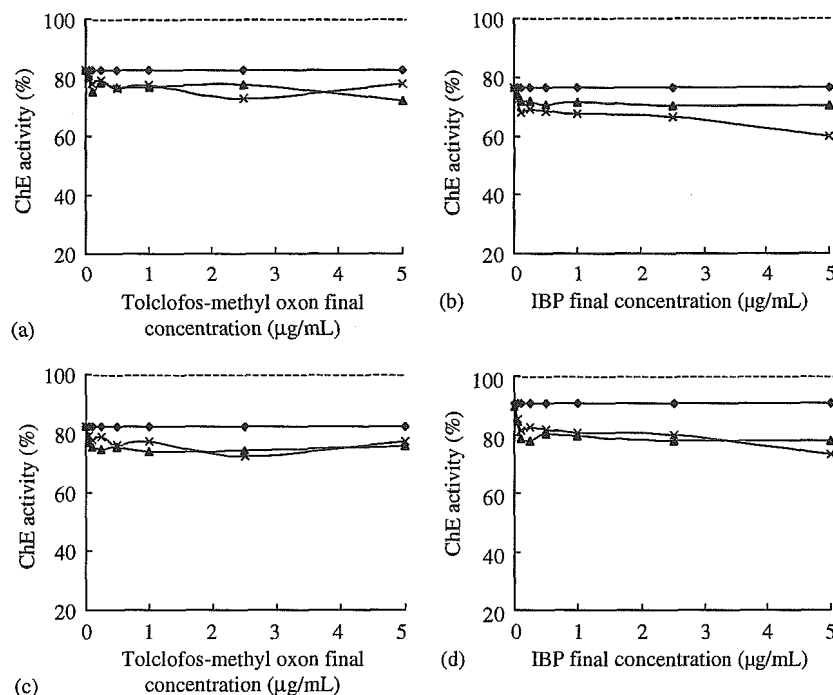Fig. 6. Chemical structures (a) butamifos oxon, (b) acephate, and (c) isofenphos oxon.

Fig. 7. The combined influence of two pesticides is shown: (a) chlorpyrifos oxon and tolclofos-methyl oxon, (b) DDVP and IBP, (c) isofenphos oxon and tolclofos-methyl oxon, (d) dcephate and IBP, —♦—; X were added into the reaction at the fixed concentration, respectively, —▲—; combined influence under the condition of the fixed concentration of X and eight concentrations of Y, —■—; predicted value of the combined influence, X: (a) chlorpyrifos oxon, (b) DDVP, (c) isofenphos oxon, and (d) acephate Y: (a) tolclofos-methyl oxon, (b) IBP, (c) tolclofos-methyl oxon, and (d) IBP.

structure of the pesticide and the mechanism of ChE inhibition is unknown.

3.3. The evaluation of OPs combinations

We examined the inhibition activity of the following two-compound combinations: chlorpyrifos oxon + isox-

athion oxon, DDVP + EDDP, chlorpyrifos oxon + tol-clofos-methyl oxon, DDVP + IBP, isofenphos oxon + tolclofos-methyl oxon, and IBP + acephate. No synergistic effects were observed; all combinations were additive with respect to ChE inhibition. Chlorpyrifos oxon + tolclofos-methyl oxon, DDVP + IBP, isofenphos oxon + tolclofos-methyl oxon and IBP + acephate
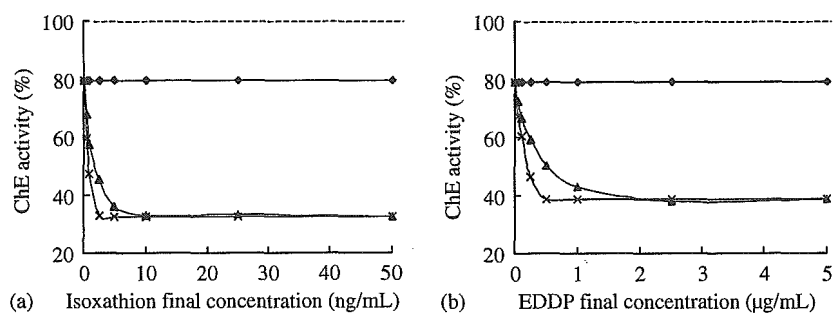
Fig. 8. The competing influence of two pesticides, including one strong inhibitor found in group A is shown: (a) chlorpyrifos oxon and Isoxathion oxon, (b) DDVP and EDDP, —♦—; X were added into the reaction at the fixed concentration, respectively, —▲—; combined influence under the condition of the fixed concentration of X and eight concentrations of Y, —■—; Predicted value of the combined influence X: (a) chlorpyrifos oxon, (b) DDVP, Y: (a) isoxathion oxon, (b) EDDP.
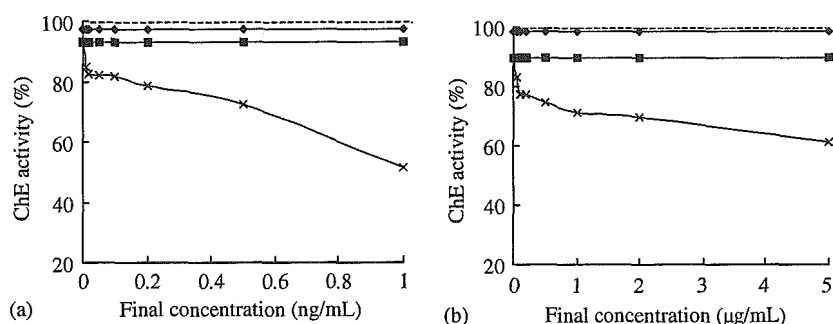

Fig. 9. The combined influence of three pesticides is shown: (a) chlorpyrifos oxon, isoxathion oxon and diazinon oxon (b) isofenphos oxon, tolclofos-methyl oxon and butamifos oxon, —♦—; X were added into the reaction at the fixed concentration, respectively, —✕—; X and Y were added into the reaction at the fixed concentration, respectively, —■—; combined influence under the condition of the fixed concentration of X, Y and eight concentrations of Z. X: (a) chlorpyrifos oxon, (b) isofenphos oxon, Y: (a) isoxathion oxon, (b) tolclofos-methyl Oxon, Z : (a) diazinon oxon, (b) butamifos oxon.

showed additive effects approximately corresponding to the sum of the predicted values for individual OPs (Fig. 7). However, the total activity of chlorpyrifos oxon + i-soxathion oxon and DDVP + EDDP (combinations of members from group A) showed less additive activity than that predicted from the activity of the individual components (Fig. 8).

Previously, Richardson et al. (2001) evaluated in vitro interaction between two OPs on ChE activity. When chlorpyrifos oxon and azinphos methyl oxon were added simultaneously to brain tissue in vitro, an additive effect was noted. Sequential exposure led to an additive effect at low concentrations but greater than additive inhibition at higher concentration. This study did not include the effect of three-compound combination exposures. There is no paper that examined the effect of three-compound combination exposure otherwise.

In our study, three-compound combinations, chlorpyrifos oxon + isoxathion oxon + diazinon oxon and isofenphos oxon + tolclofos-methyl oxon + butamifos oxon, were examined (Fig. 9). An additional inhibitory

effect was observed upon addition of the third compound when compared to the previously observed two-compound results. This suggests that exposure to multiple OPs might have adverse influences on human and wildlife due to an additive effect, even if each pesticide is present at concentrations under recognized NOAEL.

4. Conclusions

Our results indicate that the comprehensive evaluation of multiple pesticides is essential to assess the actual toxicity risks posed by exposure to mixtures of environmental pollutants having common biomolecular targets. In the present work, we demonstrate the utility of our simple, inexpensive, and robust in vitro evaluation system to examine the combined influence of OPs commonly encountered in the aquatic environment. Of note, the relative strength of ChE inhibition observed for these pesticides in our model system are well correlated with recognized values for ADI. Of course,

the adverse effect of environmental pollutants, including multiple compounds, should be evaluated in appropriate animal models. Future in vivo results could be compared to the results obtained using the in vitro method in order to gain a greater understanding of how to measure an apparent environmental exposure and establish daily toxicity monitoring. However, the reported in vitro method is useful for evaluating the toxicity of OPs and OP mixtures, and can be easily applied to risk assessment of complex OPs mixtures commonly observed as environmental pollutants. Consequently we suggest that this method be applied as a monitoring technique in order to preserve water quality and reduce the risk of pesticides upon the ecosystem.

Acknowledgements

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Effect of uncertainties in agricultural working schedules and Monte-Carlo evaluation of the model input in basin-scale runoff model analysis of herbicides

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Abstract In the prediction of time-series concentrations of herbicides in river water with diffuse-pollution hydrological models, farming schedules (the dates of herbicide application and drainage of irrigation water from rice paddies) greatly affect the runoff behavior of the herbicides. For large catchments, obtaining precise data on farming schedules is impractical, and so the model input inevitably includes substantial uncertainty. This paper evaluates the effectiveness of using the Monte-Carlo method to generate sets of estimated farming schedules to use as input to a GIS-based basin-scale runoff model to predict the concentrations of paddy-farming herbicides in river water. The effects of using the Monte-Carlo method to compensate for uncertainty in the evaluated parameters for herbicide decomposition and sorption were also evaluated.

Keywords Farming schedule; herbicide; modeling; pollutograph; runoff prediction

Introduction

Pesticides used in agriculture can enter hydrological catchment systems and contaminate rivers, which are primary sources of drinking water in many regions. Hydrological diffuse-pollution models are designed to simulate the movements of water and pollutants in river basins and thereby aid in assessing water quality. Several models for predicting pesticide concentrations in river water have been proposed and applied. The Hydrologic Simulation Program-FORTRAN (HSPF) (Johanson et al., 1983, 1997) is a comprehensive model of watershed hydrology and water quality that enables integrated simulations of runoff, sediments, and nutrient transport. It can also model pesticide transport (Moore et al., 1988; Laroche et al., 1996; Dabrowski et al., 2002), but this requires accurate agricultural as well as hydrological, meteorological, and geographical data as input. Accurate hydrological, meteorological, and geographical data are collected throughout Japan and are available to researchers. For large target catchment areas, however, acquisition of precise data on farming schedules, including the amounts of pesticides used and the dates of application, is impossible; the data acquired inevitably involves substantial uncertainty. Moreover, many factors affect the processes of sorption and decomposition of pesticides in soil and water. Owing to a lack of information on the reaction environment, however, it is impossible to quantify specific reaction rates. Generally, reported values are subject to various kinds of uncertainties.

Accordingly, the purpose of our work was to study the effects of uncertain input data regarding agricultural work schedules on model-based predictions of herbicide concentrations in river water. This paper evaluates the effectiveness of using the Monte-Carlo method to create estimates of input data, using a river-basin model composed of a large area divided into small compartments. The effects of uncertainty in the input parameters for herbicide decomposition and sorption are also discussed.