Respiratory strategy is a major determinant of [^{3}H]water and [^{14}C]chlorpyrifos uptake in aquatic insects

D.B. Buchwalter, J.J. Jenkins, and L.R. Curtis

Abstract: Despite the extensive use of aquatic insects to evaluate freshwater ecosystem health, little is known about the underlying factors that result in sensitivity differences between taxa. Organismal characteristics (respiratory strategy and body size) were used to explore the rates of [^{3}H]H_2O and [^{14}C]chlorpyrifos accumulation in aquatic insects. Ten aquatic insect taxa, including ephemeropteran, trichopteran, dipteran, hemipteran, and coleopteran species, were exposed to [^{14}C]chlorpyrifos (240 ng·L^{-1}) and [^{3}H]H_2O for up to 12 h. Because exchange epithelial surfaces on the integument are permeable to water, [^{3}H]H_2O was used as a quantitative surrogate for exposed cellular surface area. [^{14}C]Chlorpyrifos uptake rates were highly correlated with water permeability in all 10 taxa tested and largely covaried with body size and respiratory strategy. Rates were highest among smaller organisms on a per-weight basis and in taxa with relatively large external cellular surfaces such as gills. Air-breathing taxa were significantly less permeable to both [^{3}H]H_20 and [^{14}C]chlorpyrifos. A method for labeling exposed epithelial surfaces with a fluorescent dye was developed. This technique allowed discrimination between exchange epithelium and barrier tissue on the integument. Fluorescent dye distributions on the body surface provided a rapid method for estimating exposed epithelium consistent with [^{3}H]H_20 and [^{14}C]chlorpyrifos accumulation.

Résumé : Bien que les insectes aquatiques aient beaucoup servi à l'évaluation de la santé des écosystèmes d'eau douce, on connaît mal les facteurs sous-jacents aux différences de sensibilité entre les taxons. Des caractéristiques des organismes (stratégie respiratoire et taille du corps) nous ont servi à explorer les taux d'accumulation de [^{3}H]H_2O et de [^{14}C]chlorpyrifos chez les insectes aquatiques. Dix taxons d'insectes aquatiques, dont des espèces d'éphéméroptères, de trichoptères, de diptères, d'hémiptères et de coléoptères, ont été exposés au [^{14}C]chlorpyrifos (240 ng·L^{-1}) et à [^{3}H]H_2O pour des périodes pouvant atteindre 12 h. Parce que les surfaces épithéliales d'échange du tégument sont perméables à l'eau, nous avons utilisé [^{3}H]H_2O comme indice quantitatif de la surface cellulaire exposée. Les taux d'absorption de [^{14}C]chlorpyrifos sont en forte corrélation avec la perméabilité à l'eau chez les dix taxons étudiés et varient généralement avec la taille du corps et la stratégie respiratoire. Les taux par unité de masse sont maximaux chez les plus petits organismes et chez ceux qui possèdent des surfaces cellulaires externes relativement grandes, telles que des branchies. Les taxons à respiration aérienne ont une perméabilité significativement réduite à [^{3}H]H_2O et au [^{14}C]chlorpyrifos. Nous avons mis au point une méthode pour marquer les surfaces épithéliales exposées à l'aide d'un colorant fluorescent. Cette technique nous a permis de distinguer l'épithélium d'échange et le tissu-barrière sur le tégument. La répartition du colorant fluorescent sur la surface du corps permet d'estimer rapidement l'épithélium exposé impliqué dans l'accumulation de [^{3}H]H_2O et de [^{14}C]chlorpyrifos.

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Introduction

Ecologists have developed a variety of indices, metrics, and other tools that attempt to assess freshwater ecosystem health via surveys of resident insect taxa (e.g., Hilsenhoff 1988; Plafkin et al. 1989). Remarkably, none of the current survey-based bioassessment techniques is based on understanding how insect physiological and morphological attributes affect responses to specific environmental stressors. Rather, they are based on a wide assortment of observational studies that, by nature, cannot adequately define causal relationships. This paper examines organismal characteristics that are important in determining uptake of environmental pollutants.

Aquatic insects arose from numerous invasions of aquatic habitats by air-breathing terrestrial ancestors (Kristensen 1981). The radiation of aquatic insect species is accompanied by significant developments and modifications in respiratory and associated osmoregulatory systems. Several aquatic respiratory strategies have emerged from the basic open tracheal system, including epithelial gas exchange systems utilizing body walls and gills (Eriksen et al. 1996). In some orders, such as Diptera, more than one strategy is seen including air breathing and dissolved oxygen (DO) breath-

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Table 1. Test organisms, respiratory strategies (air vs. dissolved oxygen (DO)), body weights, chlorpyrifos uptake (slopes, ranks, r^2), water uptake (ranked times to ½ steady state), and number of organisms used in each experiment.

Order	Family	Genus, species	Respiratory strategy	Mass (mg)	Chlorpyrifos, slope, rank, r^2	Water rank	Number of organisms
Diptera	Chironomidae	*Chironomus riparius* (3)	DO	2.4	13.53, 1, 0.96	1	18
Diptera	Chironomidae	*Psectrotanypus* sp.	DO	3.8	2.67, 4, 0.90	5	18
Diptera	Chironomidae	*Chironomus riparius* (4)	DO	3.8	7.04, 2, 0.85	2	29
Hemiptera	Corixidae	*Sigara washingtonensis*	Air	4.9	1.96, 6, 0.75	6	29
Ephemeroptera	Heptageniidae	*Cinygma* sp.	DO (gills)	7.1	3.90, 3, 0.70	3	29
Coleoptera	Hydrophilidae	*Berosus* sp.	Air	16.7	1.97, 7, 0.91	8	18
Ephemeroptera	Baetidae	*Callibaetis* sp.	DO (gills)	22.4	2.45, 5, 0.91	4	30
Hempitera	Notonectidae	*Notonecta kirvyi*	Air	45.3	0.91, 9, 0.93	9	18
Diptera	Ptychopteridae	*Ptychoptera* sp.	Air	57.8	0.47, 10, 0.88	10	30
Trichoptera	Limnephilidae	*Dicosmoecus gilvipes*	DO (gills)	65.1	1.84, 8, 0.83	7	28

Note: (3) and (4) following *C. riparius* denote instar.

ing. All taxa in the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT) have closed tracheal systems and are dependent on aqueous gas exchange. The integuments of aquatic insects are covered by protective surfaces of waxes, chitin, and scleretin that are relatively impermeable to gasses and water. However, insects that breathe dissolved oxygen have exposed epithelial surfaces that exchange gasses and salts, including respiratory tissue, chemosensory cells, and chloride cells. Insects are extremely heterogeneous with respect to the relative epithelial surface area used for gas and salt exchange, which is determined in part by age, sex, size, life history, and water chemistry.

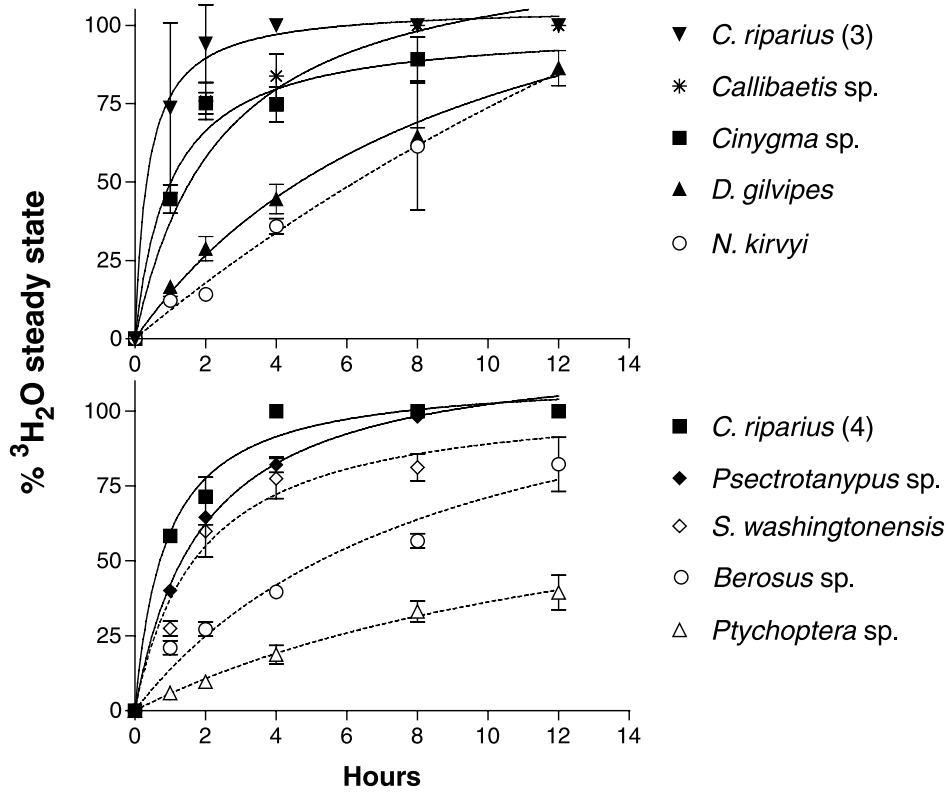
Our working hypothesis is that insects with relatively large areas of exchange epithelium are potentially more vulnerable to poor water chemistry conditions than those with smaller areas of exchange epithelium. Because organisms with larger exchange epithelial surface areas are more physiologically connected to the water column, they may be more sensitive to a variety of water column associated stressors relative to organisms that do not have large exchange epithelial surfaces. For example, in low dissolved oxygen scenarios, air-breathing insects would largely be unaffected, whereas DO-breathing insects would be stressed. The experiments presented in this paper examine the importance of respiratory strategy and associated exchange epithelium in determining accumulation of the insecticide [^{14}C]chlorpyrifos. Water permeability is used as a quantitative surrogate for estimating relative differences in exchange epithelium that we predict are important in determining exposure to contaminants. In addition, we report a technique for characterizing taxa based on exchange epithelium via the use of the fluorescent membrane dye DPH (diphenylhexatriene). The goal of this technique is to aid in our predictive capacity to determine differences in species–instar sensitivity to stressors that exert their negative influences via exposed cellular surfaces.

Materials and methods

All insects used in this study, with the exception of *Chironomus riparius*, were field collected from sites in Oregon, using a D-frame kick net (Table 1). Insects were transported in damp moss in ice-filled coolers. *Chironomus riparius* egg masses were obtained from Environmental Toxicology and Testing, Inc., Superior, Wis. No collecting was done from obviously impacted sites. Before experimentation, insects were held for a minimum of 5 days in a temperature-controlled chamber at 8.5°C with a 16 h light – 8 h dark photoperiod of indirect full-spectrum lighting. All insects were held in Instant Ocean® (San Marcos, Calif.) recirculating aquaria containing approximately 75 gal of soft well water collected from U.S. Environmental Protection Agency's (EPA) Willamette Research Station in Corvallis, Oregon. Insects were fed a diet consisting of wheat, alfalfa, and TetraMin® "Baby fish food L" (TetraWerke, Melle, Germany) ad libitum before experimentation.

Artificial water® (Fisher Scientific, Pittsburgh, Pa.; 0.67 mM $CaCl_2$, 0.3 mM $MgSO_4$, 1.2 mM $NaHCO_3$, and 0.5 mM KH_2PO_4) was used for all experiments. Fifty millilitres of Artificial water, 20 μL [^{3}H]H_2O, and 20 μL of [^{14}C]chlorpyrifos (in 50:50 acetone–water) were added to Erlenmeyer flasks. This yielded final specific activities for [^{3}H]H_2O and [^{14}C]chlorpyrifos of approximately 6.77 μCi·L^{-1} and 0.023 μCi·L^{-1}, respectively. This [^{14}C]chlorpyrifos activity corresponds to a concentration of 240 ng·L^{-1}. Individual insects were placed in each flask, with the exception of experiments with the organisms *C. riparius* and *Psectrotanypus* sp., in which two individuals were placed in each flask. Individuals were held for 0, 1, 2, 4, and 8 h and, in some cases, 12 h. When available, five insects per time point were used. After exposure, insects were removed from exposure flasks, rinsed profusely with water, weighed, and placed in 20 mL scintillation cocktail vials. Amersham (Oakville, Ont.) NCS II® tissue solubilizer was added to each vial, and digests were held at 50°C overnight and neutralized with glacial acetic acid to obtain pH 7. Eighteen millilitres of Amersham BCS-NA® nonaqueous scintillation cocktail were added, and samples were held in a refrigerator in the dark for at least 4 days to minimize chemiluminescence. These samples were well mixed and counted with a Beckman LS 6500 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Five control insects of each taxon were placed in water containing the acetone carrier for 1 h, removed, digested, and analyzed as described above. The average of control [^{3}H] and [^{14}C] activities was taken to be background for each species tested and subtracted from the counts of subsequent time points.

Fig. 1. Water permeability in aquatic insects is expressed in terms of percent body water based on the accumulation of ^3H_2O relative to total body water composition. Broken lines and open symbols represent air-breathing taxa. Solid lines and closed symbols represent dissolved oxygen breathing taxa. Error bars represent the standard errors of the means at each time point. Time courses were run to 8 h or 12 h, depending on the availability of organisms. The (3) and (4) following *Chironomus riparius* denote larval instar.



Five individuals per taxon were blotted dry, weighed, and transferred to preweighed aluminum weigh boats to be dried at 50°C for 72 h to determine average percent body water composition. Average percent body water was used to estimate body water volumes for each taxon exposed to $[^3H]H_2O$ and [^{14}C]chlorpyrifos. The accumulation of $[^3H]H_2O$ is expressed in terms of percent apparent steady state. We define percent apparent steady state as the percentage of the organisms' body water that has been exchanged with external $[^3H]^3H_2O$. At 100% apparent steady state, the internal and external concentration of $[^3H]^3H_2O$ is equivalent.

To fluorescently label external cellular surfaces on *C. riparius* and *Psectrotanypus* sp., the following procedure was used. Diphenylhexatriene (DPH) was obtained from Molecular Probes, Eugene, Oregon. A 43 mM stock solution was prepared in 100% dimethylformamide (DMF). DPH stock (0.25 mL) was added to 10 mL of a 250 mM mannitol – 10 mM HEPES buffer solution at pH 7.4. This aqueous suspension was sonicated and mixed before the addition of live larvae. One larva of each species was jointly incubated in this mixture as it was stirred slowly. The larvae were removed after a 15-min incubation, rinsed thoroughly with tap water, and anesthetized with CO_2. The larvae were then photographed with a Diagnostics Instruments (Sterling Heights, Mich.) SPOT 2® camera through a Leica (Northvale, N.J.) MZFL111® stereoscope equipped with a 100-watt mercury vapor lamp and ultraviolet fluorescent filter.

Statistical methods used include linear regression, nonlinear regression, and Spearman's rank correlation coefficients (Sokal and Roth 1995).

Results

Water permeability

There were large differences in the accumulation of water between taxa (Figs. 1*a*, 1*b*). Accumulation of $[^3H]H_2O$ was described in terms of the percentage of the individual's body water that was incorporated from the external media. As an arbitrary descriptor of water permeability, the 50% steady-state point was compared among taxa (Fig. 2). Both body size and respiratory strategy appeared to be important determinants of water uptake rates. Smaller taxa generally had higher water turnover rates than did larger organisms, and DO breathers had higher water turnover rates than did air breathers. Body weights and times to 50% water steady state were ranked. For all taxa, a negative correlation between body weight and time to 50% steady state was observed (Spearman's rank correlation coefficient = –0.75; p = 0.01). All air-breathing taxa had lower permeability than expected based on body weight alone (Table 1). The only DO breather that had lower permeability than expected based on body weight was the chironomid *Psectrotanypus* sp. (Table 1).

Fig. 2. Water permeability in aquatic insects is a function of both body size and respiratory strategy. The open symbols represent organisms with open tracheal systems (air breathers). Closed symbols represent organisms with closed tracheal systems (dissolved oxygen breathers). The (3) and (4) following *Chironomus riparius* denote larval instar.

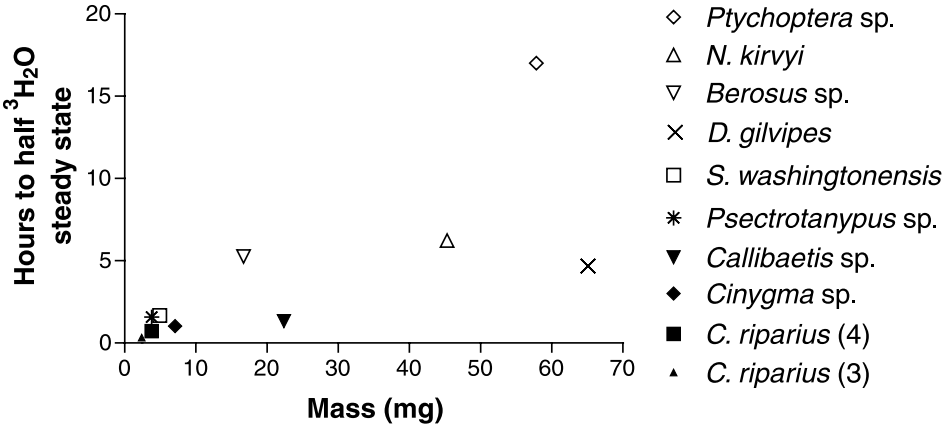
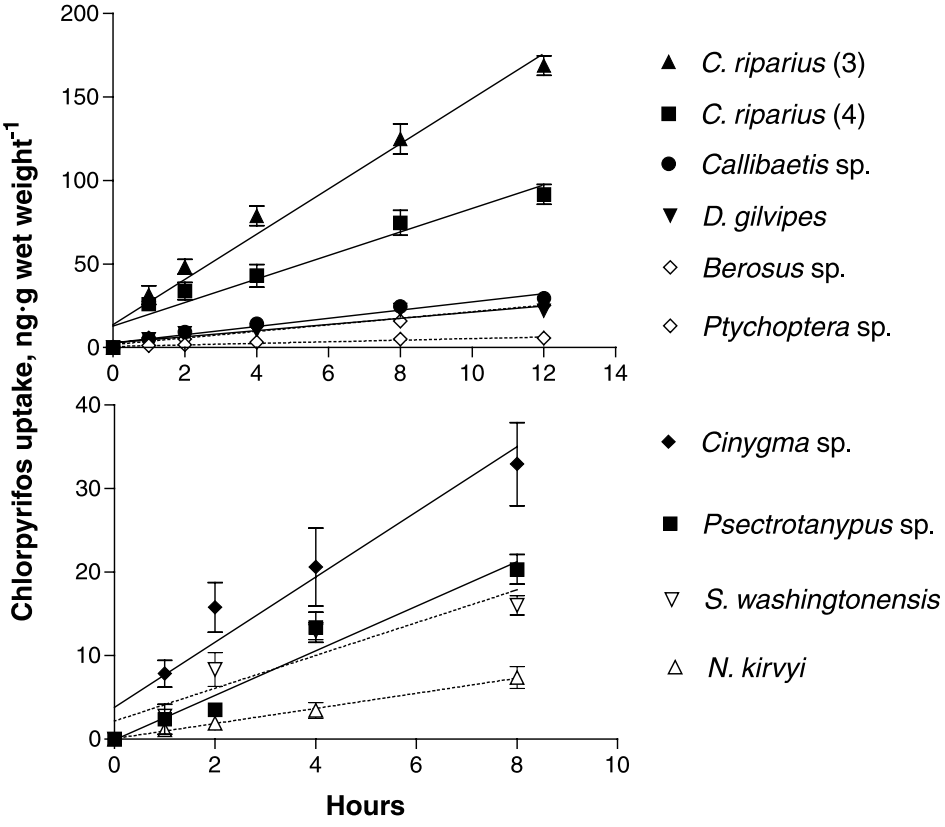


Fig. 3. Chlorpyrifos uptake rates in aquatic insects are determined by body size and respiratory strategy. Dashed lines and open symbols represent air-breathing taxa. Solid lines and closed symbols represent dissolved oxygen breathing taxa. Error bars represent the standard errors of the means at each time point. Time courses were run to 8h or 12 h, depending on availability of organisms. The (3) and (4) following *Chironomus riparius* denote larval instar.



Chlorpyrifos uptake

[^{14}C]Chlorpyrifos accumulation increased with time in all taxa (Figs. 3*a*, 3*b*). The decision to include 12 h as a time point was based solely on organism availability. As was the case with water permeability, body size was an important determinant of [^{14}C]chlorpyrifos accumulation. For all taxa, a negative correlation between ranked body weights and chlorpyrifos uptake rates was observed (Spearman's rank correlation coefficient = –0.86; p = 0.005). All air-breathing taxa had relatively low chlorpyrifos uptake rates based solely on body size (Table 1). The only DO breather that had a lower than expected chlorpyrifos uptake rate was the chironomid *Psectrotanypus* sp. (Table 1).

Water turnover rates were jointly determined by body size and respiratory strategy and were strong predictors of [^{14}C]chlorpyrifos uptake. A Spearman's rank correlation coefficient of 0.98 (p = 0.001) was observed between chlorpyrifos uptake ranks and ranked times to 50% water

Fig. 4. Chlorpyrifos uptake rates vs. water permeability in aquatic insect taxa. Water permeability is described in terms of the length of time required for an organism to accumulate 50% body water steady state. The open symbols represent organisms with open tracheal systems (air breathers). Closed symbols represent organisms with closed tracheal systems (dissolved oxygen breathers). Error bars represent the chlorpyrifos uptake slope standard errors. This exponential decay relationship has an $r^2 = 0.98$.

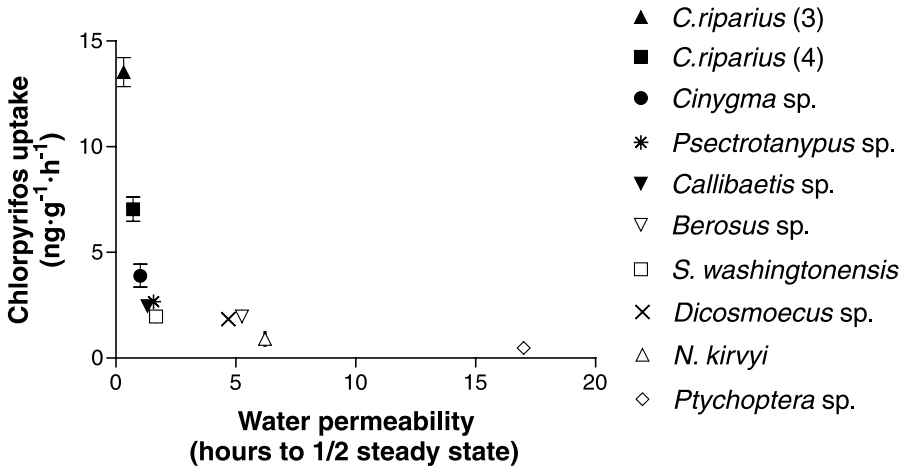
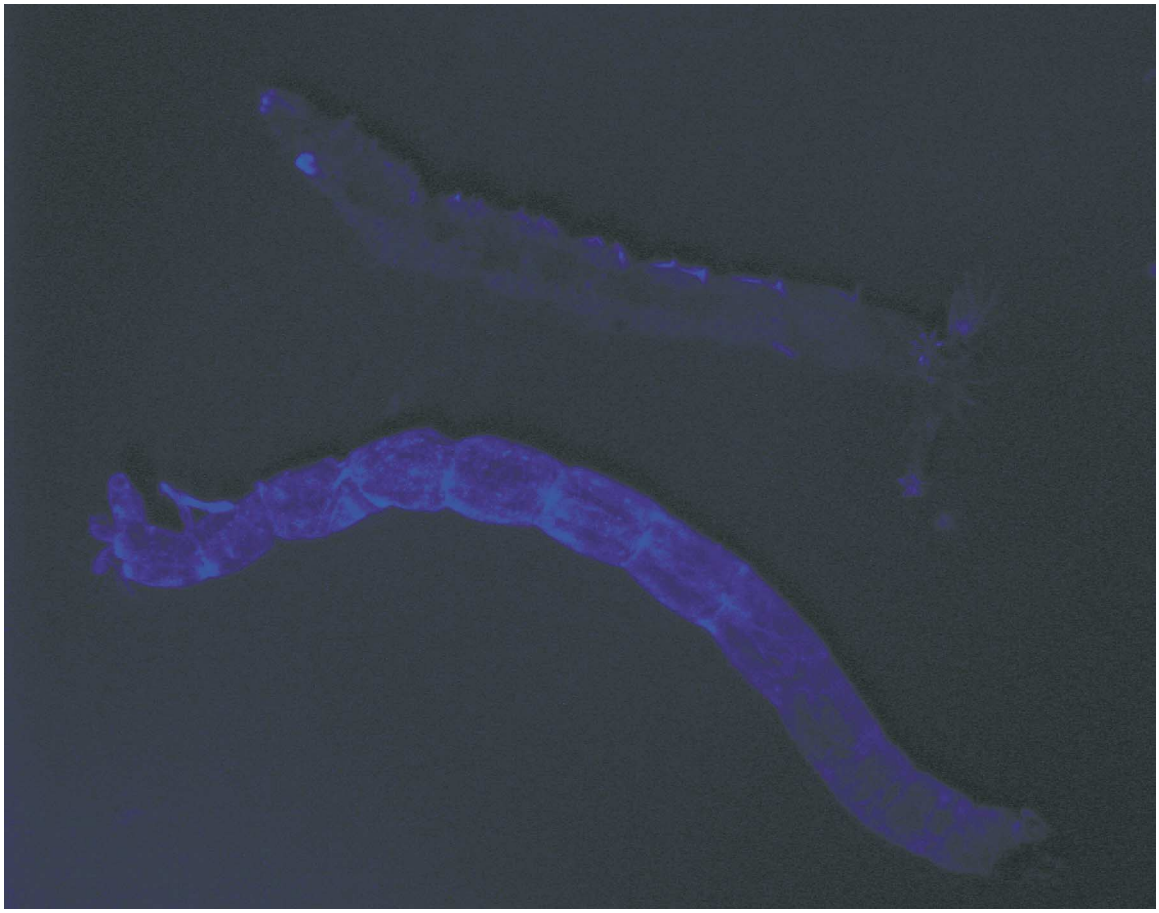


Fig. 5. Diphenylhaxatriene (DPH) labeled *Chironomus riparius* (below) and *Psectrotanypus* sp. (above). These larvae were jointly incubated for 15 min in DPH and photographed on the same slide at 12.5× magnification. No additional modifications were performed on this image.



steady state across all taxa. The relationship between the magnitudes of [^{14}C]chlorpyrifos uptake rates and water permeability was described by simple exponential decay with an $r^2 = 0.98$ (Fig. 4).

Fluorescence characterization

Fluorescence of DPH was more broadly distributed on the body surface of *C. riparius* than on that of *Psectrotanypus* sp. (Fig. 5). These larvae were jointly incubated, rinsed, and

photographed. This image was not adjusted or altered in any manner. The fluorescence distributions in these larvae indicated that *C. riparius* has a relatively large cellular interface with the water column compared with that of *Psectrotanypus* sp. DPH has several characteristics that make it useful for examining insect body surfaces. DPH is a cylindrically shaped molecule that is essentially nonfluorescent in water. However, fluorescence excitation and emission dipoles are oriented roughly parallel to the axis of the cylindrically shaped molecule, resulting in increased fluorescence when incorporated into the plasma membranes of epithelial cells. With this in mind, images of DPH-labeled insects can be compared based on overall brightness, relative size of fluorescing surfaces, and photographic attributes such as magnification and exposure time (Haugland 1996).

Discussion

Studying traits at the organismal level has advanced our understanding of how freshwater systems function. For example, functional feeding guild approaches (Cummins 1974) have helped scientists understand how communities of organisms change from stream headwaters to mouth (Vannote et al. 1980). To date, no such structural–functional approach has adequately determined which organismal characteristics are important in determining responses to specific water chemistry problems, although biological traits are beginning to be explored in this context (Charvet et al. 1998).

Water turnover

Based on results reported here and basic physiological principles, we suggest that body size and respiratory strategy (including associated exchange epithelia) are primary determinants of water turnover rates in aquatic insects. Smaller individuals have larger surface area to volume ratios. Additionally, larger organisms typically contain more water than smaller organisms. However, size alone did not determine water turnover rates in these studies. Respiratory strategy also played a key role. All air-breathing taxa had lower water turnover rates than DO-breathing taxa within a given size range. This was expected, as there is more exchange epithelium in DO-breathing taxa.

It is likely that highly permeable taxa are more vulnerable to osmoregulatory distress than other taxa. Based on the metabolic costs associated with removing excess water and retaining salts, we suggest that there are some disadvantages in having a water-permeable integument, particularly in degraded water chemistry conditions. Exchange epithelia are directly involved in osmoregulation, as water (passively) and ions (passively and actively) pass in both directions through exchange (Frisbie and Dunson 1988; Kirschner 1991; Cooper 1994). These fluxes can be altered by environmental stressors in insects (Havas and Hutchinson 1983; Lechleitner et al. 1985; Peters et al. 1985) and crustaceans (Havas and Advokaat 1995; Havas et al. 1984).

Chlorpyrifos accumulation

Several studies have indicated the importance of exchange epithelium as determinants of responses to environmental pollutants. For example, crude oil (Simpson 1980) and chlorine (Simpson 1980; Camargo 1991) were found to damage gills in freshwater insects. Respiratory and osmoregulatory epithelium is a critical target site in the toxicity of metals such as copper (Hare 1992), mercury (Bodou et al. 1991), and aluminum (Gunderson and Curtis 1995). However, direct uptake via the water column is not always the predominant exposure pathway in aquatic insects. (Hare 1992). Research with the air-breathing insect *Chaoborus* demonstrates that cadmium exposure via food is a more important exposure pathway than direct uptake via the water column (Munger and Hare 1997; Munger et al. 1999).

To date, we are aware of no other published studies that have examined the role of aquatic insect respiratory strategies in determining differences in uptake of organic pollutants. Many organic contaminants are known to partition to the lipid-rich environments of cell membranes in aquatic organisms. In their extensive work with the lampricide TMF (3-trifluoromethyl-4-nitrophenol), Maki and Johnson (1977) observed that "macroinvertebrate species with soft, relatively permeable integuments accumulate significantly higher residue concentrations…than those species with hard chitinized or calcareous exoskeletons". Our results indicate that accumulation of [^{14}C]chlorpyrifos is highly correlated with water turnover rates. However, we did not observe a relationship between the hardness of the integument and water permeability or [^{14}C]chlorpyrifos accumulation. Soft-bodied taxa were both highly (*C. riparius*) and minimally (*Ptychoptera* sp.) permeable. Rather, we observed that respiratory strategy and body size were more important factors in determining both [^{3}H]H_2O and [^{14}C]chlorpyrifos uptake.

Maki et al. (1975) also observed differences in TMF LC_{50} values between younger and older individuals of the same taxa, with younger instars being more sensitive than older instars. These results are consistent with those of Stuijfzand et al. (2000), who observed more than a 1000-fold difference in diazinon LC_{50} values for 1st and 4th instar *C. tentans*. Although we did not determine LC_{50} values in our experiments with *C. riparius*, we did observed that third instar individuals had almost a twofold higher [^{14}C]chlorpyrifos uptake rate than that of the fourth instar individuals. This could be attributed to larger surface area to volume ratios in earlier instars. Additionally, higher growth rates and metabolic demands can result in relatively larger areas of exchange epithelia in earlier instars. We are currently exploring the extent to which chlorpyrifos sensitivity differences among *C. riparius* instars are driven by differences in target site sensitivity, metabolic processes, or simply a function of differences in uptake rates.

Uptake differences among taxa do not necessarily translate to differences in contaminant sensitivity, particularly when comparisons are being made across unrelated taxa. Differences in target site sensitivity, metabolic capabilities, and detoxification mechanisms can be expected to vary widely at the ranks of order and family and, in some cases, genus. Despite the fact that *C. riparius* had high chlorpyrifos accumulation rates, it is not particularly sensitive to this compound. We have determined that this lack of sensitivity is due to the relatively slow biotransformation of chlorpyrifos to the oxon metabolite, which is the more toxic form (D.B. Buchwalter, J.J. Jenkins, and L.R. Curtis, unpublished data). However,

within closely related organisms, differential sensitivity may possibly be predictable based on exposure potential.

A major challenge in characterizing an organism's epithelial surface area is that these surfaces are not readily identifiable. We used water permeability as a quantitative surrogate to estimate the relative differences between taxa in terms of exposed cellular surface. Water permeability differences are driven by body size and exchange epithelial surfaces. We rule out the effect of drinking in these studies. It is generally thought that drinking is an osmoregulatory strategy limited to primitive forms (Komnick 1977). In addition, we suspect that large exchange epithelial surface areas are primarily driven by respiratory requirements and, to a lesser extent, osmoregulatory and chemosensory functions.

To facilitate the categorization of taxa based on exchange epithelial surface area, we offer the DPH fluorescence technique as a precursor to quantitative measures of exchange epithelia surface areas. The membrane dye DPH offers an inexpensive and rapid way of discriminating between taxa in terms of epithelial surface area differences, which may be used to predict differences in contaminant uptake rates. Two species of Chironomidae were chosen for comparison based on their phylogenetic relatedness, the simplicity, size, and similarities of the body plan, and the lack of gill coverings or complex three-dimensional gill structures. The more extensive fluorescence seen on the body surface of *C. riparius* vs. *Psectrotanypus* sp. was consistent with the higher water permeability and chlorpyrifos accumulation seen in *C. riparius* vs. *Psectrotanypus* sp. A higher incidence of fluorescence was associated with higher flux rates. Because of the limited availability of *Psectrotanypus* sp., we were unable to compare the sensitivities of these taxa.

The DPH fluorescence approach has the potential to add predictive power in assessing differences in species' exposure potential. The degree to which exposure potential and sensitivity are related should be examined further within a phylogenetic context to minimize confounding factors resulting from physiological differences among unrelated taxa. DPH is potentially useful in describing the attributes of resident biota and can be developed into diagnostic tools to discriminate between stressors that manifest their effects via epithelial surfaces and stressors that are based on physical habitat problems.

Aquatic insect organismal characteristics and life history attributes have not been adequately incorporated into either toxicological or ecological approaches to studying water pollution. In the results reported here, body size and respiratory strategy were important determinants of both water permeability and chlorpyrifos uptake. We suggest that these attributes could be incorporated into bioassessment protocols. For example, a percent air-breather metric could be particularly useful in wetland and other lentic systems to discriminate between water chemistry and physical habitat degradation. Additionally, in areas where pulses of contaminants are present, we suggest that smaller, DO-breathing organisms would be more likely to be impacted than larger and (or) air-breathing taxa. In systems with dissolved oxygen problems, we suggest that larger DO-breathing organisms would be at a disadvantage relative to smaller and (or) air-breathing taxa. Finally, the DPH technique may provide the basis for testing hypotheses regarding the potential sensitivity differences among taxonomically related organisms. This could refine techniques such as EPT-based metrics to evaluate stressor-specific responses that currently do not exist.

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