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USE OF THE STREAM MAYFLY *CLOEON TRIANGULIFER* AS A BIOASSAY ORGANISM: LIFE HISTORY RESPONSE AND BODY BURDEN FOLLOWING EXPOSURE TO TECHNICAL CHLORDANE

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Abstract—The stream mayfly *Cloeon triangulifer* is well suited as a bioassay organism because it has a relatively short egg and larval stage and can be readily cultured under laboratory conditions. Moreover, the species is widely distributed and reproduces as parthenogenetic clones. Nineteen distinct clones have been isolated from natural populations with Nei's genetic distance between clones ranging from 0.004 to 0.183. Clonal reproduction gives research scientists the option of including or excluding genetic variation from the experimental design. The present study involved exposing individuals from a single clone to initial technical chlordane levels ranging between 4.3 and 56.3 μg/L. No effect of chlordane was observed for the egg stage; eggs required 9 d to hatch at 20°C, and >95% of the eggs hatched in all controls and treatments. Egg hatch success was also tested at higher concentrations (89.0 and 158.3 μg/L), with no effect being observed. In contrast, larval survivorship decreased significantly from about 80% for control groups to 18.0 and <0.6% when larvae were exposed to initial chlordane levels of 4.3 μg/L and 9.4 μg/L, respectively. All larvae died when initial concentrations of chlordane were >15.4 μg/L. Sublethal effects on larvae exposed to 4.3 μg/L of chlordane included significantly longer developmental time (37 vs. 34.8 d) and larger adult size (1.5 vs. 1.1 mg) relative to controls. Body burdens of chlordane in adult tissue for individuals reared in the 4.3-μg/L treatment ranged from 51 to 140 ppb. Chlordane-related compounds found in adult mayfly tissue were *cis*-nonachlor (6%), *trans*-nonachlor (45%), *cis*-chlordane (4%), *trans*-chlordane (5%), oxychlordane (21%), and heptachlor epoxide (18%).

Keywords—Stream bioassay Chlordane Toxicity Aquatic insect Parthenogenetic

INTRODUCTION

Biological monitoring and toxicity testing have become extremely important means for establishing the presence, persistence, biological availability, and biological effect of chemical contaminants in ecosystems. Because the mode of action for most toxicants is similar in insects compared with other higher animals, insects have played a prominent role in toxicity testing and biological field monitoring for terrestrial systems [1]. Indeed, standard methods for mass laboratory rearing and experimenting with a wide variety of terrestrial insect species are available. In contrast, toxicity testing for protecting or setting criteria for freshwater environments, especially stream and river ecosystems, has emphasized fish and a few noninsect invertebrates, mainly Crustacea (e.g., *Daphnia magna, D. pulex, Ceriodaphnia* sp., *Artemia* sp., *Hyalella azteca, Gammarus lacustris*). The main problem with using many of these species is that they do not represent the most important (i.e., in terms of species richness, relative abundance, or productivity) and potentially most vulnerable group of organisms inhabiting streams and rivers, namely, the aquatic insects.

Only a few species of aquatic insects have been routinely used in bioassay studies (e.g., the nonbiting midges *Chironomus tentans* and *C. riparius* and the burrowing mayflies *Hexagenia rigida* and *H. limbata*). Life history characteristics and culture methods for these species have been reviewed and described [2,3], as well as bioassay procedures [4–8]. Although these species have been used widely for both acute and chronic studies, they are often less than ideal. For example, *Hexagenia* is difficult to culture and at most reproduces once per year, making whole-life bioassays practically impossible [3]. Species such as *C. tentans*, which has shorter developmental periods, are also difficult to work with due to problems with collecting and mating adults,

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initiating experiments with uniform size larvae, handling small eggs, and so forth [3]. Moreover, both *Hexagenia* and *Chironomus* tend to be sediment dwellers, inhabiting large lakes and rivers (*Hexagenia*) or predominantly lentic (*Chironomus*) rather than lotic habitats. Thus, they are well suited for sediment bioassays but less suited for other types of assays.

The paucity of stream insect species available as standard test organisms for laboratory and field bioassay procedures is noteworthy because insects are the principal group of consumer organisms in most stream and river ecosystems throughout the world. We propose that the following characteristics of stream and river insects have usually precluded the establishment of laboratory colonies and contributed to this situation: (a) their larvae typically require a flowing water system for rearing in order to satisfy respiration and/or feeding requirements; (b) they generally have relatively long life cycles (e.g., 6–12 months); and (c) the adults often have unusual behavioral requirements for reproduction (i.e., elaborate mating swarms or flights) that usually preclude the establishment of laboratory colonies.

In this paper, we focus on the response of various life stages of the stream mayfly (Insecta: Ephemeroptera) *Cloeon triangulifer* McDunnough to exposure to different levels of technical chlordane. We show that the basic life history and genetic characteristics of this species make it well suited as a bioassay organism. Furthermore, the species inhabits flowing water ecosystems and, as a mayfly, represents a widespread and important group of aquatic insects. Finally, we document the sensitivity of various life stages of *C. triangulifer* to technical chlordane and provide an analysis of the body burden of the parent toxicant compound and its metabolites by the mayfly.

TEST SPECIES

Cloeon triangulifer has several characteristics that make it well suited as a bioassay organism (Table 1) [9]: (a) it has a relatively short life cycle for an aquatic insect (about 43 d from egg to adult at 25°C [Table 1]), which makes it feasible to perform complete life cycle experiments several times during a given year; (b) female adults are parthenogenetic, lack elaborate reproductive behavior, and readily oviposit under laboratory conditions in small 30-ml jars; (c) egg and larval stages can be readily reared to eclosion or metamorphosis, respectively, under a variety of laboratory conditions (e.g., temperature, food quality, photoperiod) in small, static vessels and do not require flowing water, even though they inhabit lotic systems; (d) important life history characteristics such as egg development, larval growth, adult size and fecundity, and so forth can be readily quantified under controlled laboratory conditions; and (e) *C. triangulifer* inhabits small- to intermediate-size streams in northeastern North America [10] and represents

Table 1. Developmental time (days) for eggs and larvae and various body measurements for *Cloeon triangulifer* at various constant temperatures (data modified from [9])

	Experimental temperatures (°C)				
	10 $\bar{x}$ (SE)	15 $\bar{x}$ (SE)	20 $\bar{x}$ (SE)	25 $\bar{x}$ (SE)	30 $\bar{x}$ (SE)
Developmental time					
Eggs (days)[a]	90.0 (3.0)	28.0 (2.6)	10.5 (0.5)	6.9 (0.5)	5.0 (–)[b]
Larvae (days)[c]	189 (179–248)	≠[d]	52 (45–62)	36 (27–43)	died
Adult characteristics					
Wing length (mm)	6.9 (0.06)	≠	5.5 (0.03)	4.8 (0.04)	died
Body length (mm)	6.9 (0.07)	≠	5.4 (0.06)	4.6 (0.05)	died
Dry mass (mg)	1.8 (0.07)	≠	1.0 (0.01)	0.6 (0.01)	died
Fecundity (eggs/female)	1,275 (85.7)	≠	937 (25.7)	574 (16.8)	died

[a]All eggs died at 35°C.
[b]All replicates hatched synchronously.
[c]All data for larvae reared on an algal diet; actual datum per treatment is the median (and range) number of days required for first-instar larvae to grow, develop, and emerge as adults; sample sizes were 32, 50, and 73 for treatments 10, 20, and 25°C, respectively.
[d]Experiment terminated at day 127 when larvae were about one-third grown due to high mortality.

an order of aquatic insects (Ephemeroptera) whose species are important components of the fauna of most natural streams and rivers worldwide and are generally considered to be very sensitive to environmental perturbation [11,12].

METHODS

Allozyme electrophoresis for assessing genetic structure of C. triangulifer

Adults were stored individually at −80°C until examined electrophoretically. Allozymes were separated by horizontal stored gel electrophoresis, using methods described elsewhere [13–17]. Briefly, gels consisted of 11% potato starch (Starch Art Corporation) in 250 ml of gel buffer and measured 18.5 × 14.6 × 0.6 cm thick. Each adult was ground in a porcelain depression plate containing at least 20 μl deionized water. Soluble proteins were absorbed onto six paper wicks (2 × 7 mm; Whatman [Clifton, NJ] No. 3 filter paper). One wick was applied to each of six gels at a slice made 5.5 cm from the cathode end of the gel. Gels were cooled during the experiment by placing aluminum trays containing ice on the gel surface, which was covered with Reynolds 910 film for electrical insulation. After electrophoresis, each gel was sliced into four horizontal slabs, each of which was stained separately for one or more enzymes. At least 30 enzymes, representing a total of 38 loci, were assayed for each specimen. Staining solutions for most enzymes were applied as 2% agar overlays, except for aspartate aminotransferase, which was immersed in stain.

Thirty adults of *C. triangulifer*—25 from the test clone and five from a control clone, which served as a reference for all experiments—were examined electrophoretically on each gel. Bands of enzyme activity at a given locus were assigned numbers corresponding to their distances from the origin relative to the common allele of the control populations. Bands of a particular mobility were interpreted as alleles, and frequencies were calculated for each allele. All presumed allelic homologies were verified in subsequent tests by comparing a few representatives from each clone on the same gel.

Chlordane preparation

Technical chlordane was obtained from Accustandard, Inc., New Haven, Connecticut (cat. no. P-017N, lot 59). It is an insecticide consisting of a mixture of chlorinated cyclohexane compounds (dominated by compounds containing seven to nine chlorines [18,19]; see Appendix). Although chlordane continues to be used throughout much of the world for pest control, its use in the United States was banned by the Environmental Protection Agency (EPA) in 1988. Chlordane is nonpolar, persistent, and likely to bioaccumulate. It has been measured in the tissues of organisms worldwide [20–23] and has recently been added to the EPA's RCRA list of regulated toxic compounds in waste and wastewater [24].

For all experiments, technical chlordane was dissolved in methanol and added to enough filtered (0.45 μm) stream water to give the desired test concentrations. Methanol is an accepted solvent and carrier for nonpolar compounds such as chlordane (Clyde Goulden, personal communication). Two control treatments of stream water were used: one with 50 μl methanol and one without (hereafter referred to as the nonmethanol control). This allowed separate evaluation of direct chlordane effects from indirect effects of the solvent. It should be noted that survivorship in the methanol control was the same as that in the nonmethanol control; we concluded that methanol did not contribute significantly to toxicity in the experiment (see below).

Egg and larval development

Eggs were placed in glass jars (6.5 cm deep; 5.5 cm diameter) containing 30 ml of test solution. All eggs in a given jar were deposited by a single female; 54 females of clone WCC 2 (an iso-female parthenogenetic line started from an adult female collected from White Clay Creek, Chester County, Pennsylvania, in 1989) were used to establish six replicates each of the two control and seven treatment conditions (i.e., chlordane concentrations of 5.0, 8.9, 15.8, 28.1, 50.0, 89.0, and 158.3 μg/L). Each jar contained about 1,000 to 2,000 eggs. Chlordane was added immediately following oviposition, using micropipettes. Eggs were incubated at 20 ± 0.1°C. Eggs were examined daily for the onset of hatching. After hatching had ceased, each clutch was examined to determine hatch success.

For larvae, preliminary experiments performed at 0.005-, 0.05-, 0.5-, 5.0-, and 50-μg/L concentrations indicated that larval mortality occurred largely between concentrations of 5 and 50 μg/L (B.W. Sweeney and D.H. Funk, unpublished data). The experiment reported here included six replicates for each of two control (methanol and nonmethanol) and five initial treatment concentrations (5.0, 8.9, 15.8, 28.1, and 50.0 μg/L). For each replicate, chlordane was added at the beginning of the experiment. In three of the replicates for a given concentration, we changed the water and added

fresh chlordane halfway through the experiment (day 16). Water samples (100 ml) were taken from each jar at the start of the experiment, frozen, and later analyzed by GC electron-capture negative ionization mass spectrometry (ECNI-MS) to show the actual initial concentrations for the 5.0-, 8.9-, 15.8-, 28.1-, and 50.0-μg/L treatments to be 4.3, 9.4, 15.4, 27.4, and 56.3 μg/L, respectively.

Thirty newly hatched, first-instar larvae of *C. triangulifer* were placed in each of 42 experimental vessels (six replicates for each of seven treatments). Vessels were 1.9-L mason jars containing 1.5 L of unfiltered White Clay Creek water (n.b. total hardness, alkalinity, sulfate, and chloride levels in White Clay Creek water at the time of the experiment averaged 82.5, 59.3, 14.5, and 9.6 mg/L respectively, with an average pH of 7.4; background levels of chlordane in White Clay Creek were 0.00011 μg/L). Each vessel was submerged halfway in a 20 ± 0.1°C circulating water bath. Light was provided by two Durotest Vita Light® fluorescent lights with a timer providing a photoperiod of 13.5:10.5 h light:dark. An airstone kept dissolved oxygen levels at saturation. A small cylindrical plastic cage with a 1-mm Nitex® netting top was placed over each jar to capture emerging adults.

Periphyton was grown on clear acrylic plates (70 × 200; 3 mm thick) in an artificial stream system enclosed by a greenhouse [9]. One of these plates was placed in each vessel at the start of the experiment to provide a food source for the *C. triangulifer* larvae. Fresh plates were added twice during the experiment to ensure an adequate food supply.

Surviving *C. triangulifer* individuals from the various control and treatment groups were collected daily as subimagos (pre-adults) from emergence cages. Most subimagos emerging from a given vessel were immediately killed, measured, dried, and weighed. The viability of eggs obtained from females that had been chronically exposed as larvae to the various control and treatment conditions was also assessed. Thus, a few subimagos from certain treatments were kept alive for 24 h (n = 8, 5, 8, 6, 10, and 6 for the 4.3-, 9.4-, 15.4-, 27.4-, and 56.3-μg/L and control groups, respectively), during which time they molted to the adult stage and were allowed to oviposit into individual glass jars for assessing hatch success as described above. A few adults from the 4.3-μg/L group were frozen intact at −80°C for later analysis for chlordane residue by ECNI-MS. We also analyzed chlordane residue in adults reared as larvae in 0.005-, 0.05-, and 0.5-μg/L treatments (B.W. Sweeney and D.H. Funk, unpublished data).

Chlordane analysis of test water and adult C. triangulifer

Chlordane was extracted from water samples by liquid–liquid extraction in a separatory funnel with three aliquots of hexane. PCB204 was added as a surrogate standard before extraction. Each hexane aliquot was rinsed into the plastic sample bottle after transfer of the water sample and before its addition to the separatory funnel containing the water sample to ensure removal of chlordane adsorbed to the walls. The three hexane aliquots were combined and evaporated down to 100 μl with a rotary evaporator and then under nitrogen for the final step. Chlordane components and metabolites were quantitated from the relative responses of the surrogate standard and the authentic standards of heptachlor; *trans*- and *cis*-chlordane; *trans*- and *cis*-nonachlor; and the two metabolites, heptachlor epoxide and oxychlordane (Accustandard, Inc., and Ultra Scientific). Adult body burdens and water concentrations of chlordane residue were calculated by comparison of the component areas with that of the surrogate standard to correct for losses during extraction and purification. Absolute recovery was not determined.

Adult mayflies from a given replicate vessel per treatment were dried and combined in lots of five to 13 organisms for extraction and analysis. The mayflies were ground in 0.5 ml methanol after addition of the surrogate standard PCB204 (2,2′,3,4,4′,5,6,6′-octachlorobiphenyl) and extracted by sonication with two 10-ml aliquots of a 1:1 methanol: methylene chloride mixture. Extracts were filtered through pasteur pipettes loaded with approximately 1.0 g of Na_2SO_4 and clean glass wool. Chlordane components and the surrogate standard were separated from other components in the extract on 1% deactivated silica (0.8 g) microcolumns (organochlorines were eluted with 20% methylene chloride in hexane).

Five principal chlordane components and two metabolites (heptachlor, *cis*- and *trans*-chlordane, *cis*- and *trans*-nonachlor, heptachlor epoxide, and oxychlordane; see Appendix for structures) were identified and quantitated by ECNI-MS, using a Hewlett Packard 5988/RTE-A mass spectrometer equipped with a Hewlett Packard 5890 GC. Instrumental conditions were as follows: GC—0.25-mm, 30-m DB-5 column (J&W Scientific) held at 60°C for 2 min, then ramped to 180°C at 20°/min, to 230°C at 2°/min, to 280°C at 10°/min, and then

held at 280°C for 10 min; MS–CH_4 pressure 0.45 torr, source temperature 100°C.

RESULTS

Genetic aspects of C. triangulifer

Genetic variation within two natural populations of *C. triangulifer* in Pennsylvania was quantified (White Clay Creek [39°51′N; 75°47′W] and Chillisquaque Creek [41°03′N; 76°41′W]). Genetic variability was substantial in *C. triangulifer*, with 19 distinct genetic clones determined from the two streams (Fig. 1). The magnitude of genetic variation among clones (i.e., genetic distance [25]) ranges from 0.004 to 0.183. This is very similar to the range of genetic distances observed between local populations of bisexual mayflies but considerably less than the typical range of genetic distance between closely related (e.g., sibling) species of mayflies. The clonal nature of parthenogenetic reproduction has been confirmed in our laboratory by the absence of allozyme differences between a given mother and her offspring, as well as in the field, where individuals in natural populations cluster biochemically into distinct genotype classes or clones that have been found year after year (Fig. 1). All the experiments in this paper were performed using the WCC 2 clone isolated from White Clay Creek.

Egg development and hatch success, larval survivorship, growth, and development rate

Eggs began hatching 9 d after oviposition in all 54 jars representing the two control and seven treatment conditions (i.e., 5.0, 8.9, 15.8, 28.1, 50.0, 89.0, and 158.3 μg/L). Hatch success exceeded 95% for all replicates representing all treatments.

For larval experiments, at *t* test revealed no significant difference between the methanol and nonmethanol control groups, so only the methanol group was used for subsequent analyses of chlordane treatment effects. Also, analysis of variance (ANOVA) showed that for a given treatment level, there was no significant difference for any of the study parameters between the groups treated with

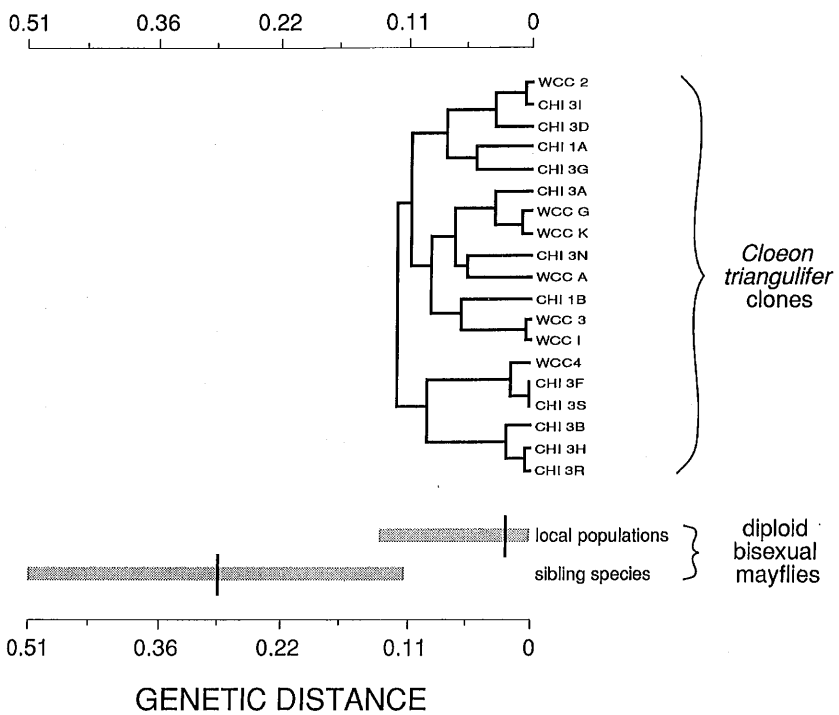

Fig. 1. A phenogram using Nei's [25] genetic distance (a measure of the accumulated number of gene substitutions per locus) and showing the genetic relationships between 19 clones of *Cloeon triangulifer* that occur in White Clay Creek and Chillisquaque Creek, Pennsylvania. Distances were calculated from allele frequency data for 30 enzyme loci. For comparison, the average (vertical bar) and range (horizontal stippled bar) of genetic distance between local, intraspecific populations (i.e., located within 200 km of one another) as well as populations of sibling species are shown for bisexual mayflies.

chlordane only once (day 1) and those retreated on day 16. For this reason, both groups were combined for all subsequent analyses.

Larval survivorship averaged about 80% for both the methanol and the nonmethanol control groups, 18% for the 4.3-μg/L treatment, <0.6% for the 9.4-μg/L treatment, and zero for chlordane concentrations ≥15.4 μg/L (Fig. 2a). These differences were statistically significant (ANOVA), with survivorship for the 4.3-μg/L treatment group (and thus higher concentrations) being significantly lower than the controls (Dunnett's t test; $p \leq 0.05$).

The average duration of the larval period or developmental time (Fig. 2b) and size of the adult at metamorphosis (Fig. 2c) were significantly longer (37 vs. 34.8 d) and larger (1.5 vs. 1.1 mg), respectively, for the 4.3-μg/L treatment group relative to the control groups (Dunnett's t test; $p \leq 0.05$). Statistical analysis of data for the 9.4-μg/L treatment group was not possible due to the low number of individuals.

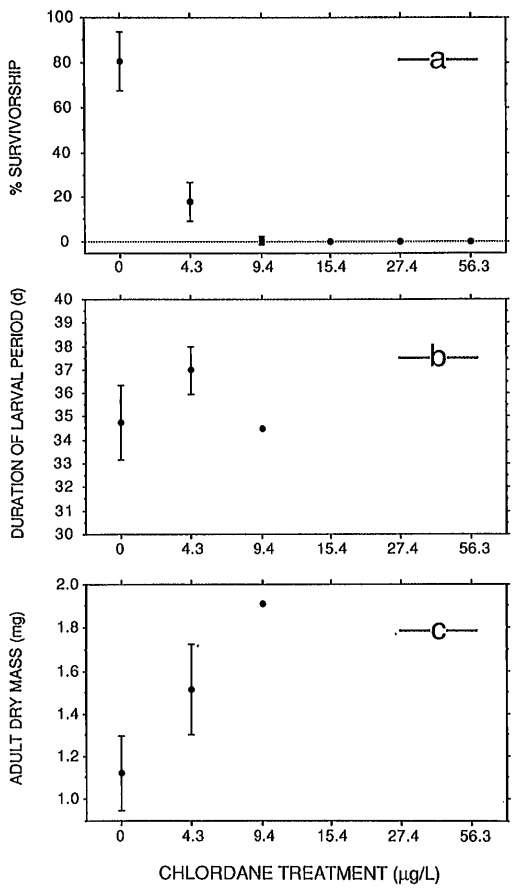

Fig. 2. Larval survivorship (**a**), duration of the larval growth period (**b**), and adult female dry mass (**c**) for *Cloeon triangulifer* as a function of various levels of chlordane treatment. Mean (solid dot) and 95% C.I. are shown for all data. Note that only one individual survived in the 9.4-μg/L treatment.

Egg hatch success exceeded 95% for all egg clutches obtained from adults who survived exposure as larvae to the two control and five treatment conditions (namely, 4.3, 9.4, 15.4, 27.4, and 56.3 μg/L). The development time of these eggs was not quantified for this portion of the experiment.

Adult body burden of chlordane residues

Body burdens of chlordane compounds and metabolites were determined for adult *C. triangulifer* that survived as larvae when reared at various concentrations of technical chlordane. Adults from three replicate-rearing vessels treated with 4.3 μg/L technical chlordane contained 100 ± 45 ppb total chlordane residue. The distribution of chlordane components and metabolites for these adults is shown in Fig. 3a, along with the distribution of compounds in adults reared at concentrations <4.3 μg/L (n.b. data for 0.005- to 0.5-μg/L treatments are from preliminary experiments that were performed using the same methods described earlier but are not reported here because treatment concentrations were too low [<4.3 μg/L] to cause significant mortality). Note that adult mayflies reared at the lower concentrations were indistinguishable from the controls with respect to pattern and concentration of components. The relative composition of principal chlordane components or metabolites found in mayflies reared at 4.3 μg/L chlordane was as follows: *trans*-nonachlor (45 ± 11%), oxychlordane (21 ± 13%), heptachlor epoxide (18 ± 7%), *cis*-nonachlor (6 ± 2%), *trans*-chlordane (5 ± 3%), and *cis*-chlordane (4 ± 3%). In contrast, the principal components in technical chlordane were *trans*-chlordane (26%), *trans*-nonachlor (23%), *cis*-chlordane (21%), heptachlor (19%), and *cis*-nonachlor (11%) (Fig. 3b). For comparison, Figure 3b also shows the relative composition of chlordane components found in other animals (e.g., porpoise [PORPS] [21], lake trout [TROUT] [26], salmon [SLMON] [21], and zooplankton [ZPLKTN] [21].

DISCUSSION

Genetic structure of C. triangulifer *and other bioassay organisms*

The extent and nature of genetic variability in laboratory populations of standard bioassay animals (i.e., *Chironomus* and *Daphnia*) have recently

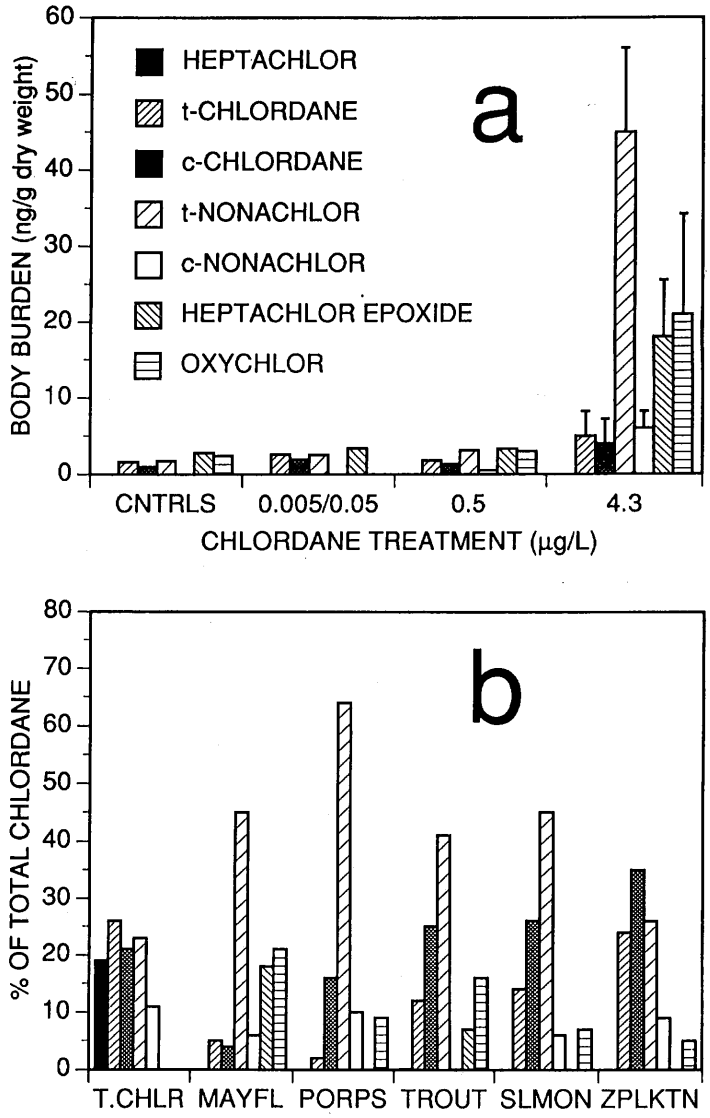

Fig. 3. (**a**) Body burden of chlordane components and metabolites found in adult *Cloeon triangulifer* mayflies whose larval stages were exposed to different concentrations of technical chlordane; (**b**) relative abundance of various components and/or metabolites of chlordane in *C. triangulifer* mayflies (this study; 4.3-µg/L treatment), porpoise [21], lake trout [26], salmon [21], and zooplankton [21]. Note that heptachlor epoxide was not measured in the salmon or zooplankton, and heptachlor was not measured in any organisms other than the mayfly. Vertical bars for the 4.3-µg/L treatment are one standard error.

been shown to be significant factors underlying the lack of repeatability of standard bioassay results between laboratories [27,28]. In fact, it has been recommended that regular genetic monitoring become standard protocol for laboratories engaged in this type of research [27]. It is clear that more attention will be paid to the genetic structure of bioassay populations in order to maintain repeatability, which remains an essential property of the laboratory bioassay. However, some of this problem could be alleviated if studies were restricted to specific clones of test organisms like *C. triangulifer*, for which genetic characterization of only a few randomly selected individuals would provide

adequate quality assurance/quality control for the genetic component of entire experiments using only one clone.

The three primary reasons that *D. magna* has been widely preferred as a bioassay organism are its ease of laboratory culture, its high sensitivity, and its clonal method of reproduction. The mayfly *C. triangulifer* shares all three advantages, including clonal reproduction. The clonal nature of parthenogenesis in *C. triangulifer* allowed us to eliminate genetic variability as a confounding variable for our experiments on all life stages. Thus, when we observed variation in average body burden of chlordane among adults reared in different replicates of our 4.3-μg/L treatment (i.e., 51–140 ppb), we were able to eliminate the possibility that one or more genotypes having different propensities to bioaccumulate chlordane was a causative factor and focus on other issues. This attribute should also make *C. triangulifer* an especially attractive organism for elucidating the physiological mechanism of a toxicant where genetic variation is simply unwanted noise that might obscure the mechanism under study [27]. Conversely, we have already isolated 19 distinct genetic clones (i.e., genotypes) of *C. triangulifer* from wild populations and have measured genetic distances [25] of as much as 0.183 between pairs of clones, which indicates substantial genetic heterogeneity within the species. Thus, we could address the role of genetic variation in susceptibility to a toxic substance if the issue was pertinent by including more than one clone in the experimental design. Future research on clonally reproducing organisms such as *C. triangulifer* and *D. magna* should focus on differences among clones in their tolerance to specific toxicants. For example, it would be extremely valuable to know if the sensitivity of different clones reared under the same exposure to toxins differed significantly and whether there was a consistent rank order in the response [28]. These types of experiments would begin addressing the problem of interactive effects between genotype and environment, a rather unexplored but very important area of bioassay research.

Response of C. triangulifer *to technical chlordane*

It is recommended that toxicity studies involving freshwater aquatic insects emphasize long-term tests on a wide variety of organisms of known source and history (i.e,. cultured organisms) that are abundant in unpolluted streams or rivers and sensitive to low concentrations of pollutants [4]. Such tests must be logistically straightforward and repeatable to be useful. Our data suggest that the mayfly *C. triangulifer* fits the recommended criteria as well as, if not better than, other available cultured stream insects and has additional unique advantages that make it even more attractive as a test organism.

Although our experiments lasted several weeks, the test organisms did not receive a constant exposure to the test concentrations. Water samples taken at the end of the experiment indicate that chlordane levels in all treatments had declined to <11 ng/L (range 0.3–11.0). We have no information as to how rapidly the chlordane levels declined following the initial dose. A significant portion of it was probably taken up by the algae, and thus experimental animals remained exposed through their diet. Ongoing studies show, for example, that algae exposed to 5 μg/L of technical chlordane rapidly accumulate a body burden of 26 ± 5 ppb. An unknown quantity was probably lost by volatilization and adsorption onto the walls of the glass vessel. A valid chronic test for chlordane would have to involve constant replenishment of toxicant throughout the duration of the experiment. However, even though we cannot use probit analysis of our survivorship data to calculate a specific LC50 for our experiment (because chlordane concentrations were not kept constant throughout the experiment), it is clear from the survivorship data that the value cannot exceed 4.3 μg/L. This suggests that *C. triangulifer* larvae are substantially more sensitive to technical chlordane than other aquatic macroinvertebrates that have been tested to date (e.g., *Chironomus* and *G. lacustris* with LC50 values of 15 and 26 μg/L, respectively [29]).

Our study also demonstrated that experiments lasting the duration of both the egg and the larval stages are both feasible and characterized by acceptable levels of survivorship in controls (>95% for eggs, about 80% for larvae). The larval control data, although not ideal, compare very favorably with survivorship values ranging from 57.0 to 83.5% in control populations of *C. riparius* during 30-d exposure tests [5]. Moreover, we have demonstrated that larval growth and development of *C. triangulifer* can be readily quantified for use in assessing sublethal effects of toxic substances. Survivors in the 4.3-μg/L chlordane treatment exhibited an increase in both larval development time and size at maturity. These data are mutually consistent because individuals having a longer development time would have a longer period to grow and, hence, could achieve a larger size at maturity. The

fact that a 6% increase (2.2 d) in developmental time resulted in a 30% increase (0.33 mg) in individual size is also not surprising because larvae grow at a rate of 0.22 mg/d during the last 5 d of their larval stage at 20°C (B.W. Sweeney, unpublished data). At this time we have no insight as to how chlordane interferes with *C. triangulifer* development.

Our data on the developmental rate and hatch success of eggs indicate that the chorion of *C. triangulifer* eggs protects the embryo from external chlordane exposure of at least 158.3 μg/L. Furthermore, even though females accumulate a substantial body burden of chlordane (up to 140 ppb) during chronic exposure as larvae, these concentrations do not seem to interfere with the rate and success of embryonic development for their offspring. This aspect of the study was important because it demonstrated that tests for evaluating the potential toxicity of compounds, either directly by water exposure or indirectly through bioaccumulation during feeding of the parental generation, can be readily designed and implemented with *C. triangulifer*.

Although we did not perform short-term (<48-h) tests on first-instar larvae, they could be easily performed as static tests in small 30-ml jars similar to those used for the egg studies. For these studies, periphyton food would probably not have to be provided for the larvae because they still have some residual yolk sac left as an energy source. We have observed newly hatched larvae to survive without food for >48 h at 20°C.

Body burdens of chlordane residues

Although recent field data suggest that the metabolic transformation capability of the mayfly *H. limbata* for chlorinated hydrocarbons is "probably insignificant" [30], our data show this not to be true for all mayflies. For *C. triangulifer*, our results for the technical chlordane experiments suggest that (a) heptachlor was completely metabolized by the mayflies into heptachlor epoxide; (b) *cis*- and *trans*-chlordane were metabolized in part to oxychlordane; and (c) *cis*- and *trans*-nonachlor were poorly metabolized. The pattern of metabolism in *C. triangulifer* is strikingly similar to available data on fish (e.g., see Fig. 3b for data on lake trout and salmon). Because heptachlor and heptachlor epoxide were not measured in the salmon [21] and only heptachlor epoxide was measured in the lake trout [26], we could compare only the chlordanes, the nonachlors, and the metabolite oxychlordane. Reduced quantities of *cis*- and *trans*-chlordane relative to the nonachlors were observed in the mayfly and salmon (but not in zooplankton), as compared to the original distribution of components in technical chlordane. Indeed, the mayflies exhibited a greater degree of transformation of the chlordanes to oxychlordane than the lake trout and salmon and an even greater level of metabolism than the zooplankton, which are organisms occupying an equivalent trophic level. To our knowledge, these are the first data showing the metabolic fate of technical chlordane once ingested or absorbed by a stream- or river-dwelling macroinvertebrate.

The mayfly *C. triangulifer* seems to have completely metabolized the heptachlor to heptachlor epoxide. A comparison with the other organisms in Figure 3b is not possible because heptachlor and/or its metabolite heptachlor epoxide were not reported [21,26]. The degree to which excretion and further metabolism of heptachlor to other by-products are occurring is uncertain because we did not carry out a mass balance of the chlordane components during the experiment.

The ratio of *trans*-chlordane to *cis*-chlordane is altered from an equal distribution in technical chlordane to a 2:1 or greater predominance of the *cis*- over the *trans*- isomer for the aquatic organisms shown for comparison purposes in Figure 3b. However, the mayfly appears to be capable of metabolizing or excreting both isomers equally (Fig. 3b). It has been hypothesized that the predominance of the *cis*- isomer may be due to the deposition and subsequent exposure of aquatic organisms to atmospherically altered chlordane residues rather than a metabolic discrimination of isomers by the analyzed organisms [26]. Our data support this hypothesis, because the mayfly larvae in these experiments were exposed to unaltered technical chlordane and treated both isomers equally.

It is also noteworthy that *trans*-nonachlor, which accounts for 35 to 56% of the stored chlordane components and metabolites in *C. triangulifer*, is also the principal component found in several species of fish and lobster collected off the east coast of Canada [31], in bald eagles [32], and in human blood [33]. In fact, the ratio of *trans*-nonachlor to *cis*-nonachlor in *C. triangulifer* (range 6.2–8.6) overlaps substantially with the ratio of 5:7 observed for marine fish and lobsters [31].

Ultimately, there is a clear trend for the mayfly to bioaccumulate *cis*- and *trans*-nonachlor as well as the metabolites heptachlor epoxide and oxychlordane. Because the metabolites are several times more toxic than the original compounds [34], this emphasizes the limitation of assessing toxicity by chemical assay of the precursor pollutants. Or-

ganisms such as the mayfly *C. triangulifer* may be much more sensitive to chlordane residues than other animals due to their greater ability to metabolize the primary components of technical chlordane.

CONCLUSIONS

The need to employ a range of species in bioassay testing has been greatly emphasized in order to evaluate and/or predict adequately the impact of a chemical on a particular environment [7,8]. In this paper, we demonstrated both the utility and the effectiveness of the mayfly *C. triangulifer* for whole life cycle bioassays. The data reveal sensitivity equal to or better than other standard bioassay species and show how various life history parameters can serve as response indexes. We conclude that the parthenogenetic mode of reproduction for *C. triangulifer*, combined with a relatively short embryonic and larval developmental period and overall ease of maintaining laboratory cultures, provides a useful and effective bioassay system for evaluating toxicants.

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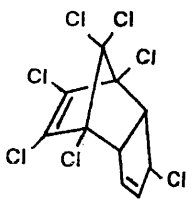
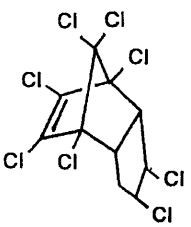
APPENDIX

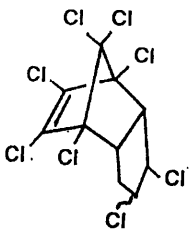
COMPONENTS OF TECHNICAL CHLORDANE:



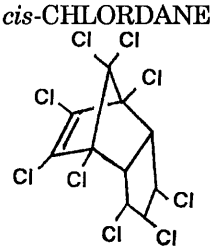
HEPTACHLOR



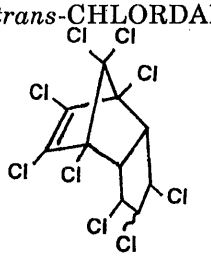
cis-CHLORDANE



trans-CHLORDANE

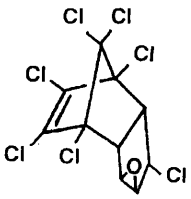


cis-NONACHLOR

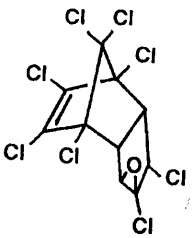


trans-NONACHLOR

METABOLITES OF TECHNICAL CHLORDANE:



HEPTACHLOR EPOXIDE



OXYCHLORDANE.