Evaluation of Glass, Nylon Fabric and Filter Paper as Substrates in Insecticide Bioassays of Cat Fleas (Siphonaptera: Pulicidae)[1]

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Abstract Four bioassays for use in detecting and measuring insecticide resistance in newly-emerged, unfed adult cat fleas, *Ctenocephalides felis* (Bouché), were evaluated: horizontal glass, horizontal Nylon 6,6 fabric disk, horizontal cellulose filter paper disk, and vertical cellulose filter paper strip (WHO bioassay). Each bioassay was evaluated using five insecticides: carbaryl, chlorpyrifos, malathion, permethrin, and pyrethrum. LC_{50}s, LC_{90}s, probit line slopes, and slope standard errors were compared. The LC_{50}s on glass were lower than those obtained with the other substrates. This difference was at least an order of magnitude with carbaryl, malathion, permethrin, and pyrethrum. The paper disk and paper strip bioassays produced the highest LC_{50}s and LC_{90}s for fleas treated with carbaryl, malathion, and pyrethrum. With chlorpyrifos and permethrin, the paper strip resulted in the highest LC_{50}s. The nylon disk tended to produce LC_{50}s intermediate between glass and filter paper. On glass, chlorpyrifos generated higher LC_{50}s (2.00 mg[AI]/m^2) than permethrin (0.927 mg[AI]/m^2) or pyrethrum (0.913 mg[AI]/m^2), yet on the paper strip was lower (65 mg[AI]/m^2) than permethrin (214 mg[AI]/m^2) or pyrethrum (466 mg[AI]/m^2). Overall, probit line slopes were highest for glass and WHO. Standard errors of the slope were not significantly different among bioassays. Although nylon disk assay possibly simulates chemical-substrate interactions on carpet, which is a common substrate where cat fleas occur, no single substrate gave acceptably precise probit lines for all chemicals tested, and chemical efficacy depended on the substrate used. Chemical-substrate interactions confound detection of insecticide resistance and chemical efficacy.

Key Words *Ctenocephalides felis*, bioassay, substrate, efficacy, insecticide resistance, nylon

Substrates on which insecticide residues are deposited affect the relative efficacies of chemicals against cat fleas, *Ctenocephalides felis* (Bouché), (El-Gazzar et al. 1986, Olsen 1993) and may contribute to actual control failures. Moyses (1995) found chlorpyrifos more effective than permethrin on filter paper and with topical application, but less effective than permethrin on nylon carpet. Many household surfaces, such as carpet and wood, hinder control efficacy, but others, such as tile, do not (Chadwick 1985, Koehler et al. 1986, Osbrink et al. 1986).

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Bioassays of adult and larval cat fleas have used a variety of substrates. The World Health Organization (WHO) standard bioassay uses a cellulose filter-paper strip (Whatman #1) that is oriented vertically in a test tube (WHO 1981). Other cat flea bioassays have utilized substrates such as glass (Schwinghammer et al. 1985), nylon carpet (Koehler et al. 1986), cotton fabric (Rust and Reierson 1988), soil (Palma and Meola 1990), fiberglass (Olsen 1993), wool fabric (Olsen 1993), turf grass (Metzger et al. 1996), direct application (Moyses and Gfeller 2001), and nylon fabric (Bossard et al., in press).

Comparing results from these different bioassays, or relating resistance values obtained in such bioassays to actual control failures, is difficult without determining representative lethal doses for each bioassay under similar conditions (Bossard et al. 1998). Horizontally-oriented nylon (i.e., carpet) is the substrate most frequently encountered in the United States when conducting premises application for flea control (Koehler et al. 1986), but mortality on a nylon-carpet substrate cannot be assessed easily. Glass or filter paper substrates, though, may cause different responses compared to those with nylon carpet.

We developed a bioassay to more closely simulate exposures of cat fleas to insecticide-treated carpet. This bioassay uses a horizontally-oriented, Nylon 6,6 fabric disk in a flat-bottomed test tube. We compared it to three other bioassays using test tubes, differing mainly by the surface treated with the insecticide residues: a horizontal glass surface, a horizontally-oriented cellulose filter-paper disk, and a vertically-oriented filter-paper strip (the standard WHO bioassay).

The evaluation criteria used considered the best bioassay to have steep probit line slopes to increase differentiation among strains, low standard errors of the slope, ease of use, and realism in simulating field application (Sheppard and Hinkle 1987, ffrench-Constant and Roush 1990, Scott 1990). Our main objective was to evaluate and determine representative lethal doses for each bioassay.

Materials and Methods

Fleas. Fleas from the "Kansas1" laboratory strain, established from fleas collected in Manhattan, KS, in July-August 1990, were reared and maintained on cats, as described by Dryden (1989). Pupae were sifted from sand media and placed on trays suspended 10 cm above the bottom of 4-L wide-mouth jars. Emerging fleas were collected from the bottoms of jars as they jumped from the suspended tray. Pupae and emerging adults were maintained in environmental chambers at 26°C (25 to 29°C) and 70 to 90% RH, in darkness. The bioassays used unfed adult fleas aged 0 to 3 d after emergence. Neither CO_2 nor cold were used to immobilize fleas during handling. Specimens of the Kansas1 strain were deposited in the Museum of Entomological and Prairie Arthropod Research, Department of Entomology, Kansas State University, Manhattan, KS (Voucher specimens #073).

Substrates. The bioassays consisted of the following substrates and orientations in 25 × 150 mm glass test tubes (Fisher Scientific, St. Louis, MO):

1. Horizontally-oriented surface of a 22 mm inside diam bottom of a flat-bottomed test tube (hereafter, Glass).

2. Horizontally-oriented, 22 mm diam Nylon 6,6 disk (Cerex® Spunbonded Nylon-Type 23, Cerex Advanced Fabrics, L.P., Cantonment, FL), that covered the bottom of a flat-bottomed test tube (hereafter, Nylon Disk). Because nylon disks of this diameter could not be obtained commercially, disks were cut using a custom-made stainless steel cutter.
3. Horizontally-oriented, 22 mm diam disk (Whatman #1 filter paper), that covered the bottom of a flat-bottomed test tube (hereafter, Paper Disk).
4. Vertically-oriented, 15 × 50 mm strip (Whatman #1 filter paper) with trimmed bottom corners, similar to the WHO protocol (WHO 1981), in a round-bottomed test tube (hereafter, WHO).

Insecticides. Five insecticides were utilized: carbaryl (Carbaryl Technical, 99%; Rhone-Poulenc Ag Co., Research Triangle Park, NC), chlorpyrifos (Dursban XP, 99%; Whitmire Research Laboratories, St. Louis, MO), malathion (Fyfanon Technical, 96%; Batch# 40415-01, Cheminova Agro A/S, Lemvig, Denmark), permethrin (Permethrin Technical, 91%; Whitmire Research Laboratories), and pyrethrum (Pyrethrum Extract, 20% active, Lot #289, Whitmire Research Laboratories). The pyrethrum was mixed with piperonyl butoxide 1:10 by weight (PBO 100% active, Lot # 9536 and 9586, Whitmire Research Laboratories), similar to some commercial formulations. Because the pyrethrum formulation on Glass did not dry even after 24 h, the amount of piperonyl butoxide on Glass was reduced until droplets were not visible (about 1:5).

The insecticides were weighed into 50-ml volumetric flasks and dissolved in acetone. Initial concentrations were 1000, 100, 10, 1 mg[AI]/m^2 with the assay repeated as needed down to 0.5 mg[AI]/m^2 or up to 10,000 mg[AI]/m^2 in order to narrow the fiducial limits of the probit estimates.

Assay procedure. All surfaces were treated using insecticide solutions applied by a micropipette to produce equivalent weight deposits of active ingredients per m^2 and were dried on wire racks for at least 2 h before testing. Around 10 fleas (range 8 to 23) were placed into each bioassay tube, 80 fleas (range 30 to 150) were used for each probit line, and one to five tubes for each dose. Tubes were capped with Parafilm® (American Can, Neenah, WI) which was punctured with needles. Bioassay tubes were placed upright in an incubator at 26°C and approximately 40% RH in darkness, with mortality assessed after 24 h. At that time, fleas that were not moving or not oriented upright were recorded as dead or moribund. Glassware was washed in detergent, rinsed, and reused. Nylon Disk and Paper Disk bioassays were repeated over a one month period to evaluate variation in lethal concentrations through time.

Statistical analysis. Probit analysis of the data produced log-dosage probit lines, from which LC_{50}s and LC_{90}s, their fiducial limits (FL), slopes using $\log_{10}$ of the concentrations, and errors were calculated (SAS Institute 1990). Significant differences between bioassays were determined by non-overlap of the 95% FL of LC_{50}s and LC_{90}s. Using analysis of variance, least significant difference tests, pooled slopes and errors for each bioassay were compared using each insecticide as a replicate (n = 5).

Results

Using carbaryl, malathion, permethrin, and pyrethrum, the LC_{50}s on Glass were between 0.913 to 2.40 mg[AI]/m^2. This was an order of magnitude lower than those

obtained with the other substrates of 16.9 mg[AI]/m^2 to 466 mg[AI]/m^2) (Figs. 1-5). The LC_{90}s, also, on Glass were much lower than those on the other substrates when using chlorpyrifos (2.77 mg[AI]/m^2) (other substrates were 10.7 to 90.7 mg[AI]/m^2), and permethrin (2.27 mg[AI]/m^2) (other substrates were 191 to 1000 mg[AI]/m^2). With carbaryl, malathion, and pyrethrum, the highest LC_{50}s were on the Paper Disk of between 71.2 to 223 mg[AI]/m^2, and WHO of between 135 to 466 mg[AI]/m^2. Nylon Disk was intermediate between Glass and filter paper bioassays. WHO generated higher LC_{50}s on chlorpyrifos (65.5 mg[AI]/m^2) and permethrin (214 mg[AI]/m^2) than those of Glass, Nylon Disk, and Paper Disk bioassays (2.00 mg[AI]/m^2, 6.30 mg[AI]/m^2, 4.82 mg[AI]/m^2; and 0.937 mg[AI]/m^2, 16.9 mg[AI]/m^2, 33.3 mg[AI]/m^2, respectively). On Glass, chlorpyrifos generated higher LC_{50}s (2.00 mg[AI]/m^2) than permethrin (0.927 mg[AI]/m^2) or pyrethrum (0.913 mg[AI]/m^2), yet on the WHO paper strip, it was lower (65.5 mg[AI]/m^2) than permethrin (214 mg[AI]/m^2) or pyrethrum (466 mg[AI]/m^2).

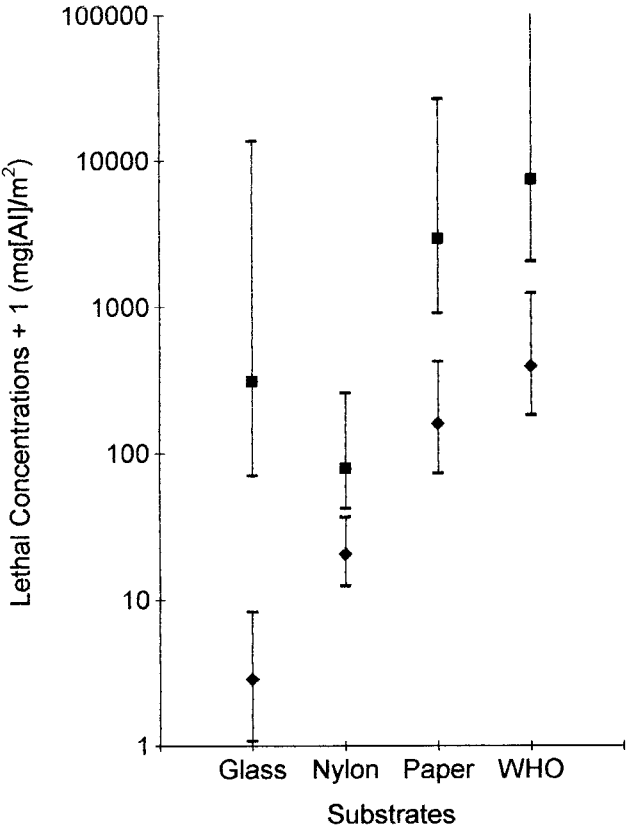

Fig. 1. Responses to carbaryl of adult *C. felis* from Kansas1 colony using four bioassay systems: Glass, horizontal glass; Nylon, horizontal nylon disk; Paper, horizontal paper disk; WHO, vertical paper strip. LC_{50} (♦), LC_{90} (■).

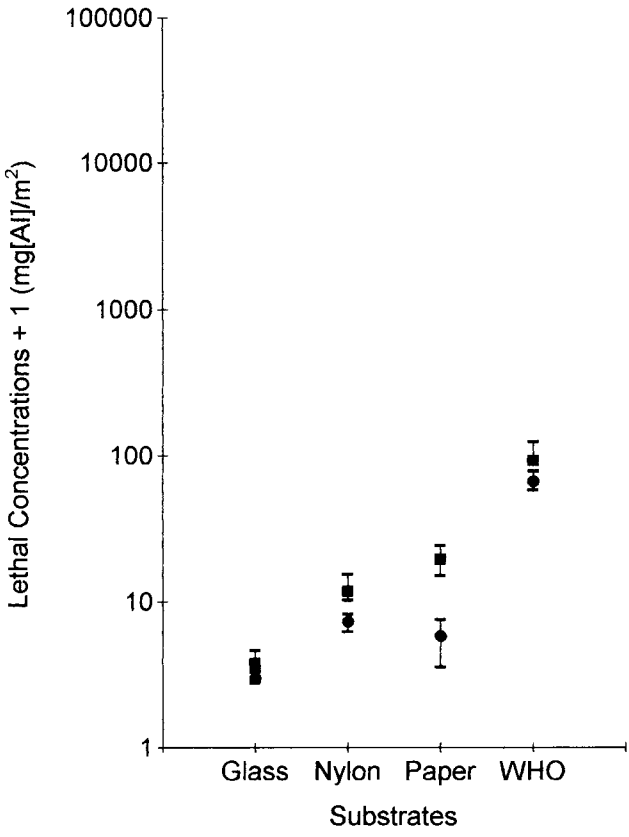

Fig. 2. Responses to chlorpyrifos of adult *C. felis* from Kansas1 colony using four bioassay systems: Glass, horizontal glass; Nylon, horizontal nylon disk; Paper, horizontal paper disk; WHO, vertical paper strip. LC_{50} (♦), LC_{90} (■).

With the pooled data, slope of the probit line was highest for Glass and WHO (Table 1), but standard errors of the probit line slope were not significantly different among bioassays. There were significant, 4-fold differences through time among LC_{50}s, and 9-fold differences among LC_{90}s, conducted on Nylon Disk and Paper Disk using chlorpyrifos (Table 2).

Glass was easy to set up and use, but required extra washing to remove chemical residues. The WHO paper strips were easier to prepare and remove for disposal than the disks. Fleas were difficult to count as they jumped behind the strip or when they crawled under the disks and, flattened, appeared dead. Occasionally, moribund fleas were only temporarily knocked down. Fleas were often unable to climb immediately onto the paper strip after having fallen off into the rounded glass bottom. Corners of the paper strip as tested may not have been rounded sufficiently against the bottom of the test tube.

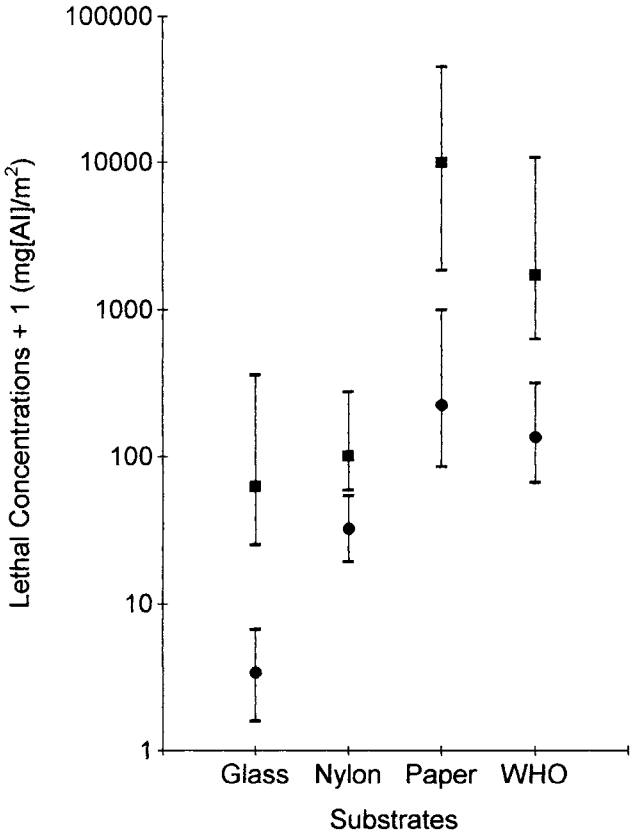

Fig. 3. Responses to malathion of adult *C. felis* from Kansas1 colony using four bioassay systems: Glass, horizontal glass; Nylon, horizontal nylon disk; Paper, horizontal paper disk; WHO, vertical paper strip. LC_{50} (♦), LC_{90} (■).

Discussion

The low LC_{50}s obtained with glass bioassays relative to bioassays on fabric is probably an effect of the increased availability of insecticides on the glass (Rust and Reierson 1988). In contrast, Burg et al. (1995) found mortality of horn flies to be usually lower on glass than on filter paper with diazinon, permethrin, and pirimiphos methyl, and about the same with fenvalerate, but they did not explain the difference. Glass may simulate non-fibrous substrates, such as tile, more effectively than nylon or paper, but an additional complication is that the bioassay solvent was acetone rather than water, so the droplet sizes obtained in bioassay probably do not duplicate the field application. These differences could affect insecticide transfer to the insect.

The higher lethal doses on the vertically-oriented surface than on the horizontally oriented surface probably result from fleas spending time on untreated surfaces ran-

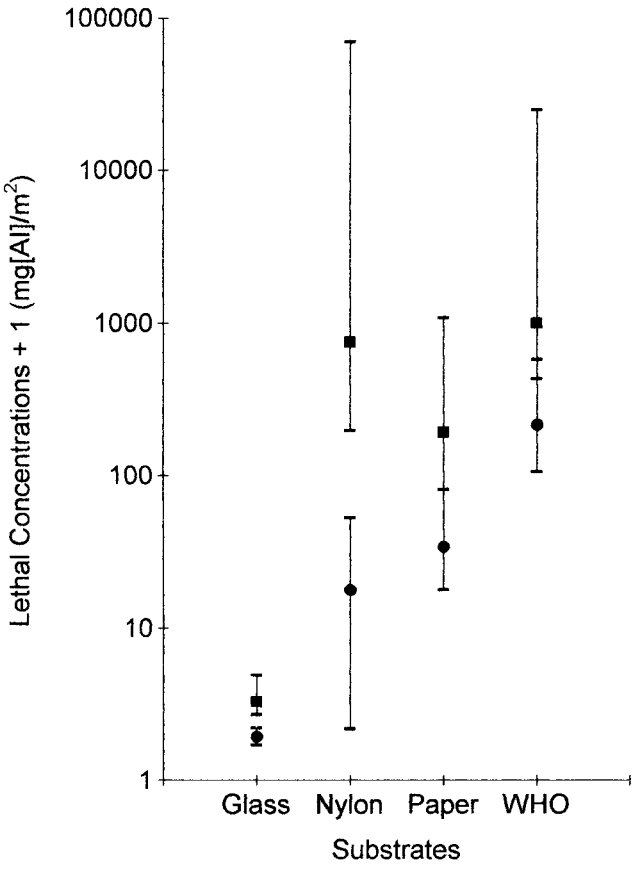

Fig. 4. Responses to permethrin of adult *C. felis* from Kansas1 colony using four bioassay systems: Glass, horizontal glass; Nylon, horizontal nylon disk; Paper, horizontal paper disk; WHO, vertical paper strip. LC_{50} (♦), LC_{90} (■).

domly, kinetically, or due to repellency. The horizontal disk bioassays force the fleas into almost continuous contact with treated surfaces.

The most precise bioassay, as indicated by the steepest probit line slope, depended on the chemical used. Nylon Disk was most precise for carbaryl and malathion, Glass and WHO for chlorpyrifos and pyrethrum, and Glass for permethrin. Sheppard and Hinkle (1987) found glass bioassays with house flies to have lower variability than topical-application bioassays.

The 4-fold fluctuation of LC_{50}s seen in repeated chlorpyrifos bioassays is within the 7-fold variation of LC_{50}s observed within cat flea strains (Moyses 1995), and may indicate strain variability (Bossard et al. 2000). The realism necessary, if any, for treated bioassay surfaces to mimic variation on actual control surfaces is unknown (Sheppard and Hinkle 1987).

Our results do not support the contention that filter paper bioassays are

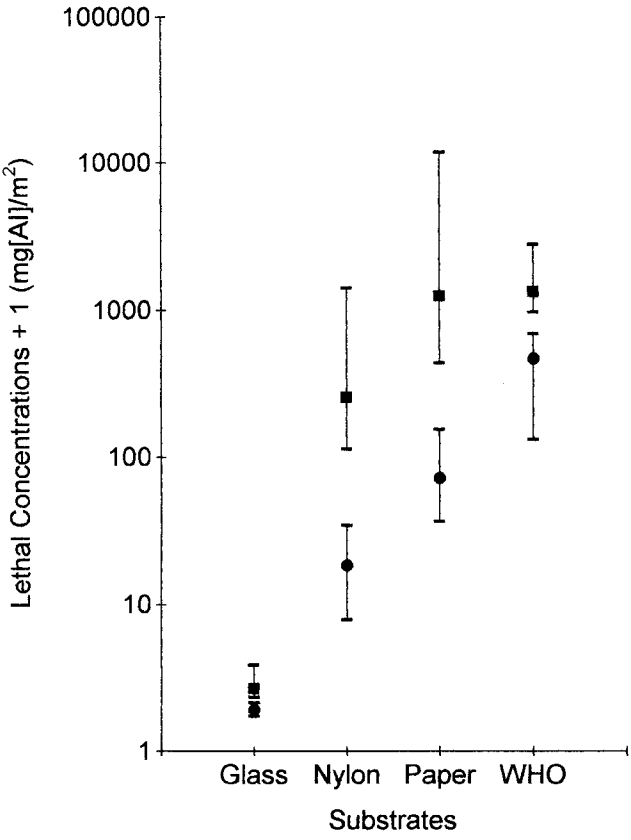

Fig. 5. Responses to pyrethrum of adult *C. felis* from Kansas1 colony using four bioassay systems: Glass, horizontal glass; Nylon, horizontal nylon disk; Paper, horizontal paper disk; WHO, vertical paper strip. LC_{50} (♦), LC_{90} (■).

necessarily more accurate than glass substrates (Koehler et al. 1986) because Paper Disk probit line slopes were lower than those of Glass. Burg et al. (1995) concluded that both glass and filter paper bioassays were often unable to detect resistance differences among populations of horn fly using diazinon, fenvalerate, permethrin, or pirimiphos methyl. The Nylon Disk bioassay, though as precise as Paper Disk, may be more appropriate than either glass or paper because the substrate is of the same material as carpet on which cat fleas are often found developing.

The changes in relative efficacy of an insecticide on different substrates may be caused by physical and chemical interactions affecting the availability of the chemical (Dryden and Reid 1996, Metzger et al. 1996, Bossard et al. 1998). Because of chemical-substrate interactions, no single substrate was most precise for all chemicals tested and resistance is confounded with chemical efficacy.

Table 1. Number of test tubes per experiment and probit line slopes of four bioassay systems using five insecticides

Insecticide	Bioassay	n	Slope ± SE
Carbaryl	Glass	8	0.576 ± 0.157
	Nylon	8	2.13 ± 0.421
	Paper	8	1.01 ± 0.184
	WHO	8	1.00 ± 0.195
Chlorpyrifos	Glass	10	9.17 ± 1.86
	Nylon	15	5.56 ± 1.087
	Paper	15	2.20 ± 0.488
	WHO	8	9.09 ± 1.92
Malathion	Glass	8	0.911 ± 0.195
	Nylon	8	2.54 ± 0.536
	Paper	8	0.777 ± 0.165
	WHO	8	1.16 ± 0.208
Permethrin	Glass	10	3.30 ± 0.624
	Nylon	6	0.779 ± 0.247
	Paper	7	1.69 ± 0.346
	WHO	3	1.92 ± 0.623
Pyrethrum	Glass	8	4.86 ± 1.12
	Nylon	8	1.10 ± 0.239
	Paper	8	1.03 ± 0.215
	WHO	10	2.81 ± 0.892

Table 2. Responses of adult *C. felis* (Kansas1 colony) to chlorpyrifos assayed over time, using nylon disk and paper disk bioassays

Date	Bioassay	n*	Slope ± SE	LC_{50}**	95% FL	LC_{90}**	95% FL
2 July 1994	Nylon	15	5.56 ± 1.087	6.30	5.22-7.16	10.7	9.21-14.1
2 July 1994	Paper	15	2.20 ± 0.488	4.82	2.54-6.50	18.4	14.0-32.1
7 July 1994	Nylon	12	1.87 ± 0.249	20.9	14.5-31.0	100	61.8-213
7 July 1994	Paper	12	1.63 ± 0.221	14.7	10.2-22.0	90.1	52.4-211
10 July 1994	Nylon	12	3.41 ± 0.559	7.17	5.53-9.50	17.0	12.3-29.4
29 July 1994	Nylon	12	2.57 ± 0.387	21.6	16.3-29.1	68.0	46.9-124

* Number of test tubes in experiment.
** mg[AI]/m^2.

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