

AN ABSTRACT OF THE THESIS OF

NANCY BRIGGS WEHR for the M. S.
(Name of student) (Degree)
in MICROBIOLOGY presented on April 24, 1969
(Major) (Date)
Title: HERBICIDE EFFECTS ON BDELLOVIBRIO BACTERIOVORUS
Abstract approved: [REDACTED]
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Bdellovibrio bacteriovorus is a small parasitic bacterium present in soil, mud, and aquatic habitats. Due to its lysis of Gram-negative bacteria, an important role in soil ecology has been suggested for this organism. A study of effects of herbicides upon Bdellovibrio might consequently be of considerable value.

In this study a Bdellovibrio bacteriovorus strain parasitic on a Pseudomonas species indigenous to local soil was isolated and characterized. Seventeen herbicides were screened for activity against these and ten additional cultures. General effects of linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea], diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and benefin (N-butyl-N-ethyl- α,α,α -trifluoro-2,6-dinitro-p-toluidine) on the Bdellovibrio isolate and three saprophytic bacteria were evaluated in liquid culture. The effect of linuron on Bdellovibrio activities was studied in depth.

Herbicide antimicrobial activity appeared to show a positive correlation with presence of an aromatic ring with a polar side chain

linked by an electronegative atom. However, DNBP (4, 6-dinitro-o-sec-butylphenol) inhibited all cultures regardless of its lack of some postulated structural requirements.

Picloram (4-amino-3, 5, 6-trichloropicolinic acid); 2, 4-D amine salt (2, 4-dichlorophenoxyacetic acid, dimethylamine salt); 2, 4, 5-T (2, 4, 5-trichlorophenoxyacetic acid); diuron; linuron; IPC (isopropyl N-phenylcarbamate); and RoNeet (3-ethylcyclohexylethylthiolcarbamate), listed in order of decreasing activity, inhibited a majority of the cultures.

EPTC (ethyl N, N-dipropylthiolcarbamate), two substituted-uracil herbicides, and MCPA ester (2-methyl-4-chlorophenoxyacetic acid, isooctyl ester) were less active. Benefin, two substituted-triazine herbicides, and the sodium salts of two chlorinated aliphatic acids produced no observable inhibition.

Bdellovibrio exhibited sensitivity to 11 of the 17 herbicides tested. The host, two known Pseudomonas species, and Serratia marcescens were the most resistant of the cultures tested on solid media. In liquid cultures the host species and Pseudomonas fluorescens were relatively resistant to effects of linuron, diuron, and benefin. Bacillus cereus growth, determined by optical density measurements, was inhibited 50% by 50 µg/ml benefin and totally inhibited by the two urea herbicides at the same concentration.

The net multiplication of Bdellovibrio in liquid cultures was

decreased by linuron; diuron and benefin appeared less active.

Motility, attachment rate, and penetration rate of the parasite appeared to be unaffected by linuron at 50 $\mu\text{g/ml}$. Rapid death ensued in linuron-treated non-multiplying Bdellovibrio cultures.

Linuron's principal site of action against Bdellovibrio thus appears to be at the level of maintenance of cellular integrity. Chelation apparently is not involved. It is postulated that linuron disturbs semipermeability of the Bdellovibrio cell membrane due to adsorption phenomena, resulting in an increased death rate.

Application of conclusions derived from in vitro studies to the soil environment must be made with caution. In soil, competition between linuron-binding sites on soil particles and on bacteria might decrease the herbicide's activity against Bdellovibrio. However results of this in vitro study should be considered in future investigations of herbicide effects on soil microbial ecology.

Herbicide Effects on Bdellovibrio bacteriovorus

by

Nancy Briggs Wehr

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

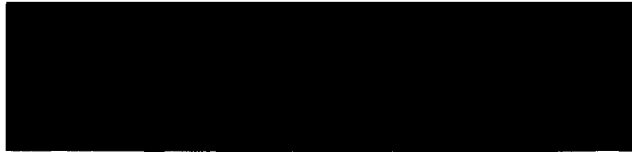
Master of Science

June 1969

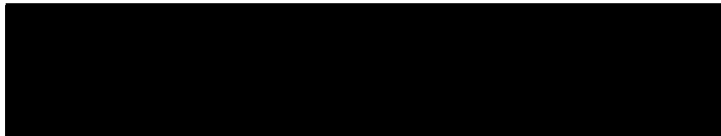
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ACKNOWLEDGMENT

The author wishes to thank Dr. D. A. Klein for his helpful suggestions and guidance throughout this study and other members of the department who made this thesis possible.

Thanks are also extended to Phil Olsen of the Department of Farm Crops for supplying the commercial preparations of the herbicides used in this study.

Finally, I would like to thank my husband Michael for his patience and constant encouragement.

This research was supported through the Agricultural Experiment Station and the Research Council of Oregon State University.

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HERBICIDE EFFECTS ON BDELLOVIBRIO BACTERIOVORUS

INTRODUCTION

Bdellovibrio bacteriovorus is a predatory bacterial parasite found in soils, muds, and aquatic environments. Principally parasitic upon Gram-negative bacteria, members of this unique group have been shown to attack Salmonella species, several plant pathogens of the genera Pseudomonas and Erwinia, as well as many other common soil microorganisms. Because of their ubiquitous occurrence and broad host range, bdellovibrios may play an important role in soil ecology. Factors affecting their growth, such as herbicides, might be expected to upset considerably balance within the soil microbial population.

Currently herbicides are being added to the soil in constantly increasing amounts to control unwanted vegetation. Until recently, secondary effects of these herbicides were given little consideration; however it has become increasingly clear that compounds added to the soil to control or eliminate one group of organisms might inhibit or stimulate the growth of others.

It was the purpose of this work to survey the effects of several representative herbicides on Bdellovibrio and other selected soil bacteria in vitro and to investigate specific effects of linuron, 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea, on a particular Bdellovibrio-host relationship.

LITERATURE REVIEW

Characteristics of *Bdellovibrio bacteriovorus*

Discovered in 1962 by Stolp and Petzold (70) and named the following year (72), *Bdellovibrio bacteriovorus* has been shown to be a widely distributed predatory bacterial endoparasite (57, 69). Tentatively placed in the family Spirillaceae (72), the organism appears on the basis of DNA base ratios and inability to utilize carbohydrates to be more closely related to *Spirillum* than to *Vibrio* (58); however no definite classification has yet been given this unique group. Host-independent and obligately saprophytic *Bdellovibrio* strains have been isolated from cultures of the parasite (64, 72). Use of these genetic variants, which possess the same DNA base ratios as their parent strains (58), has greatly facilitated the study of *Bdellovibrio* morphology and cultural characteristics (58, 64).

Morphologically, the organism is a thin, curved or straight rod, possessing a single long, thick, sheathed, polar flagellum (11). Commonly reported dimensions for newly released parasites are $0.2-0.3\ \mu \times 1.0-1.4\ \mu$ (77), with the mean length increasing to $1.7\ \mu$ (77) after several hours separation from the host. The diameter of the flagellum has been reported to be approximately 28 nm (11, 59), including the sheath which appears to be continuous with the cell wall

(11, 59). In contrast to other Gram-negative bacteria, the Bdellovibrio cell wall, which is 7.0-7.5 nm thick (59), appears to contain only two electron dense layers, separated by a layer of lesser density (69). Most other Gram-negative bacteria, such as Escherichia coli appear to possess thicker cell walls composed of three electron dense layers (48, 69). A normal double-track plasma membrane has been observed in Bdellovibrio (69).

Detailed descriptions of Bdellovibrio parasitism have been reported by several workers (11, 57, 69, 72). Initially one or two rapidly motile parasites collide with the host, possibly damaging the host cell wall, and become reversibly attached at the point of contact by the end distal to the flagellum (57). Strong surface bonding between the Bdellovibrio and host has been postulated (69). The first visible damage to the host is the formation of a swelling, or, more rarely, a pit at this site (11). The parasite, attached to the center of the bulge, is observed to twist violently, perhaps weakening the host cell wall with this motion (11, 77). In the second step, proteolytic enzymes are excreted either by the parasite (77) or possibly by the host (47, 71), causing dissolution of the host cell wall mucopeptide layer (47, 69) and often consequent spheroplast formation (69). During this process the Bdellovibrio penetrates the host cell, where it resides between the cell wall and plasma membrane (11, 69). Apparently the Bdellovibrio does not directly

attack the latter structure, but often the membrane becomes disrupted due to osmotic forces (69). The parasite multiplies intracellularly (11, 69), often forming long spirals of six to 30 bdellovibrios which normally break up and are released as single cells when the host disintegrates (57). Depending on the age of the parasite, one to 24 hours may be required to complete this process (57, 69). With strongly proteolytic Bdellovibrio strains (64) and with high Bdellovibrio multiplicities (1, 64), the spheroplast stage is apparently eliminated. In the latter case, the host cell has been observed to be disrupted without penetration (1).

Until recently Bdellovibrio was believed to be exclusively a parasite of Gram-negative bacteria; however Burger and coworkers (10) reported recently the isolation of a strain capable of parasitizing Streptococcus faecalis and Lactobacillus plantarum as well as several Gram-negative hosts. Individual Bdellovibrio isolates vary in their host range (72) and in their ability to form plaques on different hosts (10, 72). Bdellovibrios form only a temporary attachment to non-host cells or ignore these cells entirely (62, 72). To date, however, no specific attachment sites in host bacteria have been reported. Varon and Shilo (77) were able to demonstrate that heating the host inhibited attachment, possibly by altering the cell surface. Although the two outer layers of normal Gram-negative cell walls consist of lipoprotein and lipopolysaccharide (47), in contrast to the

situation with bacteriophage attack, the lipopolysaccharide composition is apparently not critical to Bdellovibrio attachment (64).

There has been considerable disagreement regarding the ability of Bdellovibrio to interact with heat- and ultraviolet-killed host cells. Shilo and Bruff (64) reported that a strongly proteolytic exoenzyme from one strain of Bdellovibrio was able to lyse heat-killed cells. Earlier, Stolp and Starr (72) had observed lysis of heat-killed host only in the presence of sufficient lysate. They concluded that an unknown factor was released from lysed host cells. In view of Shilo and Bruff's results, it appears that this factor may have been, instead, a Bdellovibrio protease. Huang (33) reported a Bdellovibrio strain which could grow in a suspension of autoclaved host cells supplemented with Ca^{++} and Mg^{++} . Varon and Shilo (77) found that attachment occurred to ultraviolet-killed but not to heat-killed cells. Lack of attachment would seem to preclude parasitic activity. It is possible that variation in results observed in these studies might in part be due to host and Bdellovibrio strain differences.

Certain Bdellovibrio strains have been reported to produce large amounts of protease while in others, production appears negligible (64). In some cases the autolytic enzymes of the host appear to be responsible for dissolution of the murein layer; the Bdellovibrio merely triggers the system (47). In contrast, Varon and Shilo (77) determined that successful invasion by one strain of Bdellovibrio was

principally dependent upon protein synthesis by the parasite.

Biochemical properties of parasitic and host-independent Bdellovibrio strains were reported recently (58, 65). Seidler and Starr (58) detected cytochromes a and c in host-independent strains, while Simpson and Robinson (65) obtained cytochrome difference spectra indicating the presence in one parasitic strain of cytochromes c, a_3 , and cytochrome oxidase. Bdellovibrio endogenous respiration was found to remain nearly constant even after low temperature storage, while that of Spirillum serpens diminished (65). Enzymes of the tricarboxylic acid cycle were present. The presence of glycolytic system enzymes, although at very low concentrations, indicates that Bdellovibrio may be capable of obtaining energy from substrate-linked phosphorylation. The glyoxylate cycle did not appear to operate in the parasite. Simpson and Robinson concluded that parasitic Bdellovibrio needs no external energy source but requires complex factors, such as proteins or nucleic acids, supplied by the host.

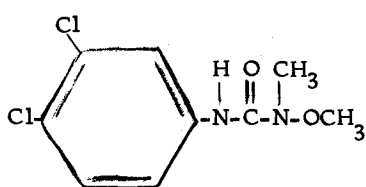
The interaction of Bdellovibrio with its host is influenced by several factors, including hydrogen ion concentration, temperature, degree of aeration, and concentration of certain ions (10, 33, 77). Optimum pH for attachment was determined to be between 8.0 and 9.0, while optimum temperature was reported as 30-35° (77). An oxygen partial pressure greater than 4 to 5 mm Hg was determined to be essential for Bdellovibrio activity (10).

Recently Mg^{++} and Ca^{++} ions were reported to stimulate Bdellovibrio multiplication but apparently not attachment rate (33). Mg^{++} was able to replace the Ca^{++} requirement entirely. One suggested role of these cations is to activate enzymes involved with cell wall maintenance by replacing disulfide bridges in the enzyme molecules (47). A second reported function is to form essential crosslinks in the cell wall material itself (47, 80). The bactericidal effect of ethylenediaminetetraacetic acid (EDTA) on Pseudomonas aeruginosa was suggested to be due to removal of these cation crosslinks from either mucopeptide (47) or lipopolysaccharide (80). Motility of Bdellovibrio was shown by Varon and Shilo (77) to be more sensitive to this agent than was motility of Pseudomonas fluorescens and Escherichia coli, perhaps due to bdellovibrio's unusual flagellar construction.

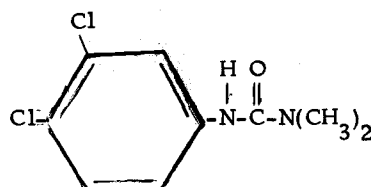
Phosphate ion at 0.05 M concentration inhibited proteolytic exoenzyme activity of Bdellovibrio (64) and reduced the ability of the parasite to attack (77) or grow (56). Varon and Shilo (77) reported that any addition of buffer to the suspending medium decreased the attachment rate, but that of the buffers tested, tris (hydroxymethyl)amino methane and succinate buffers at 0.002 M concentrations were least inhibitory. Tris buffers have been used commonly in Bdellovibrio culture media (57, 65, 69, 72).

Effects of Selected Herbicides
on Physiological Processes

Linuron 3-(3, 4-dichlorophenyl)-1-methoxy-1-methylurea (79) and diuron 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (79) are closely related substituted-urea herbicides.



Linuron



Diuron

Study of metabolic effects of this class of compounds has largely been limited to plants and to measurement of total respiration rates of treated soil microorganisms in vivo. In plant photosynthesis the urea herbicides are known to inhibit the photooxidation of OH^- that gives rise to oxygen evolution (41), possibly by combining with a "specialized" pigment in photosystem II (6). Death of susceptible plants may be caused by decreased photophosphorylation combined with non-cyclic electron flow (44). Black and Myers (6) noted that since the urea herbicides can kill plants in darkness as well, there might be more than one phytotoxic mechanism operating. A review of other reported effects would seem to be more directly applicable to the study of possible actions of urea herbicides, specifically linuron and diuron, upon heterotrophic soil microorganisms.

Alexander (2, 3) noted abnormal enzyme activity and synthesis in young sugarcane plants treated with diuron in the presence of varied nitrate levels. Leaf phosphatase, amylase, and leaf peroxidase activities were reported to be affected. Balandina (5) observed that diuron-treated cotton plants displayed more vigorous shoot development, carbohydrate hydrolysis, and protein formation, while Nashed and Ilnicki (50) found that linuron and diuron increased the respiration rate of corn by 20% in 20 hours.

Inhibition of RNA synthesis (60) and increased incidence of chromosomal aberrations (83) are other reported effects observed in plants treated with substituted-urea herbicides.

Another important area affected by linuron is plant ion metabolism (31, 49). Nashed and Ilnicki (49) found that linuron in low concentrations caused increased Ca^{++} and SO_4^{--} uptake in corn, soybean, and crabgrass; however, in corn linuron concentrations greater than 4 mg/3.5 l (approximately 1.14 $\mu\text{g}/\text{ml}$) decreased the uptake of these ions as well as that of Mg^{++} . An apparent loss of Mg^{++} from soybean with increasing linuron concentration was noted, possibly indicating changes in the permeability of the cytoplasmic membrane due to linuron treatment. A concurrent increased uptake of NO_3^- , PO_4^{-3} , and SO_4^{-2} was observed. The authors concluded that variations in ion uptake might indicate basic changes in metabolism caused by linuron.

Using tracer methods, Hogue (31) demonstrated that sublethal and lethal levels of linuron stimulated the uptake of ^{32}P and inhibited absorption of ^{45}Ca in both parsnip and tomato. The labeled phosphorus accumulated in the inorganic fraction of the leaves rather than in the nucleic acid and phospholipid. Urea herbicides might alter bacterial metabolism in a similar manner.

Also included in the current study was benefin (N-butyl-N-ethyl- α, α, α -trifluoro-2, 6-dinitro-p-toluidine) (79), a preemergence herbicide, reported to affect seed germination and associated growth processes (79). An analogue, trifluralin, (α, α, α -trifluoro-2, 6-dinitro-N, N-dipropyl-p-toluidine) (79) inhibited oxygen uptake and oxidative phosphorylation in isolated plant mitochondria (51). Either of these latter modes of action might possibly affect soil microorganisms.

Herbicides applied to the soil disappear due to several different mechanisms: microbial decomposition, photodecomposition, chemical decomposition, volatilization, leaching (79), and plant absorption and subsequent detoxification (67).

Leaching has been demonstrated to be unimportant in loss of linuron from the soil. In Keyport silt loam the concentration of adsorbed diuron in equilibrium with a 1 $\mu\text{g/g}$ soil solution at 72°F (22.2°) was 5.2 $\mu\text{g/g}$ (79). For linuron under the same conditions, the value reported was 5.5 $\mu\text{g/g}$ (79). Thus the adsorption

phenomenon alone potentially increases the effective concentration of these herbicides on soil particles more than five-fold over the average application rate.

Hance (29, 30) has observed that only a fraction of the total soil surface is available for the adsorption of substituted-urea herbicides. These compounds can compete successfully with water for adsorption sites only on organic matter and not on mineral particles or other hydrophilic surfaces. This phenomenon increases the effective concentration of the herbicide around soil organic matter. Effective concentration is also influenced by soil particle size, the soil with the larger particles having less specific surface, thus greater effective concentration (42).

An inverse relationship between concentration of soil organic matter and toxicity of urea herbicides was noted by Dubey and co-workers (20), who concluded the effect was at least partially due to increased microbial activity in soils with higher levels of organic matter. Microbial breakdown appears to be the prime factor in removal of substituted-urea herbicides from soil (79).

Benefin is also highly adsorbed to soil organic matter and clay colloids (79), and leaching of the herbicide is not considered an important means of detoxification. The compound is susceptible to volatilization and photolytic decomposition (79). Both benefin and trifluralin are gradually metabolized by soil microorganisms;

however, only two species, Lachnospira multiparus and Bacteroides ruminicola subsp. brevis, have been demonstrated to carry out the initial reactions leading to anaerobic decomposition of trifluralin (81).

Soil bacteria are generally found adsorbed to the surfaces of clay particles and humus (4, 38). The adsorbed microorganisms would thus be in close proximity to bound herbicides, leading to decomposition or inhibition. In addition to adsorbing onto the soil particles near bacteria, the herbicides might be adsorbed onto the surface of the bacteria themselves, as was observed in the case of trifluralin, in vitro (81). Adsorption and related phenomena may exert a lethal effect on bacteria (74).

At normal field concentrations linuron, diuron, and monuron [3-(p-chlorophenyl)-1, 1-dimethylurea] (79), caused no detectable permanent changes in total respiration of soil microorganisms in vivo (13, 26, 55), although transient effects were noted. In vitro, however, individual species showed considerable sensitivity (21, 26, 27, 35, 76). Tulabaev and Tamikaev (76) reported that monuron and diuron at 1.5 kg/ha (1.5 μ g/g) (7) decreased the number of ammonifying bacteria but only weakly affected cellulose-decomposing microorganisms. Lembeck and Colmer (39) concurred with this report, finding no inhibition of cellulolytic activity of Sporocytophaga myxococcoides by diuron in vitro at normal field concentrations.

In pure culture, Goguadze (26, 27) determined that diuron was

toxic to Mucor, Fusarium, Phoma, Aspergillus, and to some degree to cellulose-decomposing actinomycetes, while Penicillium, Trichoderma, and non-sporulating bacteria were more resistant. Ebner (21) also noted fungicidal activity of linuron and diuron.

Kaszubiak (35) found that Afalon (active ingredient 50% linuron) and Karmeks (active ingredient 50% diuron) were bactericidal in vitro to both fast- and slow-growing Rhizobium species. Susceptibility of the rhizobia was not influenced by addition of amino acids, vitamins, or nitrogen bases. It should be noted that Kaszubiak autoclaved the herbicide-containing media, which might have increased herbicide toxicity (18).

Rankov et al. (55) reported that under field conditions at normal application rates, Afalon had no effect on nodulation of bean plants or on the growth of either Azotobacter chroococcum or Clostridium pasteuranum. In the same study it was found that the herbicides were weakly toxic to the nitrogen-fixing bacteria in vitro.

After studies with linuron in several soil types, Lode (40) concluded that, although there was an initial depression in the number of bacteria and fungi present in the soils, the populations eventually did recover.

One of the less obvious but none the less potentially harmful aspects of herbicide application was discussed by Pinckard and Standifer (54) and Trang and coworkers (75). Treatment of cotton

with trifluralin predisposed the plants to infection with pathogenic Rhizoctonia. To combat this pathogenic fungus, PCNB (pentachloro-nitrobenzene) was added (54). Subsequently non-pathogenic fusarial species increased. A major conclusion of Pinckard and Standifer was that herbicide and fungicide applications might alter the soil microbial flora, thus encouraging potential plant pathogens.

A parallel might be drawn in relating the potential effects of herbicides upon specific Bdellovibrio-plant pathogen ecological relationships.

Lack of specific information to date on Bdellovibrio-herbicide interactions led to the development of the present study on the possible effects of herbicide application upon this unique species, which may be intimately involved with maintenance of delicate balances within the soil microbial population.

MATERIALS AND METHODS

Apparatus

Broth cultures of all organisms were grown on an Eberbach reciprocal shaker at 120 cycles per minute and approximately 4 cm displacement. Optical density of cultures was measured at 600 nm on a Beckman Spectronic 20 colorimeter. An A O Series 10 phase microscope (American Optical) was used for direct observation of cultures under phase contrast oil immersion.

Media

Liquid cultures of host bacteria and Bdellovibrio cultures were grown in TYP broth, a modification of Stolp and Starr's YP broth (72), containing 1.21 g Trizma Base tris-(hydroxymethyl)amino methane (Sigma), 3 g Bacto-Yeast Extract (Difco) and 0.6 g Bacto-Peptone (Difco) dissolved in one liter distilled water; the pH before autoclaving was 8.4 ± 0.1 .

The ratio of medium volume to flask volume used in all broth cultures was 1:5. Host inoculum was 0.1 ml of a 24 hr TYP broth culture of the organism, grown at $27^{\circ} \pm 1^{\circ}$ with reciprocal shaking. Standard 4% (v/v) Bdellovibrio inoculum was from a two to four day, partially lysed culture of the parasite in a TYP broth culture

of the host, grown at $27^{\circ} \pm 1^{\circ}$ on a reciprocal shaker.

TYP agar (TYP broth plus 1.5% w/v Bacto-agar) was used for maintenance of stock cultures and for standard plate counts. A double layer plating procedure similar to that of Stolp and Starr (72) was used for Bdellovibrio plaque assays and maintenance of parasite stock cultures. The basal layer consisted of 15 ml TYP agar and the overlay of 5 ml TYP semisolid agar (0.6% agar), containing 1.0 ml of a 24 hour TYP broth culture of the host. For assay of Bdellovibrio liquid cultures for plaque forming units (pfu's), sterile tap water dilutions of the culture (10^0 to 10^{-8}) were prepared. One ml volumes of the even dilutions were distributed in the molten (48°) semisolid layer. Plaques were counted after six days and ten days incubation at room temperature. Stock Bdellovibrio transfers were made directly onto the surface of the solidified semisolid layer.

Standard physiological test media (17, 68) were utilized in the determination of host cultural characteristics.

Rhizobium cultures used in this study were maintained and tested for herbicide sensitivity on Yeast-mannitol agar (10 g mannitol, 0.5 g Bacto-Yeast Extract, 0.5 g K_2HPO_4 , 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 1000 ml tap water, and 1.5% and 0.6% Bacto-purified Agar for basal layer and overlay, respectively).

Herbicides

Commercial formulations of herbicides used in the preliminary portion of this study (Table 1) were obtained from the Farm Crops Department, Oregon State University. Technical grade benefin (Elanco Products) and recrystallized linuron and diuron (E. I. DuPont) were used in the latter portion of this investigation.

Two methods of herbicide addition to sterile liquid media were used. Either 0.05 ml of a Seitz filter-sterilized acetone solution of the herbicide was added directly to the medium or the same volume of sterile solution was dispensed onto a sterile 22 × 22 mm, no. 1 coverslip (Aloe); the coverslips were then dried at room temperature in a closed Petri dish for 24 hours and added aseptically to the broth. The first method was used only when rapid dispersion of the herbicide in the broth was essential. The second method avoided the possible dangers incurred by addition of an organic solvent directly to the growth medium, but with this method dispersion of the herbicide was somewhat retarded. In all cases heating the herbicides in the media was avoided due to the possibility of chemical changes such as those reported by Doxtader (18). Controls for the two addition methods were, respectively, an equal volume of acetone added either directly to the culture flask or to a coverslip and dried before addition to the medium.

Table 1. Herbicides assayed for antimicrobial activity

Active ingredient (79)	WSA common name (79)	Trade name (79)	Formulation (24)	Solvent
4-amino-3, 5, 6, -trichloropicolinic acid	picloram	Tordon	AC ¹	A ⁶
5-bromo-3- <u>sec</u> -butyl-6-methyluracil	bromacil	Hyvar X	WP ²	A
3- <u>tert</u> -butyl-5-chloro-6-methyluracil	terbacil	Sinbar	WP	A
N-butyl-N-ethyl- α, α, α -trifluoro-2, 6-dinitro-p-toluidine	benefin	Balan	EC ³	A
2-chloro-4, 6-bis(ethylamino)- <u>s</u> -triazine	simazine	Simazine 80 W	WP	M ⁷
2-chloro-4-ethylamino-6-isopropylamino- <u>s</u> -triazine	atrazine	Atrazine 80 W	WP	M
2, 4-dichlorophenoxyacetic acid, dimethylamine salt	2, 4-Damine salt	Weedar 64	AC	W ⁸
3-(3, 4-dichlorophenyl)-1, 1-dimethylurea	diuron	Karmex	WP	A
3-(3, 4-dichlorophenyl)-1-methoxy-1-methylurea	linuron	Lorox	WP	A
2, 2-dichloropropionic acid, sodium salt	dalapon, sodium salt	Dowpon	WSP ⁴	W
4, 6-dinitro- <u>o-sec</u> -butylphenol	DNBP	Dow General Weed Killer	EC	E ⁹
3-ethylcyclohexylethylthiolcarbamate	--	RoNeet	EC	A
ethyl N, N-dipropylthiolcarbamate	EPTC	Eptam	EC	A
isopropyl N-phenylcarbamate	IPC	Chem-hoe	WP	A
2-methyl-4-chlorophenoxyacetic acid, isooctyl ester	MCPA	MCPA ester	EC	A
trichloroacetic acid, sodium salt	TCA, sodium salt	Dow Sodium TCA 95%	G ⁵	W
2, 4, 5-trichlorophenoxyacetic acid	2, 4, 5-T	--	EC	A

¹ aqueous concentrate (AC)² wettable powder (WP)³ emulsifiable concentrate (EC)⁴ water soluble powder (WSP)⁵ granules (G)⁶ acetone (A)⁷ methanol (M)⁸ water (W)⁹ ethanol 95% (E)

Cultures

With the exception of Rhizobium meliloti (ATCC No. 9930), all known saprophytic bacterial cultures used in this study were obtained from the stock culture collection in this department. The Bdellovibrio culture and its host were isolated from Woodburn silty clay loam from a local creek bed.

The host was isolated from 1 g of the soil sample using a mineral salts-dextrose medium (MSD). MSD broth composition was 1 g $(\text{NH}_4)_2\text{SO}_4$, 1 g KH_2PO_4 , 0.2 g MgSO_4 , 0.02 g CaCl_2 , 0.01 g FeCl_3 , 1000 ml distilled water; 0.05 g technical grade benefin and 10 ml 50% w/v Seitz filter-sterilized dextrose solution were added after the medium was autoclaved. Enrichment techniques were used to isolate Bdellovibrio strains parasitic on this soil isolate. A 24 hour host culture in 80 ml MSD broth, fortified with 5% YP broth (v/v), was inoculated with 2 g of the soil sample and allowed to shake at room temperature for four days, after which 10 ml of the culture was filtered through a 0.45 μ Millipore filter and the filtrate assayed for the presence of Bdellovibrio pfu. Of the several Bdellovibrio isolates obtained by this procedure, the one forming plaques most rapidly on the host was chosen for further study.

Host Cultural Characteristics

TYP broth cultures of the host incubated at 30° on a shaker were used for determination of Gram stain reaction, motility, cell morphology, generation time, and for flagella stain smears (modified Liefson's method, Difco). Optimum temperature was determined on TYP slants held at 8°, 22°, 27°, 30°, and 37°. Glucose nutrient agar deep cultures and pour plates were used for determination of oxygen requirement and colony morphology.

The medium of Davis and Park (15) was used for detection of fluorescent pigment production. Indole production, hydrolysis of urea, and H₂S production (SIM medium) were tested according to procedures given in the Difco Manual (17). Citrate and ammonium utilization were demonstrated on Simmon's citrate medium (17). Oxidation of glucose and lactose were detected according to methods of Hugh and Liefson (34). The remaining tests were performed according to standard procedures (68).

Cultural Characteristics of the Bdellovibrio Isolate

The optimum temperature of the parasite was determined on TYP agar double layer plates by following the rate of appearance of plaques at 8°, 22°, 27°, 30°, and 37° over a period of ten days.

Observations on Bdellovibrio growth were also derived from TYP broth cultures incubated at several temperatures.

Cross reactions of the Bdellovibrio isolate with the following cultures were evaluated by standard plating procedures: Escherichia coli, Aerobacter aerogenes, Serratia marcescens, Alkaligenes viscolactis, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, and the host strain. Ten ml of a four day lysed broth culture of Bdellovibrio was filtered through a 0.45 μ Millipore filter and one drop of the filtrate placed on each plate. Plates were incubated at room temperature for 18 days and inspected on alternate days after inoculation for appearance of lysis.

Effect of Plating Procedure Upon Apparent pfu Concentration

Standard double layer plates were prepared and inoculated with 1.0 ml of a 24 hr culture of the host and a Bdellovibrio suspension sufficiently dilute to form isolated plaques. Following solidification of the upper layer, a second semisolid layer of graded depth was applied to half of the plate by tilting. The number of plaques appearing, their location, and their rate of growth were recorded at 7, 10, and 18 days.

To develop a more quantitative plate assay method for Bdellovibrio plaque forming units, 0.1 and 1.0 ml volumes of serial

dilutions of an active Bdellovibrio inoculum were spread onto the surface of one series of double layer plates previously inoculated with host and incorporated into the overlay of a second series. Overlay agar concentrations of 0.6%, 0.8%, 1.0%, 1.2%, 1.4%, and 1.5% were included in both plating series.

Survey of Herbicides for Antimicrobial Activity

Seventeen herbicides listed in Table 1 were tested for antimicrobial activity against 12 bacterial species believed to be present in soil. Double layer plates were prepared using an overlay inoculated with 0.5 ml of a 24 hour TYP broth culture of the test organism. Test plates of Rhizobium species were prepared in a similar manner with Yeast-mannitol agar containing 1.5% and 0.6% Bacto-purified agar in the basal layer and overlay, respectively.

Commercial herbicide preparations in liquid carriers were diluted with the solvents listed in Table 1 to the proper concentration without purification. The active ingredient in powder formulations was recrystallized from the chilled solvent. Sterile discs of Whatman No. 1 filter paper, 6 mm in diameter, were saturated with Seitz-filter-sterilized solutions of the herbicide preparations containing 100 mg/ml active ingredient. Excess herbicide solution was drained off, discs dried at room temperature for 24 hours in a closed Petri dish, and applied to the hardened, inoculated test plates. The weight

of herbicide retained on each disc was approximately 2 mg. Diameters of zones of inhibition of most species tested were measured after 24 hours incubation at 27°. Slow-growing Rhizobium species (74) were allowed to incubate five days at the same temperature.

Bdellovibrio's herbicide sensitivity was measured by inhibition of plaque formation on standard double layer plates of the host. An active TYP broth parasite culture was inoculated by needle onto six isolated points in the prepared plate. After plaque areas had developed for six days, the herbicide test discs were applied tangent to the plaque edge. Degree of increase in diameter of the plaque after four days and 14 days incubation at 27°, compared with plaque development in controls, gave qualitative evidence of sensitivity of Bdellovibrio plaque growth to each herbicide. Untreated discs and discs saturated with each of the solvents and dried were controls in both disc assay procedures.

Growth of Three Saprophytic Bacterial Species in Presence of Herbicides

Growth curve studies of three bacterial species, Bacillus cereus, Pseudomonas fluorescens, and the host species were carried out in 50 ml TYP broth containing approximately 50µg/ml linuron, diuron, and benefin added on sterile coverslips. Controls containing acetone-treated coverslips without herbicide were studied concurrently. Inocula consisted of 0.1 ml of a 24 hour TYP broth culture

of each organism. Cultures were grown at $29^{\circ} \pm 1^{\circ}$ with slow reciprocal shaking. Growth was measured by optical density at 600 nm.

Growth of *Bdellovibrio* in the Presence of Herbicides

Effects of linuron, diuron, and benefin at several concentrations were studied in liquid cultures of *Bdellovibrio* grown at $28.5^{\circ} \pm 1.5^{\circ}$ with reciprocal shaking. To 50 ml of a 24 hour broth culture of the host ($OD = 0.95 \pm 0.01$ at 600 nm), herbicide coverslips were added and allowed to equilibrate one hour with reciprocal shaking. Two ml of a partially lysed two day TYP broth culture of *Bdellovibrio* was added and pfu concentrations assayed by standard double layer plate count methods at 0, 24, and 48 hours.

Specific Effects of Linuron Upon *Bdellovibrio bacteriovorus*

Motility

Motility of *Bdellovibrio bacteriovorus* was examined under phase contrast in cultures containing 75 μ g/ml linuron and in controls without linuron after 0, 1, and 24 hours incubation at 27° on a shaker.

Attachment

Attachment rates in 50 ml volumes of TYP broth cultures

containing no solvent, 0.05 ml acetone, and 0.05 ml of a solution of linuron in acetone giving a final concentration of 50 $\mu\text{g/ml}$ linuron were measured according to a modification of the method of Varon and Shilo (77). Twenty-five ml aliquots of a 24 hour host broth culture were preincubated for 20 minutes at 27° with the acetone or linuron solution prior to addition of 25 ml standard Bdellovibrio inoculum at time 0. The number of Bdellovibrio pfu's filterable through a 0.8μ Millipore filter was measured at 0, 6, 20, and 40 minutes by immediate double layer plating of the filtrate. For each flask the percent decrease in filterability of the Bdellovibrio pfu's at each of the latter three times was determined. This value was assumed to be proportional to the number of parasites attached to the host cells.

Penetration

To standardize methods for penetration measurement, a preliminary investigation was undertaken. Ten ml of a 24 hour host culture in TYP broth was added to 10 ml of a four day TYP broth culture of Bdellovibrio and the mixture incubated at 27° with reciprocal shaking for 20 minutes to allow maximum attachment (77). After incubation, the culture was diluted to 1% of its original concentration in sterile tap water. One-hundred ml aliquots of the dilution were blended in a Waring Blendor at high speed (unimpeded blade

speed 19,500 rpm) for 0, 0.5, 1.0, and 2.0 minutes and filtered through a 0.8 μ Millipore filter. One ml aliquots of each sample were plated immediately after blending and after filtration to determine changes in concentration of pfu's due to each procedure. Results were compared with plaque counts obtained from corresponding samples in the unblended dilution, and the optimum blending time for reversal of attachment was determined.

Penetration rate, like attachment rate, was measured by a modification of the method of Varon and Shilo (77) in which, prior to the filtration step, the culture was diluted 1.0×10^{-2} in sterile tap water and agitated in a Waring Blendor for one minute. Percent decrease in number of Bdellovibrio pfu's filterable at 6, 20, and 40 minutes to those filterable after the same treatment with no incubation period was determined.

Effect of Linuron on Non-multiplying Cultures

A lysed four day TYP broth Bdellovibrio culture was filtered through a 0.8 μ Millipore filter and the filtrate dispensed in 25 ml aliquots into 125 ml flasks. Linuron in 0.05 ml acetone solution was added to give final herbicide concentrations of 0, 4, 20, and 80 μ g/ml. A control flask with no additions was also included. Bdellovibrio pfu's and host cells viable at 0, 24, and 48 hours were measured by standard plate assays and the pfu concentration

remaining at each time determined.

Chelation Studies

Pfu concentration was followed for 48 hours in shaken TYP broth cultures containing 6.9×10^{-4} M linuron with no added Mg^{++} , 3.65×10^{-4} M added Mg^{++} , and 1.14×10^{-2} M added Mg^{++} . Linuron was added in 0.05 ml sterile acetone solution to 25 ml of a 24 hour TYP broth culture of the host, one hour prior to addition of the 0.5 ml Bdellovibrio inoculum. Magnesium was added in 0.1 ml aqueous solution of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ to give the required final concentrations. Pfu concentrations in experimental flasks were compared to those in controls containing 0.05 ml sterile acetone in place of the linuron.

RESULTS AND DISCUSSION

Host Cultural Characteristics

The host organism, designated 1-26-4, is a motile, Gram-negative, obligately aerobic rod, which grows in pairs in young cultures and singly in older cultures. Motility is lost after 48 hours. Dimensions of the host cells grown 18 hours in a shaken TYP broth culture at 27° are $0.75-1.0\mu \times 1.25-1.5\mu$. The cells possess one to four polar multitrichous flagella. Optimum temperature range is 30° to 37°. At 30° generation time in TYP broth is approximately 2.3 hours. Stationary nutrient broth cultures are slightly turbid with a small amount of sediment. No pellicle is formed. No fluorescent pigment formation was demonstrated after 14 days incubation at 30°. Nutrient agar colonies are circular, slightly convex, with an entire edge. Texture is granular and color creamy white. Diameter of an average 48 hour colony is 1.5 mm. Potato slab colonies are orange-brown, turning the potato gray, without abundant or mucoidal growth.

Urease production, indole production, H₂S production, nitrate reduction to nitrites or ammonia, gelatin liquefaction, and acetyl methyl carbinol production were not observed. The methyl red test was negative. The organism utilizes citrate as a sole carbon source

and ammonium ion as the sole nitrogen source. Its reaction in litmus milk was alkaline. Glucose, galactose, mannose, maltose, sucrose, lactose, starch, inulin, mannitol, and salicin were not fermented; however, glucose and lactose were slowly oxidized.

Klinge (37) reported Pseudomonas species as a group generally to give negative reactions to the following tests: acetyl methyl carbinol production, methyl red test, indole production, and urea hydrolysis. Catalase production was reported to be positive, as was utilization of NH_4^+ in the presence of glucose or citrate. The present isolate displays all these characteristics. According to Sheewan, Hobbs, and Hodgkiss' work (61) on identification of Gram-negative rod-shaped bacteria, polar flagellation, oxidation of carbohydrates, growth at 37° , and lack of fluorescent pigment production place this organism in the group Pseudomonas II. The isolate is included in Holdings' (32) classification Pseudomonas due to its ability to oxidize glucose and utilize inorganic nitrogen in the form of ammonium. Holding notes that for identification of various groups, the only essential carbohydrate oxidation test is glucose.

Results presented in the current study are consistent with the observation of Hugh and Liefson (34) that most Gram-negative organisms are either exclusively oxidative or fermentative, and, in general, the strictly aerobic bacteria are oxidative, whereas fermentative organisms are usually facultative. In general Hugh and Liefson found

non-fermenters did not produce indole.

Thus, host strain 1-26-4 appears to be a Pseudomonas species with cultural characteristics most similar to those of Pseudomonas cruciviae, as described in Bergey's Manual (8).

Cultural Characteristics of the Bdellovibrio Isolate

Optimum temperature of the Bdellovibrio isolate used in these studies was between 27° and 30° on double layer plates. Plaques appeared slowly at 22° but not at all at 8° or at 37°. In shaken TYP broth cultures, the maximum temperature allowing net multiplication was 33° to 34°. This temperature limit probably is determined in part by the optimum temperature of the host culture. Stolp and Starr (72) reported that parasitic activity and multiplication of Bdellovibrio were favored under medium conditions restrictive to host growth. The same was implied to be true of temperature effects also. Varon and Shilo (77) have reported an optimum temperature for attachment between 30° and 35°. Saprophytic mutant Bdellovibrio strains have been reported to possess lower optima (72).

In an examination of various media supporting Bdellovibrio growth, multiplication of the isolate was shown to be inhibited by phosphate ion at a concentration of 0.012 M. The effect may be due to inactivation of parasite proteolytic enzymes (64). Strongly proteolytic Bdellovibrio strains often do not cause spheroplast formation in

host cells (64). The lack of spheroplast formation in the present host is a second indication that this Bdellovibrio isolate contains proteolytic enzymes.

The isolate did not form plaques in lawns of any of the seven additional Gram-negative cultures tested for susceptibility. Stolp and Starr (72) divided their Bdellovibrio isolates into five groups by host range. Two of the groups were composed to bdellovibrios able to form plaques only on very restricted groups of pseudomonads. Results obtained in the present work indicate this Bdellovibrio isolate possesses a similarly restricted host range. It should be noted that one of Stolp and Starr's two groups able to attack only pseudomonads contained as its sole member a Bdellovibrio isolate A3.12, whose host-independent mutant subsequently was shown to be strongly proteolytic, extremely sensitive to phosphate concentration, and unable to induce host spheroplast formation (64). Shilo and Bruff (64) reported a wider host range for Bdellovibrio A3.12 based on attachment in liquid cultures.

Effect of Plating Procedure Upon Apparent pfu Concentration

On gradient plates the plaque appearance rate and number of plaques formed were inversely proportional to the depth of the agar covering. After seven days a concentrated Bdellovibrio culture

causing confluent lysis in the uncovered half of the plate developed only two plaques in the covered half; these were in the area with the minimum additional overlay. At 18 days, an average of eight new plaques had appeared.

The results of this experiment concur with earlier reports on the aerobic nature of the organism (65, 72). No previous statement was found relating depth of agar covering to plaque development on plates. This is evidently a critical factor in quantitative plate assays for Bdellovibrio. It might be noted that previous interpretations of nonuniform rate of plaque development have ascribed the phenomenon to physiological variation within the parasite culture (36). Present findings indicate that the variability may be largely a laboratory artifact due to the position of the Bdellovibrio in the agar overlay and consequent oxygen limitation.

A decrease rather than an increase in the number of plaques and their rate of appearance was observed by spreading the Bdellovibrio suspension onto the surface of the overlay instead of incorporating it into the layer. These results are in apparent conflict with those reported above; however, the critical factor on the surface of the gel may be moisture rather than aeration. Over the time period required for visible plaque formation, sufficient moisture may be lost from the surface by evaporation to restrict parasite motility. Plaque size and number also decreased with increasing agar

concentration.

When Bdellovibrio pfu's were incorporated into overlays of standard thickness but of varying agar concentrations, an inverse relationship was observed between plaque diameter and agar concentration. At overlay concentrations less than 1% (w/v), the total number of plaques developing was not influenced by agar concentration. Above 1%, the number of plaques developing decreased in proportion to agar concentration increase. An average of 54 plaques developed on plates with up to 1.0% agar overlays. With a 1.4% overlay the plaque count had decreased to 14, and at 1.5% to nine. Both concentration effects were presumably due to impeded motion of the parasites in the more dense gel network.

Present studies indicate that the most quantitative plating procedure involves incorporation of the Bdellovibrio inoculum into a thin, uniform overlay of semisolid agar containing not more than 0.6% agar. These are features of the currently used method of plaque enumeration.

Survey of Herbicides for Antimicrobial Activity

Herbicide disc assay results are summarized in Table 2. The results are qualitative due to variations in herbicide water solubilities and diffusion rates, which vary inversely with molecular size (12). Zone diameters are also affected by the growth rate of the test

Table 2. Sensitivity of various bacterial species to herbicides. 1, 2

Herbicide	Molecular weight	H ₂ O solubility at 20-30° C (µg/ml)	<i>Bdellovibrio bacteriovorus</i>	<i>Sarcina lutea</i>	<i>Bacillus cereus</i>	<i>Achromobacter</i> sp.	<i>Aerobacter aerogenes</i>	<i>Rhizobium trifolii</i>	<i>Rhizobium japonicum</i>	<i>Rhizobium meliloti</i>	<i>Serratia marcescens</i>	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas fluorescens</i>	host 1-26-4
DNBP	240.2	52	++ ³	21.5 ⁴	37	32	20	10	12	8	10	8	8	10
2, 4 D amine salt	266.1	3.0 x 10 ⁸	+	8.5	9	14.5	9	11	8	8.6	9.5	10	8.5	--
picloram	241.5	430	sl+	15.5	10.5	16	8	8.5	8	--	8	--	--	--
2, 4, 5-T	255.5	238	+	8	8.5	11.5	8.5	8	8	8	--	--	--	--
diuron	233.1	42	+	14	11	13.5	8	8	sl+	sl+	--	--	--	--
linuron	249.1	75	++	9	8.5	10	8.5	9	sl+	--	--	--	--	--
IPC	179.2	250	+	9	--	15.5	8	9	sl+	sl+	--	--	--	--
RoNeet	215.4	100	+	9	9	13.5	9	--	8	--	--	8.2	--	--
EPTC	189.3	375	+	9	9	10.5	8	--	--	--	--	--	--	--
bromacil	261.1	815	sl+	11.5	--	20	9	9	--	--	--	--	--	--
terbacil	216.7	710	-- ⁶	11	--	16	9	9	--	--	--	--	--	--
MCPA ester	313.6	-- ⁵	+	10	10	13.5	--	--	--	--	--	--	--	--
benefin	335.3	70	--	--	--	--	--	--	--	--	--	--	--	--
simazine	201.7	5	--	--	--	--	--	--	--	--	--	--	--	--
atrazine	215.7	70	--	--	--	--	--	--	--	--	--	--	--	--
dalapon, Na ⁺ salt	165.0	5.02 x 10 ⁵	--	--	--	--	--	--	--	--	--	--	--	--
TCA, Na ⁺ salt	185.4	1.2 x 10 ⁶	--	--	--	--	--	--	--	--	--	--	--	--

¹ approximately 2 mg herbicide per disc

² acetone, ethanol, methanol, and water-treated disc controls caused no inhibition zones

³ *Bdellovibrio* sensitivity measured qualitatively only: slight inhibition (sl+), moderate inhibition (+), complete inhibition (++)

⁴ inhibition zone diameter (mm)

⁵ not stated

⁶ no observable inhibition

organism and size of the inoculum. In general a higher growth rate is a protective factor due to reduction in the ratio of available herbicide to cells (12).

Aerobacter aerogenes and Achromobacter sp. differed in absolute sensitivity to only one compound, MCPA ester, although in all cases the Achromobacter species displayed a much larger zone of inhibition. The greater apparent sensitivity of this culture is probably due in part to its slower growth and possibly to a lower inoculum level. Another Gram-negative eubacterium, Serratia marcescens, displayed a high degree of resistance to the majority of herbicides tested.

The host isolate 1-26-4 and the known Pseudomonas species included in the investigation showed similar resistance to the majority of herbicides tested by this method. The only compound displaying inhibitory activity against the host species was DNBP, a general weed killer which is reported to cause plant cell necrosis (79). This compound was active against all 12 cultures tested.

The three rhizobia gave varied responses. Paradoxically, the fastest growing species of the three, Rhizobium trifolii (35, 74) appeared most sensitive to herbicide addition. The other fast-growing species, R. meliloti, displayed greater resistance. Compounds active against all three rhizobia were diuron, IPC, 2, 4-D amine salt, 2, 4, 5-T, and DNBP. A common feature of all five of these compounds is a substituted aromatic ring. With the exception of

DNBP, all the compounds possess a polar side chain attached to the non-polar aromatic moiety by an electro-negative atom, either oxygen or nitrogen. The only additional aromatic ring substituents in these compounds are chlorine atoms. Linuron and MCPA ester also possess these features but were less active against the rhizobia. The latter compound is the iso-octyl ester of the parent compound and, thus, would be considerably less polar than the parent compound. In this work linuron inhibited two of the three rhizobia and possibly should be classified as active. Kaszubiak (35) reported that linuron inhibited all three species in vitro.

Structural similarities of these herbicides to phenolic disinfectants suggest that they might also act as surfactants (28, 74). With one exception, the members of this herbicide group inhibited the two Gram-positive organisms tested and a majority of the Gram-negative eubacteria; the pseudomonads were considerably more resistant. Without exception, these compounds inhibited Bdellovibrio plaque development.

Complete lack of detectable activity of benefin and the two triazine herbicides tested, simazine and atrazine, may partially be caused by their lack of solubility in water, resulting in minimal diffusion. The activity spectrum of the two substituted uracils appeared similar except against Bdellovibrio. With this one exception, only eubacteria were inhibited.

The two thiolcarbamate herbicides, RoNeet and EPTC were slightly active against Gram-positive organisms but showed no consistent inhibition of Gram-negative species. Picloram appeared highly active against both Gram-positive and most Gram-negative eubacteria; however, its relatively high water solubility should also be noted.

The sodium salts of the chlorinated aliphatic acids, TCA and dalapon, although both readily soluble in water, showed no bactericidal activity against any species tested. In vitro TCA and other anions of organic acids (23) precipitate proteins. TCA and dalapon, which has a structure differing from TCA by one methyl group, probably would act in a similar manner. If either anion were able to penetrate bacterial cell membranes, lethal protein precipitation might perhaps occur within the cells.

Growth of Three Saprophytic Bacterial Species in Presence of Herbicides

Table 3 summarizes the effects of linuron, diuron, and benefin upon the growth of three representative soil organisms. In liquid media Bacillus cereus exhibited acute sensitivity to diuron and linuron and less sensitivity to benefin at approximately equal concentrations. The disc assay procedure indicated similar effects for the first two compounds but showed no inhibition by benefin, probably in part due

Table 3. Herbicide effects on growth of three saprophytic soil bacteria.

Organism	Herbicide ¹	Length log phase (hr)	Maximum OD at 600 nm	Growth rate (division x hr ⁻¹)
<i>Bacillus cereus</i>	acetone	5.2	0.90	0.53
	benefin	6.4	0.43 ²	0.20
	diuron	-- ³	--	--
	linuron	--	--	--
<i>Pseudomonas fluorescens</i>	acetone	4.4	0.84	0.67
	benefin	4.8	0.84 ²	0.59
	diuron	4.1	0.88	0.71
	linuron	4.8	0.87	0.67
1-26-4	acetone	8.1	0.92	0.37
	benefin	8.6	0.66 ²	0.29
	diuron	6.6	0.75	0.36
	linuron	8.0	0.74	0.31

¹ All herbicides added on sterile coverslips to give an approximate final concentration of 50 µg/ml

² OD values corrected for turbidity due to benefin emulsion

³ No growth observed

to insufficient diffusion. In earlier efforts to enrich for soil organisms able to grow in the presence of benefin, no Gram-positive organisms were obtained in evaluating 30 isolates. It appears that benefin may be inhibitory to Gram-positive organisms in general.

Pseudomonas fluorescens showed no detectable sensitivity to the three herbicides tested, corroborating evidence obtained earlier by disc assay. Host strain 1-26-4 was slightly more sensitive to the three herbicides than P. fluorescens. In all cases of herbicide addition to cultures of the host, both growth rate and maximum optical density, thus total cell mass (46), were decreased to some extent.

Apparent discrepancies between liquid culture results and those obtained in the disc assay may be due to herbicide application methods. In a discussion of a similar phenomenon in antibiotic sensitivity assays, Cooper (12) reported that the critical concentration necessary to produce zones of inhibition on plates is usually two to four times the minimum inhibitory concentration observed in liquid media. Several factors are involved, the most important being time. In liquid cultures the agent and bacteria come into almost instantaneous contact. The inoculum is not allowed to multiply first. This is not the case in solid media where there is a time lag between inoculation and diffusion of the antimicrobial substance to the location of multiplying bacteria. Thus the ratio of herbicide to organism tends to be decreased in plate assays compared to liquid assays. The slower the

diffusion rate, the more pronounced this phenomenon becomes.

After analysis of herbicide effects on these cultures, linuron was selected for more detailed study. For purposes of comparison, in certain portions of the study diuron and benefin were also included.

Growth of *Bdellovibrio* in Presence of Linuron

In liquid cultures the quantitative effect of linuron at increasing concentrations upon *Bdellovibrio* multiplication was observed as shown in Figure 1. The intersection of the 0, 24, and 48 hour curves at approximately 16 µg/ml linuron marks the minimum inhibitory concentration of this agent on this particular *Bdellovibrio* strain. At higher concentrations of the agent, net death occurred, while at lower concentrations a net increase in pfu's occurred, indicating that the multiplication rate of the organism was higher than the combined death rates due to natural causes and due to linuron's effect on the culture. No threshold concentration was observed below which some inhibition did not occur, although at low application levels, the detrimental effect on the *Bdellovibrio* culture was slight in the first 24 hours of contact, becoming more pronounced with lengthened exposure time.

Controls in these studies indicated that either the multiplication rate decreased between 24 and 48 hours or the death rate increased during this period. No distinction can be made between the two in

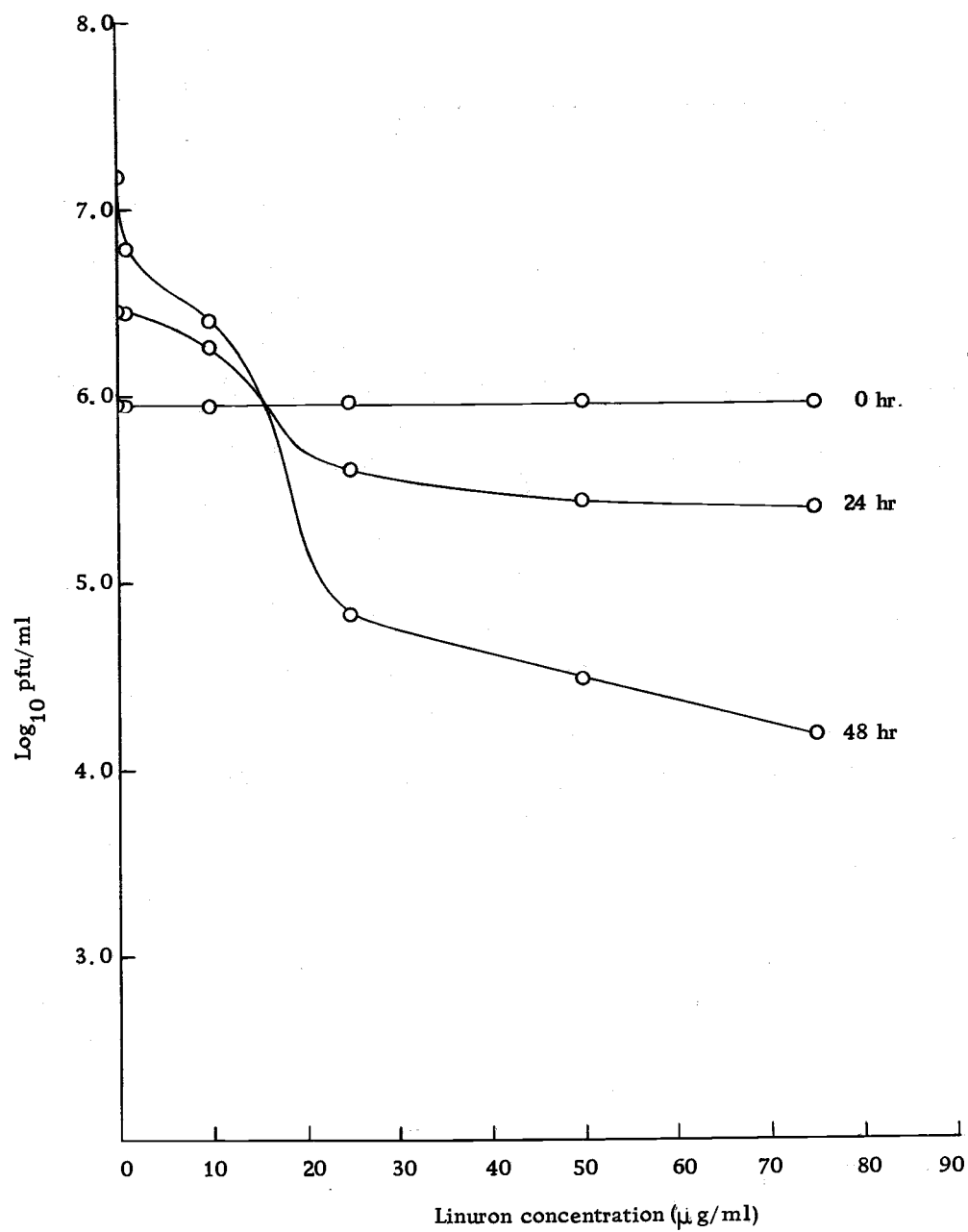


Figure 1. Effect of linuron concentration on multiplication of Bdellovibrio.

total population counts of this type. Possible causes might be decline in concentration of essential nutrients, decrease in parasitizable host cells (77), or accumulation of harmful metabolic wastes.

In broth cultures diuron and benefin at 40 $\mu\text{g/ml}$ displayed essentially no effect on the multiplying Bdellovibrio culture, although diuron did appear qualitatively to inhibit plaque formation on double layer plates.

Linuron does appear to exert some detrimental effect upon Bdellovibrio activities. The herbicide may modify the host-parasite interaction in some manner, either by altering the host cell's ability to be attacked or by restricting bdellovibrio's ability to parasitize. Parasitic activity can be altered by reducing motility, attachment rate, or penetration rate. Separate from the effect on the host-parasite interaction is a second site of possible sensitivity to herbicides. This is at the level of maintenance of Bdellovibrio cellular integrity.

Specific Effects of Linuron Upon Bdellovibrio bacteriovorus

Motility, Attachment Rate, Penetration Rate

Motility of Bdellovibrio did not appear to be affected significantly by linuron, either after short incubation periods or after 24 hours in the presence of 75 $\mu\text{g/ml}$ concentration of the herbicide. Phenol

at 500 μ g/ml was reported to inhibit Bdellovibrio motility (77); thus linuron's effect may not be identical to the effect of this compound, regardless of any superficial structural resemblance.

Linuron was shown to have no detrimental effect on the rate of attachment of the parasite to the host (Figure 2).

Results of the preliminary investigation on the effect of blending showed that the decline in viable bdellovibrios was directly related to the length of the blending treatment. However, the filterability of the culture displayed an approximate threefold increase after one minute of blending compared to the unblended control. Two minutes of continuous blending resulted in a decline in total viable bdellovibrios sufficiently great to offset the advantage of increased filterability. The extended treatment thus resulted in a marked decrease in percent viable pfu's in the filtrate relative to the percent pfu's filterable in the unblended control. Blending caused a reversal of attachment, although the treatment decreased the total parasite viability. On the basis of these results, a blending time of one minute was selected for all measurements of Bdellovibrio penetration rate.

Figure 3 shows that linuron concentrations of 50 μ g/ml did not decrease significantly the rate of Bdellovibrio penetration into the host. A comparison of the Bdellovibrio attachment curve with the penetration curve obtained indicates that penetration occurred at a faster initial rate than did attachment. Since attachment is the

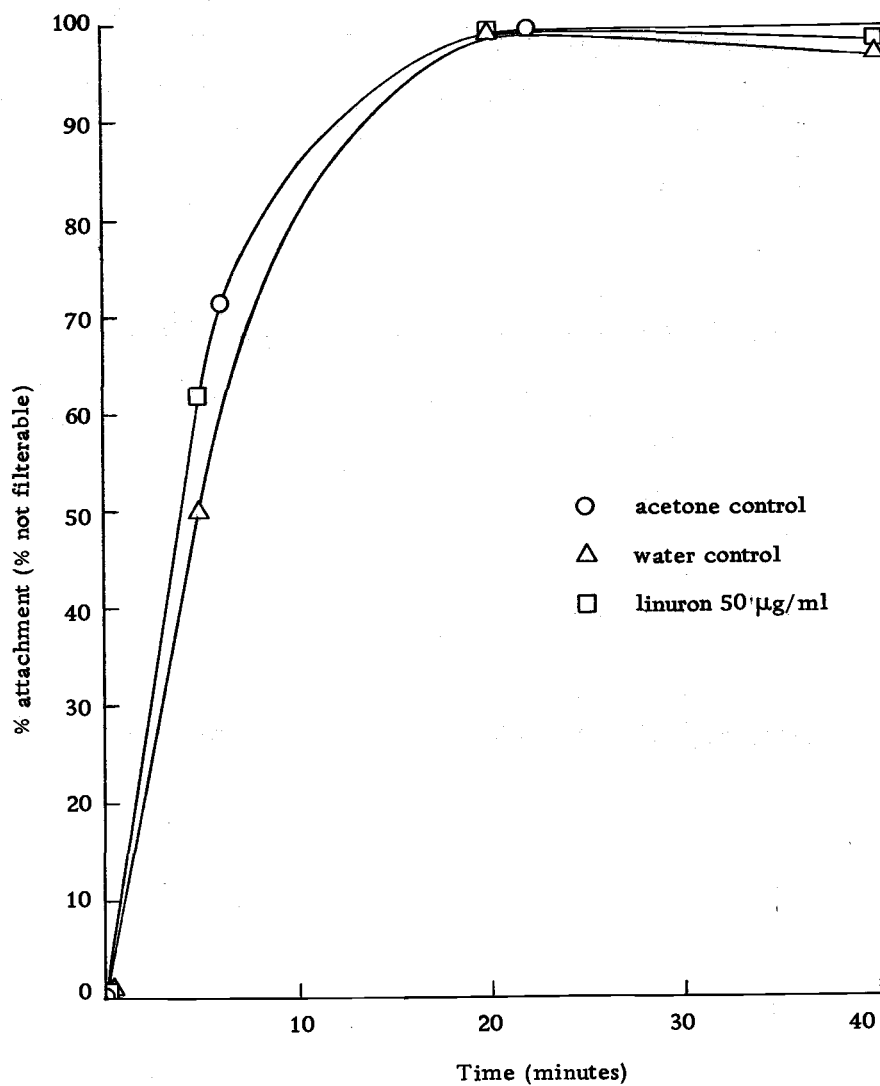


Figure 2. Effect of linuron on attachment rate of *Bdellovibrio*.

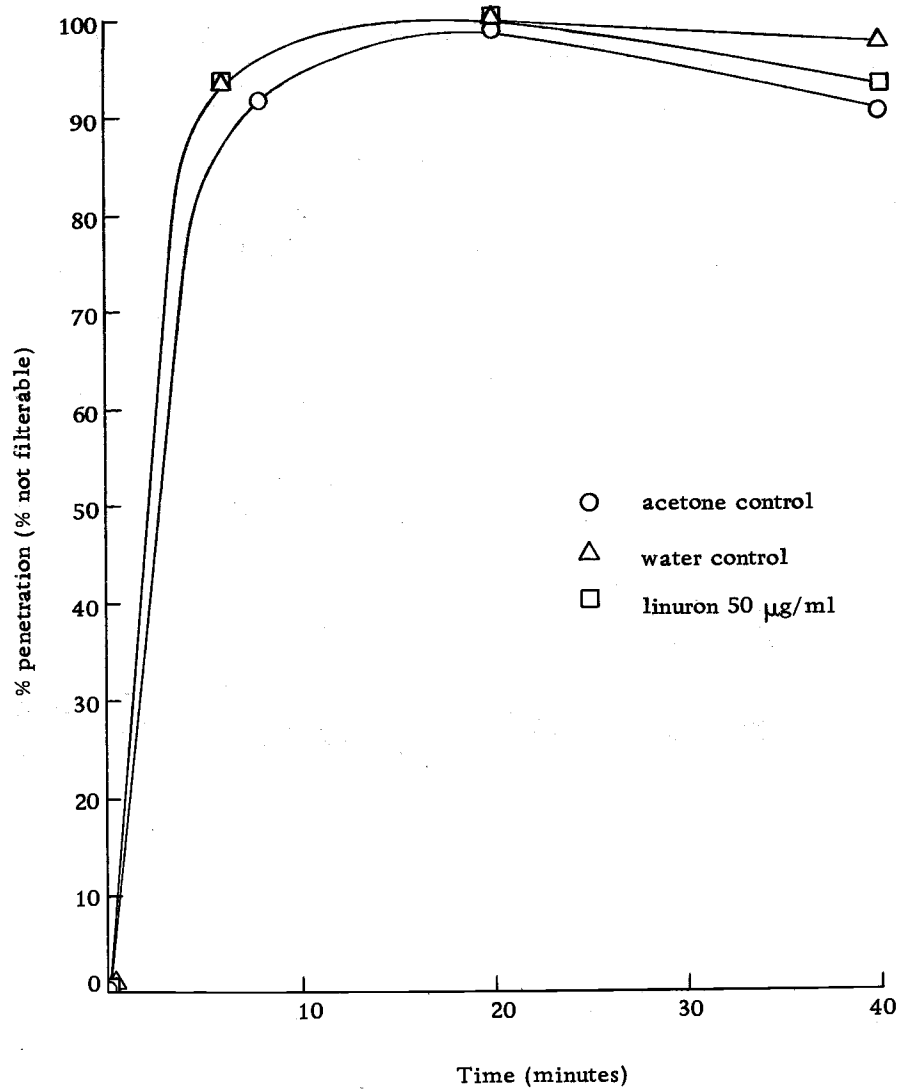


Figure 3. Effect of linuron on penetration rate of *Bdellovibrio*.

initial step, the procedure must be introducing an artifact. The only procedural difference between the attachment measurement and the penetration measurement is the blending step. Therefore this is probably at fault. It appears possible that blending may be more damaging to the bdellovibrios when they are attached or in early stages of penetration than when they are free or completely penetrated. Such a phenomenon would introduce the type of artifact observed. In the assay procedure used, a lower pfu concentration in the filtrate was interpreted to mean that a higher proportion of the parasites was unfilterable due to penetration. In actuality, they might have been killed in the blending procedure. Conversely, a decreased vulnerability to blending at later stages in the parasitic process would also explain the apparent decline in the proportion of bdellovibrios penetrated between 20 and 40 minutes. The maximum number of parasites should have entered the host cells by this time (77) but they should not have been released in this time interval. No net increase in pfu concentration was noted over the 40 minute period, which would strengthen this postulation.

Sensitivity of attached bdellovibrios might be due to increased chance of blade contact with the larger parasite-host complex. More likely, it is related to physical disruption of the bonded Bdellovibrio cell in the "holdfast" region due to medium agitation.

Present observation supports in part the findings of Burnham

and coworkers (11), who reported failure in attempts to reverse early stages of attachment by either agitation or sonication. More extreme treatment was reported to disrupt cells of both host and parasite.

Attachment apparently was reversed in the present work as evidenced by results of the preliminary investigation but is masked at certain stages of the parasitism by what appears to be a mortality due to the agitation procedure. By preincubating the culture 20 minutes before treating the samples, apparently this effect was avoided in the preliminary work by allowing the majority of the culture to be advanced past the more vulnerable stages of the parasitism. In future quantitative work one might be able to utilize this apparent vulnerability of the parasite in certain stages of the parasitic process, for measurement of proportion penetrated rather than to rely on procedures aimed at separating the two organisms in a viable form prior to measurement.

Qualitatively the attachment curve obtained in the present work agrees with the results of Varon and Shilo. However their curve shows a lower overall percent of parasites attached at each sampling time. Several reasons for this quantitative disagreement are apparent. Bdellovibrio strains vary in their ability to form plaques and attach to different hosts (8, 64). Attachment efficiency also depends on the age of the host and parasite cultures (77). Thus either strain

differences or treatment of cultures might explain the discrepancy.

The smaller diameter of cells of the host used in this study necessitated the use of 0.8 μ Millipore filters rather than the larger 1.2 μ filter discs used by Varon and Shilo. This factor should be of minimal concern since calculations in both methods were based on the initial percent filterable.

Differences in assay procedures might explain in part why Varon and Shilo obtained a sigmoidal penetration curve while the present work indicated logarithmic penetration. Any fragments of attached parasites killed by the agitation methods would have been washed into the filtrate. This portion of parasites would not have been counted in Varon and Shilo's method, which was based on radiometric determination of carbon-14 labeled parasites retained on the filter disc at each sample time. However, by assaying for pfu's in the filtrate, one would not have detected this group either.

It was assumed that parasites not detected in the filtrate were caught on the filter disc due to penetration into non-filterable host cells. Thus, the possible lethality of the blending procedure at early stages of attachment may have caused these results to vary from Varon and Shilo's. Regardless of possible procedural difficulties, the major observation of these studies is still valid. Linuron-treated cultures exhibited no significant differences from controls in ability either to attach to or to penetrate host cells.

Effect of Linuron on Non-multiplying Cultures

Viability of host 1-26-4 was only slightly affected by linuron in maximum stationary cultures. Over a 24 hour period linuron concentrations of 20 μ g/ml and 50 μ g/ml caused viable counts to drop by less than 0.2 log units.

In contrast, the viability of non-multiplying Bdellovibrio was greatly decreased in the presence of increasing linuron concentrations (Figure 4). Diuron at 50 μ g/ml exhibited approximately the same activity against Bdellovibrio as linuron at 20 μ g/ml, while benefin at the same concentration was as lethal as 4 μ g/ml linuron.

Before linuron was added to the flasks, the concentration of viable host cells had been adjusted to approximately 40% that of Bdellovibrio by allowing maximum lysis, then differential filtration of the culture. This low level of host would ensure that parasitic activity was reduced (77). In addition, the host cells remaining in a lysed Bdellovibrio culture very possibly may not support parasitic activity due to their physiological condition. Thus the effective concentration of host to Bdellovibrio was further reduced, and consequently, parasitic activity was presumed to be of minimal importance in the treated culture.

The observed decrease in viable parasites corresponding to increased linuron dosage (Figure 4) appears, therefore, to reflect

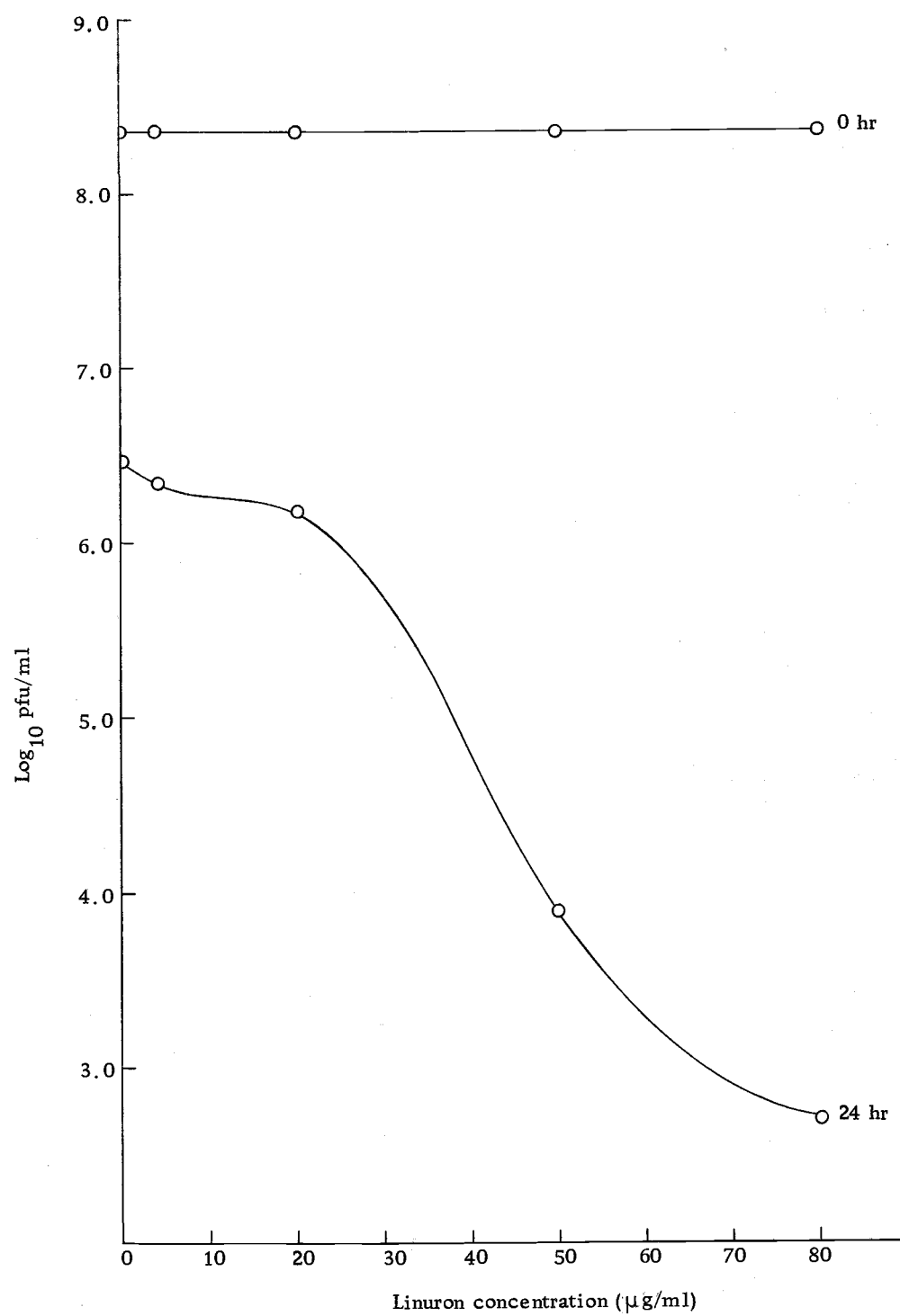


Figure 4. Effect of linuron concentration on resting *Bdellovibrio* culture.

the herbicide's direct effect upon viability of the Bdellovibrio cell and not inhibition of host-parasite interactions. In the absence of available host cells, Bdellovibrio dies rapidly (77), thus, the observed drop in pfu's even in control flasks after 24 hours incubation.

A comparison of Figures 1 and 4 shows that the majority of the parasite's sensitivity to linuron in multiplying cultures is due to the herbicide's enhancement of death rate rather than due to any influence on parasitic processes. The slopes shown in Figures 1 and 4, although not identical, are similar; differences might result from variations in herbicide concentration and continued ability of the linuron-treated parasites to reproduce in the presence of host.

Linuron may affect viability of resting Bdellovibrio cells by altering endogenous respiration or osmotic integrity. Endogenous respiration of Bdellovibrio is maintained at an unusually high level even after storage at 3° for four days (65). Thus, this might be a site of herbicide sensitivity. If, on the other hand, osmotic integrity is affected, cell wall structure variations might explain bdellovibrio's unusual sensitivity.

The Bdellovibrio cell wall appears to be composed of only two electron dense layers, while most Gram-negative bacteria possess thicker cell walls with three such layers (69). Bdellovibrio and another member of the family Spirillaceae, Vibrio fetus, have been reported to display a rigid layer closely associated with the plasma

membrane, where most Gram-negative organisms appear to have a "gap" (47). Another unusual feature is *Bdellovibrio*'s extreme sensitivity to fixation procedures in electron micrograph preparations. The unusually severe separation of wall components may be caused by autolytic enzymes of the Bdellovibrio. From these reports it appears that Bdellovibrio cell walls display some unusual characteristics. It is possible that their structure facilitates access of herbicide molecules to the osmotically sensitive plasma membrane, while the thicker cell walls of most Gram-negative organisms provide a relatively greater degree of protection.

Chelation Studies

Several isolated observations led to the investigation of linuron as a possible chelator of essential ions. Bdellovibrio was reported to be very sensitive to magnesium and calcium concentrations in the growth medium (33, 56), as well as to EDTA (33, 77), a compound which could bind these ions. As was mentioned earlier, magnesium and calcium are believed to activate essential enzymes involved in cell wall maintenance and to provide crosslinks in the cell wall material, as well (47, 80). EDTA was shown to destroy the mucopeptide layer (47) and the polysaccharide layer (80) in Pseudomonas aeruginosa. Thus, a chelating agent might exert a detrimental effect on non-multiplying cells. Linuron was

reported to upset cation uptake in certain plants (31, 49), a phenomenon which would be consistent with chelating effects. The structure of linuron did suggest a possibility of its binding magnesium and calcium.

Chelation is a form of coordination in which a ring structure, generally five- or six-membered, is formed between the bound metal ion and at least two ligands in the same molecule, which are functional groups containing unshared electron pairs (45). Carbonyl, amine, or amide groups often function in such binding, and open chain or heterocyclic ethers may also be involved (25). Several such potential electron donors are present in the linuron molecule. At physiological pH, magnesium, calcium, and manganese bind primarily to oxygen-containing ligands such as the oxygens of the carbonyl and methoxy groups in linuron, while ferrous iron, cobalt and nickel, in general, bind to mixed oxygen and nitrogen donors (78). Chelate rings need not be symmetrical (45).

Chelation would not appear sterically possible without the methoxy group of linuron, which diuron lacks. This would give a convenient explanation as to diuron's ineffectiveness as a Bdellovibrio bactericide. However, present experimental evidence (Figure 5) does not appear to support the chelation theory. Magnesium ion, even at molar concentrations more than 16 times greater than linuron (assuming magnesium ion concentration in TYP broth negligible in

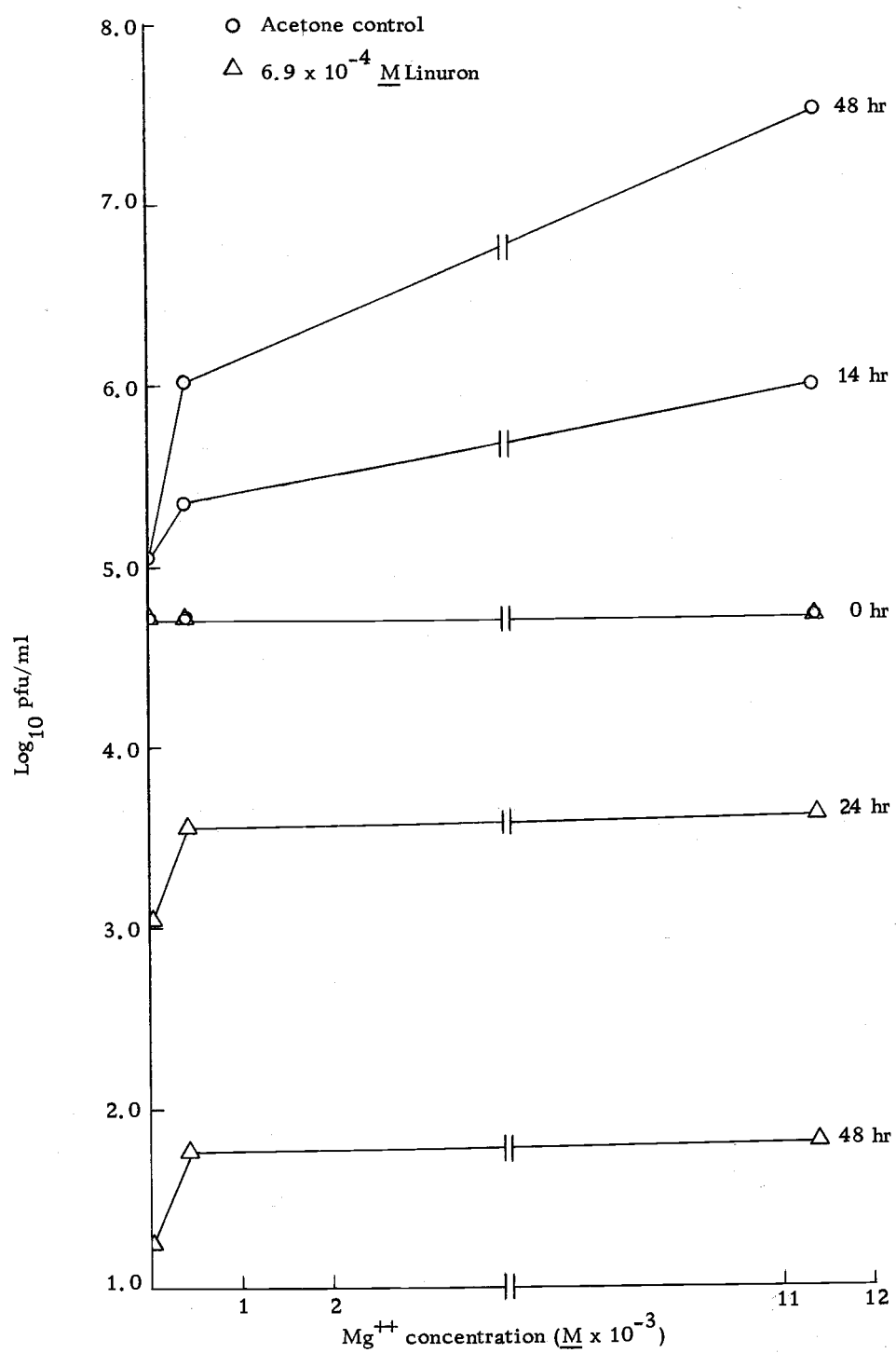


Figure 5. Effect of Mg⁺⁺ on multiplication of Bdellovibrio in the presence of linuron.

comparison), were unable to offset the herbicide's lethal effect on Bdellovibrio. Parallel increases in pfu concentration with increasing additions of magnesium ion were seen in flasks containing linuron and in acetone controls. This indicates that the effect of linuron on Bdellovibrio is not in removing essential Mg^{++} from the medium. These results, however, do not unequivocally preclude activity of the herbicide as a chelator of some other untested essential ion.

It might be noted here that the medium used in all previous experiments appears to be limiting in available magnesium. Addition of 3.6×10^{-4} M Mg^{++} to the culture medium maintained Bdellovibrio growth rate in the second 24 hour period at the level of the first 24 hours (Figure 5), while total pfu concentration in controls without added Mg^{++} declined slightly between 24 and 48 hours. The addition of approximately 4.0×10^{-4} M Mg^{++} to the TYP medium might allow better growth of the parasite in future investigations.

The exact mode of action of linuron on Bdellovibrio has not been precisely defined by these studies. Some reported activities of compounds with structural similarities to the urea herbicides are the halophenols' effect as uncouplers of oxidative phosphorylation (66) and the phenyl carbamates' inactivation of certain enzymes (53). There are no indications that linuron selectively acts against Bdellovibrio by either of these means; however neither can be unequivocally eliminated.

Doxtader (18) has reported that 18 of 19 bacterial isolates sensitive to diuron required biotin. However this limited information does not clarify linuron's role substantially since the linuron-sensitivity of several Rhizobium strains requiring biotin (74), as well as one not requiring biotin (74), was not reversed by addition of vitamins (35). Furthermore, one host-independent Bdellovibrio strain was found not to require biotin (64). Thus, linuron is probably not actually binding the vitamin or inactivating it before it enters the cell. One conclusion appears reasonable. Since this herbicide is lethal to both resting and multiplying cultures, the primary lethal effect is probably not on cell wall synthesis.

It appears that linuron's effect may be due to adsorption phenomena, either at the cell wall or onto the cell membrane. It was mentioned earlier that substituted-urea compounds bind to hydrophobic surfaces, linuron more tightly than diuron. The superficial lipoprotein portion of the bacterial cell wall would provide such a surface (28). Similar to other polar, lipid-soluble substances, linuron might bind to the cell lipoprotein complex by its aromatic hydrophobic portion with its hydrophilic polar side chain extending outward into the medium, reducing surface tension and possibly causing cytolysis (28).

Cells need not be entirely coated with the active compound for a lethal effect to be exerted (28). Goldberg (28) suggested that surfactants may denature enzymes associated with cell walls. Increased

death rates with higher concentrations of the herbicide may be caused by the random adsorption of a quantity of herbicide molecules to each cell sufficient to cause disorganization of the cell membrane and leakage of essential low molecular weight precursors, such as organic ions, amino acids, purines, and pyrimidines (52) into the medium. Such an effect has been observed with certain polypeptide antibiotics (82) and quaternary ammonium compounds (9). The bactericidal effect which these membrane-disorganizing compounds exert against non-growing cells may be due to the leakage phenomenon or to loss of active transport capabilities, the leakage being only secondary (9).

Linuron is more toxic to Bdellovibrio than is diuron. Enhanced activity of the former must be due to substitution of the methoxy group for one of the two methyl groups of diuron. The side chain of linuron appears to have been made much more polar by the substitution of this stronger electron donating group. It is possible that the methoxy-substituted nitrogen atom would thus be made sufficiently negative to attract a hydrogen ion from the medium. The structure of this charged species might be further stabilized by the interaction of the hydrogen ion with the carbonyl oxygen. Diuron does not appear to present bonding possibilities of this type. Thus, its side group would be less likely to be charged. A positive charge would accentuate the polarity of the linuron molecule, which would, in turn, increase the

activity of the compound as a cationic surfactant.

Hogue (31) and Nashed and Ilnicki (49) reported ion uptake alterations in plants treated with linuron. Nashed and Ilnicki suggested that the compound might be affecting permeability of the plant cytoplasmic membrane. Since the chemical composition (43), dimensions (47), and permeability characteristics (22) of Gram-negative bacterial membranes are believed to be not unlike those of plant membranes, this observation gives supportive evidence for an adsorption effect in bacteria, resulting in alterations in semi-permeability.

Temporary effects on soil population due to application of linuron appear possible, although direct correlations between in vitro effects in a two-membered culture and effects in soil systems must be made with caution. Recommended soil application rates of linuron are between 0.5 and 3.0 lb. active ingredient per acre, equal to approximately 0.5 to 3.0 $\mu\text{g/g}$. Adsorption phenomena may cause the concentration on soil particles and possibly on the bacteria themselves to be as high as 5.5 times the overall application rate in certain soil types. This would allow a possibility of a 1.75 to 16.5 $\mu\text{g/g}$ concentration of linuron in the vicinity of the soil microorganisms.

In vitro, a concentration of 16 $\mu\text{g/ml}$ linuron appeared to be the minimum net inhibitory concentration for Bdellovibrio, but death rate was increased over controls at concentrations as low as 2 $\mu\text{g/ml}$.

Thus it appears from the in vitro data that linuron, especially at high

application rates, could temporarily cause a depression in the soil Bdellovibrio population. Bacillus species and other Gram-positive organisms might also be severely inhibited. Assuming other soil pseudomonads to be as resistant to the compound as the organisms tested, a large proportion of the Gram-negative soil population would not be seriously affected.

Thus it would appear that the soil microbial population might be at least temporarily disturbed until the compound is dissipated. If Bdellovibrio is actually involved, as postulated, in maintaining the balance between various groups of Gram-negative organisms in the soil, an application of linuron might produce a dual effect. As was noted in the case of phytopathogenic fungus control (54, 75), it is possible that a Pseudomonas species, for example, pathogenic to a certain crop, might actually be encouraged by an application of linuron both due to linuron's effect on potentially parasitic Bdellovibrio and due to a reduction in Gram-positive organisms competing for available nutrients.

A balancing factor should also be considered. Adsorption of the herbicide to soil organic matter would locate it not only near sensitive bacteria, but also close to organisms able to decompose it. Detoxification of linuron by the latter organisms might occur at a sufficiently rapid rate to reduce antimicrobial effect below critical levels before serious population shifts could occur.

SUMMARY

A Bdellovibrio bacteriovorus strain parasitic on a Pseudomonas species indigenous to local soil was isolated from Woodburn silty clay loam. Seventeen herbicides were screened for activity against these and ten other bacterial cultures.

Herbicide antimicrobial activity appeared to show a positive correlation with presence of an aromatic ring with a polar side chain linked by an electronegative atom. However, DNBP (4, 6-dinitro-o-sec-butylphenol) inhibited all cultures, regardless of its lack of some of the postulated structural requirements.

Picloram (4-amino-3, 5, 6-trichloropicolinic acid); 2, 4-D amine salt (2, 4-dichlorophenoxyacetic acid, dimethylamine salt); 2, 4, 5-T (2, 4, 5-trichlorophenoxyacetic acid); diuron [3-(3, 4-dichlorophenyl)-1, 1-dimethylurea]; linuron [3-(3, 4-dichlorophenyl)-1-methoxy-1-methylurea]; IPC (isopropyl N-phenylcarbamate); and RoNeet (3-ethylcyclohexylethylthiolcarbamate), listed in order of decreasing activity, inhibited a majority of the cultures.

EPTC (ethyl N, N-dipropylthiolcarbamate), two substituted-uracil herbicides, and MCPA ester (2-methyl-4-chlorophenoxyacetic acid, isooctyl ester) were less active. Benefin (N-butyl-N-ethyl- α, α, α -trifluoro-2, 6-dinitro-p toluidine), two triazine herbicides, and the sodium salts of two chlorinated aliphatic acids produced no

observable inhibition in any culture.

Bdellovibrio bacteriovorus exhibited sensitivity to 11 of the 17 herbicides tested. The host 1-26-4, two known Pseudomonas species, and Serratia marcescens were the most resistant of the cultures tested.

General effects of linuron, diuron, and benefin on the Bdellovibrio isolate and three saprophytic bacterial in liquid culture were determined and the effects of linuron on Bdellovibrio activities studied in depth. The host culture and Pseudomonas fluorescens appeared only slightly affected and unaffected, respectively, by the urea herbicides. Benefin was less active. Bacillus cereus growth was totally inhibited by linuron and diuron and decreased approximately 50% by benefin, based on optical density readings.

The net multiplication of Bdellovibrio plaque forming units in liquid cultures was decreased by linuron; diuron and benefin showed less activity, although plaque growth was inhibited on plates by both linuron and diuron, but not by benefin. Motility, attachment rate, and penetration rate of the parasite appeared unaffected by linuron at 50 $\mu\text{g/ml}$. Rapid death ensued in linuron-treated non-multiplying Bdellovibrio cultures.

Linuron's principal site of action against Bdellovibrio thus appears to be at the level of maintenance of cellular integrity. Chelation apparently is not involved in linuron's lethal effect. It is

postulated that linuron disturbs the semipermeability of the Bdellovibrio cell membrane due to adsorption phenomena to which other Gram-negative bacteria are more resistant, resulting in an increased Bdellovibrio death rate.

Application of conclusions derived from in vitro studies to the soil environment must be made with caution. Linuron might decrease Bdellovibrio numbers in treated soils, resulting in possible disequilibrium in the soil microbial population. The effect is, however, apt to be slight due to dilution effects in the soil and might be transient due to rapid decomposition of the applied herbicide by indigenous soil microorganisms. Nevertheless, results of this in vitro study should be considered in future investigations of herbicide effects on soil microbial ecology.

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