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Bacterial-Degradation of Pesticides Residue in Vegetables During Fermentation

Aslan Azizi

Agricultural Engineering Research Institute (AERI), Karaj
Iran

1. Introduction

An application of large quantity of agricultural pesticides in rural area is a common practice in order to increase the productivity and yield, to protect the agricultural crop from pests and prevent products lost due to insect and bacterial contamination is a common practice. Resistance and mutation of some pests to chemicals are the causes of using larger quantity of pesticide in the developing countries. According to the rate of degradation of chemicals, pesticides can be categorized as sensitive or tolerant to decomposition. Their destruction might be occurred under exposing to the normal atmospheric conditions or by biological activity of the soil microorganisms such as *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, *Rhodococcus*, *Gliocladium*, *Trichoderma* and *Penicillium*. These microorganisms use the pesticides as their carbon and energy sources (Aislabie & Lloyd-Jones, 1995). Because agricultural pesticides are mostly artificial synthetic compounds without any identical in the nature, they are substantially tolerant towards degradation in natural conditions. In many cases, stability of these pesticides to the biological destruction arises from their insolubility in water, as the microorganisms are incapable of decaying such materials. Malathion, carbamate, pyrethriod, diazinon, dichloropicolinic acid and phenylalkanbic are sensitive pesticides to the hydrolytic activity of microorganism enzymes. Extracellular enzymes of the bacteria are capable of cleavage broad range of chemical pesticides. Apart from the natural structure of the pesticides, their volatility and adsorption ability to the soil compounds are also important factors affect sensitivity to the biological cleavage. These factors themselves are dependent on temperature, light, soil moisture and pH. The more fugacity of the pesticides, the more transfer of them to the atmosphere. Higher moisture of the soil ease degradation rate of the water soluble pesticides by the microorganisms, while reduce their volatility. Some of the pesticides such as diazinon are very sensitive to the low pH range and their degradation at this range dramatically occur (Muller & Korte, 1975; Freed et al., 1979). Because organophosphorous compounds are decomposed faster and easier compared to the organochlorine compounds, their application have been increasing day by day. Consumption of fruits and vegetables containing organochlorine components residue causes undesirable health disorders especially on the nerve system (Racke & Frank, 1997; Pehkonen & Zhang, 2002; Lal & Saxena, 1982). This danger is more acute in Iran because of the improper attitude that excessive application of the pesticides leads to the more efficient deterioration of the pests.

Because of the important impact of the microorganisms in the degradation of the pesticides, numerous researches have been done regarding qualitative and quantitative aspects of this phenomenon. Navab *et al.* (2003) studied the effects of isolated *Pseudomonas* spp. from soil on the DDT, DDD, DDF and HCH under the laboratory conditions. The bacteria were able to partially degrade the pesticides. It has been reported that *Flavobacterium* and *Spingomonas paucimobil* spp. decayed some types of the pesticides during 48 h of fermentation process (Dimitrios *et al.*). Comprehensive research done by Peric *et al.* (1981) about the biological decomposability of the pesticides revealed that DDT (mainly) and HCH (partially) degraded by the species of *Debariomyces*, *Micrococcus* and *Lactobacillus*. *Lactobacilli* showed the lowest effect. Peric *et al.* (1981) also perceived that adding mentioned microbial mix to the fermented sausage led to the significant decrease in HCH concentration. Similar degradation of DDT and DDE in the Roquefort blue cheese by the using different species of gram positive *Lactobacilli*, *Streptococci* and yeasts were reported by Ledford and Chen (1969) and Mirna and Coretti (1979). Therefore, isolation, identification and screening of the microorganisms which are capable of pesticides residue degradation in food materials are important issues. No research has been done about the isolation and identification of the indigenous pesticides decomposing microflora in vegetable materials. Therefore, this study intends to investigate the effects of isolated indigenous microflora from Iranian vegetable source on the degradation of containing pesticides residue during the fermentation process.

2. Experimental

In order to produce vegetable with desirable pesticides residue, broadcasting precise quantity pesticide and conducting systematic experiments, experimental farm with a surface area of 1000 m² was prepared. The land preparation stages namely: land excavation, land leveling, land grading, primary and secondary tillage operations, sowing, irrigation and cultural practices were carried out.

Types of pesticides used in cultivated vegetables: Diazinon and malathion, which are the most consuming agricultural pesticides in Iran were selected for this study (the pesticides were obtained from Plant Production Department, Ministry of Agriculture, Tehran, Iran). The pesticides were sprayed on the vegetables in a concentration of 0.002 g L⁻¹. The cultivated vegetables were tomato (Super Queen), celery (Tail Utah), green bean (Sun Ray), pea (Green Arrow), cabbage (Space Star) and cauliflower (Globe Master). The original seeds were obtained from seed and plant production institute of Iran.

Preparation of vegetable samples: Cultivated vegetables were harvested at maturation stage and were immediately transferred to the laboratory. The external waste and damaged leaves of vegetables were removed then, piled, trimmed, washed and cut to the desired sizes. Cut vegetables were mixed together and filled into glass jars and covered with 2% (w/v) hot brine (95°C). The aim of adding hot brine was to destroy the heat labile anaerobic nonspore forming microorganisms such as coliforms, improve colour and texture of final product, accelerate the acidification rate and improve nutritional property of the final product. Finally, about 4 mL of vinegar was added to the top of samples in order to prevent activity of unwanted microflora. The mouth of the jars were covered with nylon film having low permeability to water vapour and oxygen, tied with thread and kept at room temperature.

Fermentation process was immediately started. In order to isolate and identify microorganism involved in fermentation of mixed vegetable in different stages, sampling

was conducted every 12 h. Fermentation was continued till the pH of the product reached about 4.0. Fermentation process was stopped by opening the jars. Vegetables were removed from jars washed and stored at -4°C until pesticide detection experiments were done.

Culture media used for the isolation, screening and enumeration of the microorganisms: MRS broth/agar and LSDM broth/agar media (Merck, Darmstadt, Germany) were used for the isolation, partial identification, screening and enumeration of lactic acid bacteria. Media compounded, heated in a flask and boiled in a thermostatically controlled heater till cleared. Then pH of the medium was adjusted to 6.20. The media solutions were distributed in 250 mL flasks. The flasks were autoclaved at 121°C for 15 min and kept in refrigerator until used. In order to evaluate fermentation rate of the different carbohydrates, MRS broth without glucose and beef extract containing 0.05% chlorophenol red was used as a base medium. The ingredients of the medium were separately prepared from Merck (Darmstadt, Germany) and mixed carefully under controlled conditions. All the carbohydrates were sterilized using membrane filtration and added to the basal media to have final concentration of 1% in media. After inoculation of the microflora to the media, incubation process was carried out at 37°C for 7 d and colour variations within this period were studied.

Isolation and identification of microorganisms involved in fermentation of vegetables: Fermentation of vegetables started after few hours, subsequent to sealing the jars. Lactic acid bacteria were isolated from fermenting vegetables using MRS agar and LSDM at 12, 24 and 48 h of fermentation. The contents of the jars were mixed thoroughly and 10 mL of brine was withdrawn under sterile condition using a syringe. Fermenting brine 1 mL was serially diluted in saline and was plated on MRS agar and LSDM agar. The streaked plates were incubated at 30°C for 72 h. At the end of the incubation period, bacteria colonies were counted. Individual colonies were isolated based on morphology, gram reaction, cell morphology, catalase production, presence of spores and aerobic and anaerobic growth. Isolated cultures were purified by repeated streaking on MRS agar and isolation. The microflora at the end of 12, 24 and 48 h were isolated and examined for gram reaction, morphology, catalase production, presence of spore and growth under aerobic and anaerobic conditions. The general key used for the identification of gram positive bacteria was done according to the procedure given in Bergey's manuals determinative bacteriology (Sneath et al., 1986). The general morphological and biochemical characteristics of lactic acid bacteria were determined according to the procedure of (Sharpe et al., 1979). In the course of identification of microorganism, to identify microorganism capable of standing presence of two pesticides malathion and diazinon in MRS and LSDM media, the amount of carbohydrate in the media was reduced and same quantity of malation and diazonin was added. They were inoculated with the desired purified cultures. Finally, the isolates were washed under aseptic conditions and centrifuged at 3000 g for 10 min to separate the cells from culture medium. Recovered cells were washed with sterile distilled water several times and kept in a 1% sterilized brine solution for further studies.

Identification procedure was adapted based on the classical biochemical and morphological tests. Biochemical tests included fermentation patterns of the sugars (arabinose, fructose, esculin, glucose, galactose, lactose, mannose, maltose, manitol, rhamnose, raffinose, ribose, salicin, sucrose, sorbitol and xylose), capability of hydrolyzing casein and gelatin, indol production, ammonia from argenin, catalase and pseudocatalase tests, gas formation from consumption of glucose, VP and MR tests, being homofermentative or heterofermentative,

reaction to the molecular oxygen, organic acid production under aerobiosis and anaerobiosis, growth at 15, 30 and 45°C, survival at 60°C after 0.5 h, reaction to the 0.1% MB solution, growth at pH 4.0 and 9.6, growth at the 4 and 10% brine solutions and cells motility. Morphological tests consisted of considering cell appearance, cell arrangement, spore production and gram staining (Sharpe et al., 1979; Anderson, 1984).

Detecting pesticides residue in the vegetable samples: Liquid-liquid extraction method with the solvents of acetone and dichloromethane were used for extraction of the malathion and diazinon from vegetable samples. Quantification analysis of the pesticides was done by applying GC method (Shimadzu 2100, Japan) with the NPD detector and DB5 column 20.

Replications: Experiments were performed three times in duplicate and the mean of the results were considered as final data.

3. Results and discussion

Enumeration of vegetable microflora during the fermentation process: Extra care must be taken when comparing the results; *in vitro* studies are not always real situation in food products. This is due to the fact that the biodegradation process may be affected by a number of factors such as the interaction between microorganisms, the microbial concentration of the medium, whether the medium is liquid or solid and the microbial growth conditions of temperature and pH.

Table-1 indicates total counts of lactic acid bacteria in MRS and LSDM agar along with the pH drop kinetic, in 12 h of vegetable fermentation. The population of microorganisms increased during 24 h of fermentation reaching pH 4.2, then, sudden decrease of population was observed. According to the Table, the population of *Lactobacillus* and *Streptococcus* genera (which were enumerated by LSDM media) was considerably higher than other genera of lactic acid bacteria. It can be attributed to the naturally higher number of bacteria belong to the two mentioned genera in the vegetable mix.

Fermentation time (h)	Bacterial counts (log cfu/g)		pH of the vegetable mix
	MRS	LSDM	
12	9.30	8.18	4.2
24	10.81	9.09	3.7
48	8.04	8.84	3.6

Table 1. Total counts of microorganisms in MRS and LSDM during fermentation of vegetables.

Identification of isolated bacteria: According to the results obtained from classical tests (Tables 2-5), the isolated microorganisms totally belonged to the lactic acid bacteria group and consisted all four genera of these bacteria including *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc*. According to Table-2, isolates 12.2, 12.3, 12.11, 12.14 and 24.5 were from *Lactobacillus* genus including *L. delbrueckii* ssp. *Lactis* and *L. plantarum*. Apart from the *Lactobacilli*, 11 types of cocci were isolated (Table-2). These bacteria belonged to the genera *Streptococcus* species *S. lactis* (isolates 48.2 and 48.3) and *S. raffinolactis* (isolate 48.4), *Pediococcus* species *P. pentosusus* (isolate 12.7), *P. acidilactici* (isolate 12.13) and *P. damnosus* (isolate 12.11) and *Leuconostoc* species *L. mesentroides* (isolate 12.4), *L. cremoris* (isolate 48.6) and *L. oenus* (isolate 48.7). Some of the unidentified species concerned to the genus *Leuconostoc* were also recognized.

Isolate No.	Colony morphology		Gas from glucose	Catalaze	Pseudo catalaze	Motility	Growth pH 4.0	Growth pH 9.6	Salt 4 %	Salt 10 %	Survival at 60 °C for 0.5 h	Vp test	MR test
	Gram +v/-v	B/C Arrangement											
At the end of 12 h													
1	+	B/Sh	Cl	-	-			+	+	-	-	+	+
2	+	B/Sh	Cl	-	-			-		-	-	-	+
3	+	B/Sh	2's	-	-		+		+	-	+	-	+
4	+	C/Tiny	1's	-	-		+			-	-	-	+
5	+	B	2's	-	-		+	+		-	-	-	+
6	+	B	2's	-	-		+	+	+	-	+	-	+
7	+	C	Ch, 2's	-	-		-	+	-	-	-	+	+
8	+	B	2's	-	-			+		-	-	-	-
9	+	B	2's	-	--					-	-	-	+
10	+	B	2's	-	-					-	-	-	+
11	+	C/B	2's, Cl's	-	-		-	+	+	-	+	-	+
12	+	C/B	2's	-	-	+	+	+		--	+	-	+
13	+	C	2's	-	-	+	+	+	+	-	+	-	+
14	-	C/B	2's	--	---			+	+	-	+	-	+
At the end of 24 h													
1	+	B	1's, 2's	-	-		-	+		-	-	-	
2	+	C	Cl's, 4, 1's	-	-	+		+		-	-	+	+
3	+	C	Cl's, 4's	--	-	+	+	+		-	-	+	+
4	+	C/B	1.2's, Ch	-	-	-	-	+		-	-	-	+
5	+	C/B	1's, Ch	-	-	-	+	+		-	-	-	-
6	+	C/B	2's	-	-			+		-	-	-	+
7	+	C	Cl's, Ch	+	+		Nd			-	-	-	+
At the end of 48 h													
1	+	C/B	2's	-	+		-	-		-	-	-	+
2	+	C/B	2's	-	+		-	-		-	-	-	-
3	+	C	1.2's	-	-		-	-		-	--	-	-
4	+	C	1.2's	-	-	+	+	+		-	-	-	-
5	+	C	1.2's	-	+	+	+	+		-	-	-	+
6	+	C	1.2's	-	+	+	-	-		-	-	-	+
7	+	C	1.2's	-	+		-	-		-	-	-	+

B: Bacilli; C: cocci; Cis: Clusters; Sh: Short; 1's: Single; 2's: Two cells together; Ch: Chain; 1.2's: Single and two cells; 4's: Four cells together.

Table 2. Characteristics of isolates from the brine of fermented vegetables at different intervals

Characteristics	A	B	C	D	E	F	G
1. Gram reaction	+	+	+	+	+	+	+
2. Arrangement	-	-	-	-	-	--	-
3. Gas from glucose	--	-	-	--	--	--	-
4. Catalase	-	-	-	-	-	-	-
5. Pseudo catalase	--	-	-	-	-	-	-
6. Motility	-	-	-	-	-	-	-
7. pH 4	-	-	-	+	+	+	+
8. pH 9.6	+	+	+	+	+	+	+
9. Salt 4 %	+	+	+	+	+	+	+
10. Salt 10 %	-	-	-	-	-	-	-
11. Growth at 25 °C	-	-	-	+	+	+	+
30 °C	+	+	+	+	+	+	+
45 °C	+	+	+	+	+	+	+
12. Vp test	+	+	-	-	-	-	-
13. MR test	+	+	+	+	+	+	+
14. Survival at 60 °C for 0.5 h	+	+	+	+	+	+	+
15. Reaction to 0.1 % MB	+	+	+	+	+	+	+
16. NH ₃ from arginine	+	+	+	+	+	+	+
17. Indol produced	-	-	-	-	-	-	-
18. Casein hydrolyzed	-	-	-	-	-	-	-
19. Gelatin hydrolyzed	-	---	-	-	-	-	-
20. Arabinose	+	+	+	+	+	+	+
21. Esculin	+	+	+	+	+	+	+
22. Fructose	+	+	+	+	+	+	+
23. Galactose	+	+	+	+	+	+	+
24. Glucose							
25. Lactose	-	+	+	+	+	+	+
26. Maltose	+	+	+	+	+	+	+
27. Mannitol	-	-	-	+	+	+	+
28. Mannose	+	+	+	+	+	+	+
29. Raffinose	-	-	-	+	+	+	+
30. Rhamnose	-	-	-	-	-	-	-
31. Ribose	-	-	-	+	+	+	+
32. Salicin	+	+	+	+	+	+	+
33. Sorbitol	-	-	-	+	+	+	+
34. Sucrose	+	+	+	+	+	+	+
35. Xylose	-	-	-	+	+	+	+

A = Isolate 12-2; B = Isolate 12-3; C = *L. delbrukii* Sub. Sp. lactic; D = Isolate 12-11;
E = Isolate 12-14; F = Isolate 24.5; G = *L. plantarum*

Table 3. Physiological and biochemical characteristics of isolates from fermenting brine

Isolate No.	Identification
Obligate homofermentative <i>Lactobacilli</i>	
12-2	<i>L. delbrueckii</i> sub. Sp. Lactis
12-3	<i>L. delbrueckii</i> sub. Sp. Lactis
Facultative heater fermentative <i>Lactobacilli</i>	
12-11	<i>L. plantarum</i>
12-14	<i>L. plantarum</i>
12-12	<i>L. plantarum</i>
12-13	<i>L. plantarum</i>

Table 4. Characterization of Lactobacillus isolates

Characteristic	Isolate No. 48-2, 45-3	<i>S. lactis</i>	Isolate No. 12, No. 11	<i>Leuconostoc mesentroides</i>	Isolate 48.6	<i>Leuconostoc cremoris</i>	Isolate No. 25-7	<i>Leuconostoc oenos</i>	Isolate No. 12-7	<i>Pediococcus pentosaceus</i>	Isolate No. 12-3	<i>P. acidilactici</i>	Isolate No. 24-2, 24-3	<i>P. damnosus</i>
Gram reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas from glucose	-	-	+	d	+	-	+	d	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudo catalase	-	-	-	-	+	-	+	-	-	+	-	+	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH 4	-	-	+	-	-	-	-	+	-	+	+	+	+	+
At pH 9.6	-	-	+	+	-	-	ND	d	+	d	+	d	-	-
Growth in salt 4 %	-	+	+	+	-	-	+	ND	+	+	+	+	+	-
In salt 10 %	-	-	-	-	-	d	-	ND	d	d	-	-	-	-
Growth at 25 °C	-	+	+	+	-	-	-	-	+	+	+	+	+	-
at 37 °C	+	+	+	d	+	-	+	d	+	+	+	+	+	-
at 45 °C	-	-	+	-	+	-	+	-	-	-	-	-	+	-
Vp test	-	-	-	-	-	-	-	-	+	+	+	+	-	-
MR test	-	-	-	-	+	-	-	-	+	+	+	+	-	-
Survival at 60 °C for 0.5 h	+	+	-	-	+	-	-	-	-	-	-	-	-	-
Reaction to 0.1 % MB	-	+	+	-	-	-	-	-	-	-	-	-	-	-
NH ₃ from arginine	+	+	-	-	-	-	+	-	+	+	+	+	-	-
Indol produced	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Casein hydrolyzed	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolyzed	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	+	+	-	-	-	d	+	+	+	d	-	-
Esculin	-	d	+	d	-	-	+	+	-	+	+	+	+	+
Galactose	+	-	+	-	+	-	+	-	d	-	-	-	-	-
Fructose	+	-	+	-	-	-	+	-	+	-	-	-	-	-
Lactose	+	+	+	d	+	+	-	-	-	d	+	d	-	-
Maltose	+	+	+	+	-	d	+	-	+	+	+	-	+	d
Mannitol	+	-	+	-	-	-	+	-	-	-	-	-	-	-
Mannose	-	+	-	+	-	-	+	d	-	-	-	-	-	-
Raffinose	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Rhamnose ribose	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Ribose	+	-	+	-	-	-	-	-	+	-	+	d	-	-
Salicin	-	-	+	d	+	-	+	d	+	-	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	+	+	-	-	-	-	+	-	-	-	-	d
Xylose	-	-	-	-	-	-	+	-	+	+	+	+	-	-

Table 5. Physiological and biochemical characteristics of isolates from fermenting brine

Degradation of the pesticides by the lactic acid bacteria: Table 6 shows the effect of indigenous lactic acid bacteria in vegetable mix on the degradation of malathion and diazinon after 48 h of fermentation process. According to the Table-6, the initial concentrations of malathion and diazinon in the vegetable (unprocessed sample) were 3.5 and 0.6 mg kg⁻¹, respectively. This fact implies more penetration of the first pesticide into the plant tissues during the pesticide spraying stage. After 48 h of fermentation, the concentration of malathion considerably decreased and reached to 0.5 mg kg⁻¹, whereas, diazinon concentration only decreased about 0.1 mg kg⁻¹. The remarkably degradation of the malathion during the fermentation could be attributed (Freed et al.,1979) to its instability at low pH ranges, regardless of bacterial decomposition.

Sample name	Diazenon (mg/kg)	Malation (mg/kg)
Control	Not detected	Not detected
Un processed sample	0.6	3.5
Processed sample	0.5	0.5

Table 6. Dagradaation of Malation and Diazinon by lactic acid bacteria in 48 h.

4. Conclusion

According to the results obtained from this research, indigenous microflora of Iranian vegetables which are substantially consisted of different species of lactic acid bacteria, were capable of degrading malathion and diazinon, the two common pesticides used in Iran. Regardless of enhancing hygienic value of the vegetable products, fermentation led to the formation of a novel fermented vegetable with well organoleptic characteristics. Among the isolated lactic acid bacteria, *L. plantarum* was a probiotic bacterium. Because the mix microflora was able to grow fast up to about pH 4.2 (Table-1), special attention should be made on this point whether mentioned bacterium is tolerant towards low pH ranges. This fact would be very important because probiotics are generally sensitive to low pH and high acidic media. The lactic acid microflora showed synergistic relationships among the species, because single cultures were not able to reduce the medium pH compared with the mix cultures, when inoculated to the vegetable, separately (data not shown). Adapted microflora after 3 times of transfer in MRS broth containing malathion and diazinon instead of glucose, made the tolerant microorganisms which were highly capable of growing and decreasing the pH of the media. Therefore, producing such a mix culture in the form of lyophilized starter culture for production of fermented vegetable products could be an important objective. Determination of optimum fermentation time is also an important issue, because along with the increase of fermentation time, the amount of pesticides as well as the viable counts of the bacteria decreases. The second fact is not favourable. Because the number of viable cells remarkably decreases during the period of 24 to 48 h after the start of

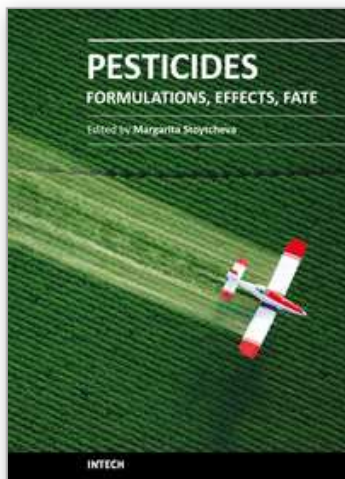
fermentation (Table-1), it is recommended that the optimum fermentation time to be identified during 24 to 48 h. Complementary research should be done about the degrading effects of fermentation on the other consuming chemical pesticides. Moreover, it might be interesting to determine the contribution of each species in the degradation of the pesticides, during the fermentation period. Finally, the components produced from degraded pesticides must be identified and evaluated from safety as well as sensory points of view.

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This book provides an overview on a large variety of pesticide-related topics, organized in three sections. The first part is dedicated to the "safer" pesticides derived from natural materials, the design and the optimization of pesticides formulations, and the techniques for pesticides application. The second part is intended to demonstrate the agricultural products, environmental and biota pesticides contamination and the impacts of the pesticides presence on the ecosystems. The third part presents current investigations of the naturally occurring pesticides degradation phenomena, the environmental effects of the break down products, and different approaches to pesticides residues treatment. Written by leading experts in their respective areas, the book is highly recommended to the professionals, interested in pesticides issues.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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