Scientific paper

Membrane Changes Associated with Exposure of *Pseudomonas putida* to Selected Environmental Pollutants and their Possible Roles in Toxicity

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Abstract

A bacterial model system (*Pseudomonas putida* DSM 50026) was used in this research to assess potential effect of five selected chemically diverse environmental pollutants on cell membranes. Long chain fatty acid profiles of cultures exposed to environmentally relevant concentrations of atrazine (ATR), metolachlor (MET), pentachlorobiphenyl (PCB), hexachlorobenzene (HCB) and fluoranthene (FL), were analyzed and compared to non-exposed cultures. To assess sensitivity of membrane-based responses, the impact of each toxicant on culture growth was also followed spectrophotometrically. Results revealed changes in fatty acid profiles when cells were exposed to PCB, HCB and FL in concentrations below the inhibitory levels. Moreover, the observed membrane responses were similar to the ones previously associated with adaptation to some membrane-active compounds. On the other hand, exposure of cells to any of the two herbicides, ATR or MET, did not induce any significant changes in fatty acid profiles. However, when combined with a commonly used fertilizer compound, NH_4NO_3 growth impairment was observed. Synergistic effect of the two herbicides with NH_4NO_3 might be a consequence of changes in fatty acid profile increasing membrane fluidity, likely induced by NH_4^+ ions.

Keywords: Atrazine (ATR), metolachlor (MET), pentachlorobiphenyl (PCB), hexachlorobenzene (HCB), fluoranthene (FL), ammonium nitrate, fatty acid methyl esters (FAME) profiles, growth inhibition

1. Introduction

In the past few decades environmental pollution has become one of the major concerns in most parts of the world. In recent decades, a wide range of modern pollutants have emerged – not least, those associated with road traffic, industry and agriculture. Most of these pollutants are rarely present in excessively large concentrations, so effects on health are usually not immediate or obvious. Long latency times, the effects of cumulative exposures, and multiple exposures to different pollutants, which might act synergistically, all create difficulties in unraveling associations between environmental pollution and health.[1] Despite the fact that the persistent organic pollutants (POPs), like pentachlorobiphenyl (PCB) and hexachlorobenzene (HCB), have been banned globally by Stockholm convention, the lands with intensive agriculture or industry remain contaminated.[2] The impact of such compounds on ecosystem is significant, especially since their lipophilic nature results in bioaccumulation and associated biomagnification effect, having the most detrimental impact on species at the top of the food chain.[3–4] On the other hand, many of less persistent pollutants, like pesticides atrazine (ATR) and metolachlor (MET), are still used in agriculture or industry in many parts of the world, despite their recognized toxic potential.[5–7]

Standard approach to monitor environmental pollution by these compounds is based on chemical analytical methods, which provide accurate information on absolute concentrations of analyzed compounds in the environ-

mental sample, but lack an adequate interpretation of their toxicity to (micro)biota. In order to obtain more realistic information for environmental risk assessment it is therefore important to complement the chemical analysis data with knowledge on biological impact of released chemicals and their mixtures.[8–9] Due to recently worldwide promoted and EU-level prioritized strategy for the reduction of the use of laboratory animals in scientific studies (3R strategy), *in vitro* studies implying microorganisms have been recognized as powerful tools, e.g., in pre-clinical stages of toxicity analysis.[10–11]

Cell membrane is the first barrier separating cellular interior from its environment and therefore represents a primary defense line against unfavorable environmental impacts. It is known that many toxic chemicals exert their toxicity by affecting cell membranes and basic metabolic processes, so *in vitro* test methods are many times of sufficiently predictive power of harmful effects of chemicals also on higher biological levels (vertebrates).[11–12]

One of the frequently used test species in screening the toxicity of water samples is a ubiquitous heterotrophic bacterium *Pseudomonas putida*. In a standardized method, the toxicity of water-soluble pollutants is assessed by monitoring the effects on *P. putida* growth.[13] On the other hand, bacteria from genus *Pseudomonas* were also shown to adapt to certain toxic compounds by modifying fatty acid composition of their membranes. The adaptive membrane changes represent an early response which is not limited to compounds with substantial water solubility. These changes might therefore be usefully applied as biomarkers in toxicity studies and environmental monitoring.[14–17] In the present work we analyzed the effect of environmentally relevant concentrations of five selected pollutants with different chemical structures and associated toxicity potentials (HCB, PCB, FL, ATR and MET), on fatty acid profile of ubiquitous bacterium *Pseudomonas putida*.

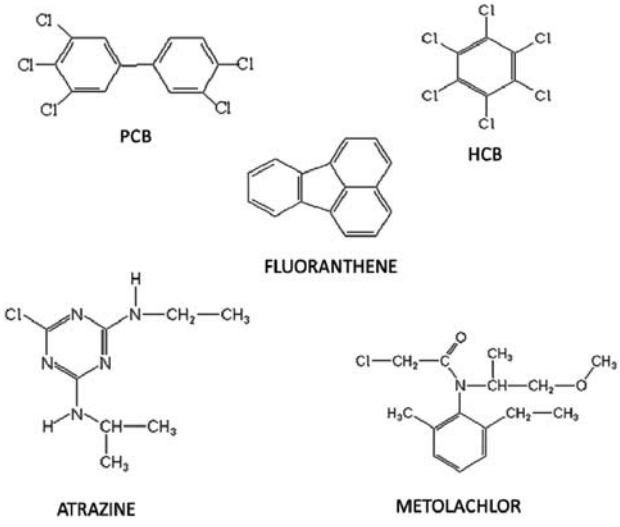

Figure 1. Chemical structures of pollutants used in the experiment

HCB and PCB are two persistent chlorinated aromatic compounds with negligible water solubility (0.005 mg/L and 0.003 mg/L at 20 °C, respectively) and high toxicity as well as carcinogenicity. HCB is a fungicide formerly used as a seed treatment, especially on wheat to control the fungal disease, meanwhile PCB is a compound formerly used in heat exchange and dielectric fluids of transformers and capacitors, hydraulic and lubricating fluids, diffusion pump oils, plasticizers, extenders for pesticides and as ingredients in caulking compound, paints, adhesives and flame retardants. Fluoranthene is another very hydrophobic compound (water solubility: 0.27 mg/L at 20 °C) which belongs to a group of polycyclic aromatic compounds (PAH). Despite the evidence of its toxicity, especially for some aquatic organisms, it is still used in industry, as an intermediate for (fluorescent) dyes, pharmaceuticals and agrochemicals.[18–19] Atrazine and metolachlor are widely used herbicides with several orders higher water solubility than the other compounds used in our experiment (530 mg/L for MET, 30 mg/L for ATR at 20 °C). They are considered as moderately toxic and possible carcinogenic (group C) compounds. Additionaly, recent evidence of endocrine disruption of atrazine is a cause of serious concern.[20] Despite the fact that their toxicity mechanisms have not yet been fully elucidated, they are commonly used in mixtures for corn, soybean and other crops for weed control.[6,11,21]

The aim of our study was to answer the question if there is any correlation between the pollutant chemical properties and its effect on membrane composition (fatty acid profile) of *P. putida*. Secondly, we evaluated the sensitivity of membrane-based responses in comparison to commonly used and standardized method of measuring growth inhibition. Since in agricultural fields the presence of pesticides, like ATR and MET, many times coincides with the presence of fertilizers containing NH_4NO_3, we also tested if a mixture of these compounds affects measured parameters any differently than each single compounds.

2. Experimental

2. 1. Test Strain

P. putida DSM 50026 cells were purchased in freeze-dried form from Deutsche Sammlung von Mikroorganizmen und Zellkulturen GmbH.[22]

2. 2. Chemicals

General reagents: dimethylsulfoxide (DMSO), methanol, n-hexane and glycerol as well as tested chemicals: atrazine, metolachlor, hexachlorobenzene, pentachlorobiphenyl, fluoranthene and ammonium nitrate (NH_4NO_3) were purchased from Sigma (St. Luis, MO, USA) or Merck (Darmstadt, Germany). The purity of all tested

compounds was above 97%. To overcome the problem of low water solubility, the selected toxicants were dissolved in 0.5 mL of DMSO before adding to the culture medium to the final concentrations stated in Tables 1 and 2. Negative controls were performed with the addition of DMSO only. Bacteriological agar and peptone were purchased from Biolife (Milan, Italy) and meat extract from Becton Dickinson (New Jersey, NJ, USA). Standard calibration mixture of bacterial fatty acid methyl esters (FAME) in methyl-caproate (BAME standard) was purchased from Sigma (St. Luis, MO, USA) and standard calibration mixture of bacterial FAME in hexane (MIDI standard) was purchased from Hewlett Packard (USA).

2. 3. Culture and Exposure Conditions

P. putida DSMZ 50026 was grown for 20 hours in medium described by DSMZ (1998) containing 3 g of meat extract and 5 g of bacteriological peptone per 1000 mL of distilled water (dH_2O). Cells were grown in 10 mL test tubes, at 27 °C and without shaking. During incubation, growth was followed by measuring optical density at 654 nm (OD_{654}) by Novaspec II Visible Spectrofotometer. Selected concentrations of tested chemical compounds (Tables 1, 2) were added to cultures of *P. putida* with OD_{654} of 0,24 ± 0,01 which were than shaken and incubated for another 22 hours at 27 °C (without further shaking).

Table 1. Concentrations of pollutant compounds used in the experiment

Tested compound	Concentrations (µg/L)			
Hexachlorobenzene (HCB)	1000	1	0.1	0.01
Pentachlorobiphenyl (PCB)	1000	1	0.1	0.01
Fluoranthene (FL)	1000	1	0.1	0.01
Atrazine (ATR)	1000	1	0.1	0.01
Metolachlor (MET)	1000	1	0.1	0.01

Table 2. Concentrations of different components in mixtures used in the experiment

Mixtures of compounds	Concentrations		
	ATR (µg/L)	MET (µg/L)	NH_4NO_3 (mg/L)
ATR+MET	1000	1000	–
ATR+ NH_4NO_3	1000	–	50
MET+ NH_4NO_3	–	1000	50
ATR+MET+ NH_4NO_3	1000	1000	50

2. 4. Lipid Extraction and Transesterification

Lipids were transesterified using modified HCl/methanol procedure.[23] Freeze-dried samples were pulverized and transferred to HACH screw cap test tubes. First, sample was mixed with 0.5 mL of hexane. Then 1 mL of 1.5 M HCl in methanol and 1mL of pure methanol were added. Test tubes were filled with N_2 and incubated at 80 °C for 10 min. Reaction was stopped by cooling the tubes on ice. Following addition of 2 mL of double distilled water, each reaction mixture was vigorously mixed for 1 min and centrifuged (30 s, 500 g). The upper, organic phase (containing extracted FAMEs) was transferred to vial and filled with N_2. The samples were stored at –20 °C until analysis.

2. 5. Gas Chromatography

Fatty acid methyl esters (FAMEs) extracts in hexane were analyzed on gas chromatograph Shimadzu GC-14A equipped with flame ionization detector (FID). Capillary column (Equity-1; Supelco, 28046-U) with non-polar stationary phase (100% poly-dimethyl-siloxane) was used for FAME separation. Analysis followed temperature program: temperature gradient from 150 to 250 °C at 4 °C min^{-1}. The flow rate of carrier gas (He) was 30 mL min^{-1}, split: 1:50. The injector temperature was held at 250 °C and detector at 280 °C. The results were registered on Chromatopac C-R6A integrator. Relative proportions of fatty acids between C10 and C20 were calculated from peak areas. Identification was done either directly by comparison of retention times of unknown peaks with standard fatty acid calibration mixtures (BAME, MIDI; SIGMA-Aldrich) or indirectly by equivalent chain length (ECL) factors calculation.[24] Membrane fatty acids which were present in less than 0.5 % of total fatty acids were signed as fatty acids in traces and were not considered for further interpretation.

2. 6. Calculations

Equation 1: ECL factors for identification of unknown FAMEs

$$ECL(x) = \frac{\log Rt(x) - \log Rt(z)}{\log Rt(z+1) - \log Rt(z)} + z$$

where *Rt(x)* is a retention time of FAME to be identified (x). *Rt(z)* is the retention time of the straight-chain saturated FAME eluting before x and *Rt(z)* is the retention time of the straight-chain saturated FAME eluting after x; *z* is a number of C-atoms in the straight-chain saturated FAME eluting before x. The ECL factors of straight-chain saturated FAMEs equal the numbers of C-atoms in their chains.

Equation 2: *Trans/cis* ratio of unsaturated fatty acids

$$trans/cis\ ratio\ of\ unsaturated\ fatty\ acids = \frac{(C16:1t + C18:1t)}{(C16:1c + C18:1c)}$$

where *t* means *trans* form of fatty acid and *c* means *cis* form of fatty acid.

2. 7. Statistical Analysis

All the experiments were performed in 4 parallel samples. The data were statistically analyzed using Student's *t*-test with significance level of 0.05.

3. Results and Discussion

Five pollutant compounds of different chemical structures and recognized toxicities (HCB, PCB, FL, ATR and MET) were used in this study to assess their effects on *P. putida* membrane composition. Despite the fact that none of the compounds inhibited growth of *P. putida* DSM 50026 in concentration range from 0.01 to 1 µg/L, significant changes in fatty acid profiles could be detected in cultures exposed to any concentration of the three most hydrophobic toxicants, namely HCB, PCB and FL (Table 3).

Observed changes were similarly expressed regardless of the chemical structure or concentration of tested toxicant – by increase in proportion of C16:1*t*9 and C18:1*t*11 and decrease in proportion of C16:1*c*9. These changes were associated with increased *trans/cis* fatty acid ratio in cellular membranes, which had previously been shown to lower membrane fluidity (permeability) due to tighter packing of *trans* acyl chains (in comparison to *cis* isomers)[25] and supposedly have a protective role when cells are exposed to some toxic compounds.[12,26–29] The described way of membrane adaptation is performed by *cis-trans* isomerase (Cti), a constitutively expressed periplasmic enzyme that, to exert its action, necessitates neither ATP nor other cofactors, and consistently, is independent of *de novo* synthesis of lipids. Due to its direct correlation with toxicity, *cis-trans* isomerization is a potential biomarker for recording acute stress due to membrane acting compounds.[30]

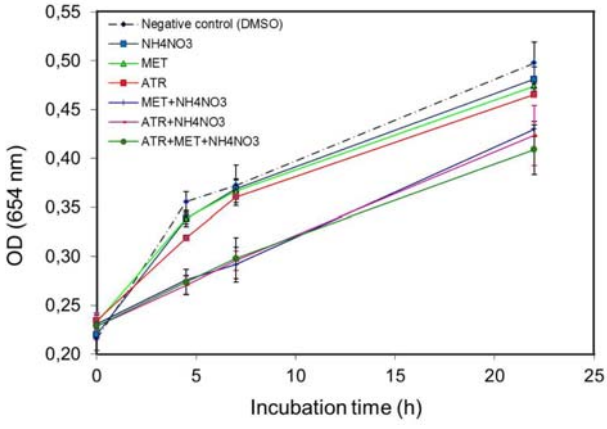

Figure 2. The effect of ATR (1000 µg/L), MET (1000 µg/L), NH_4NO_3 (50 mg/L) and mixtures of each pesticide with NH_4NO_3 on growth rate of *P. putida* DSM 50026. Incubation time applies to the time after the addition of the chemicals.

Table 3. Effect of HCB, PCB and FL on fatty acid profile of *P. putida* DSM 50026. The values represent % of each fatty acid after culture was exposed to a certain concentration of compound.

	negative control	HCB			PCB			FL		
	1 µg/L	0.1 µg/L	0.01 µg/L	1 µg/L	0.1 µg/L	0.01 µg/L	1 µg/L	0.1 µg/L	0.01 µg/L
C12:0	1.07 ± 0.01	1.22 ± 0.02	1.19 ± 0.04	1.18 ± 0.02	1.15 ± 0.05	1.21 ± 0.05	1.24 ± 0.08	1.17 ± 0.06	1.22 ± 0.03	1.17 ± 0.07
C12:0 2OH	4.29 ± 0.02	3.98 ± 0.29	3.86 ± 0.23	4.05 ± 0.10	4.06 ± 0.29	4.44 ± 0.49	4.44 ± 0.49	3.91 ± 0.15	3.92 ± 0.05	3.84 ± 0.23
C14:0	0.90 ± 0.01	0.99 ± 0.01	0.98 ± 0.07	0.93 ± 0.02	0.93 ± 0.07	3.91 ± 0.12	0.92 ± 0.01	0.92 ± 0.03	0.94 ± 0.03	0.96 ± 0.02
C15:0	0.50 ± 0.01	0.57 ± 0.05	0.57 ± 0.06	0.51 ± 0.03	0.52 ± 0.04	0.52 ± 0.02	0.52 ± 0.02	0.51 ± 0.03	0.50 ± 0.01	0.55 ± 0.01
C16:1c9	**21.69 ± 0.06**	**18.45 ± 0.77**	**18.74 ± 0.35**	**21.66 ± 0.52**	**18.73 ± 0.28**	**18.58 ± 1.00**	**19.14 ± 0.57**	**19.48 ± 0.41**	**19.95 ± 0.07**	**19.26 ± 0.47**
C16:1t9	**14.81 ± 0.08**	**18.80 ± 0.81**	**18.35 ± 0.60**	**16.19 ± 0.61**	**18.16 ± 0.64**	**18.71 ± 0.63**	**17.99 ± 0.32**	**17.96 ± 0.50**	**17.68 ± 0.34**	**18.29 ± 0.70**
C16:0	28.29 ± 0.31	28.47 ± 0.22	28.42 ± 0.13	28.13 ± 0.16	28.68 ± 0.36	28.24 ± 0.17	28.54 ± 0.19	28.42 ± 0.22	28.28 ± 0.14	28.61 ± 0.18
C15:0 3OH	0.54 ± 0.04	0.43 ± 0.04	0.43 ± 0.06	0.41 ± 0.03	0.41 ± 0.04	0.42 ± 0.00	0.43 ± 0.03	0.39 ± 0.02	0.39 ± 0.02	0.47 ± 0.13
C17:0 cyc	3.42 ± 0.13	3.76 ± 0.30	3.71 ± 0.21	3.68 ± 0.12	3.91 ± 0.13	3.54 ± 0.16	3.59 ± 0.19	3.51 ± 0.18	3.54 ± 0.11	3.31 ± 0.13
C18:1c11	16.73 ± 0.29	16.19 ± 0.42	16.22 ± 0.17	17.18 ± 0.07	16.07 ± 0.60	16.10 ± 0.47	15.90 ± 0.33	16.13 ± 0.06	16.38 ± 0.18	16.25 ± 0.26
C18:1t11	**2.22 ± 0.33**	**3.64 ± 0.43**	**3.38 ± 0.06**	**2.57 ± 0.20**	**3.59 ± 0.09**	**3.66 ± 0.48**	**3.30 ± 0.07**	**3.43 ± 0.11**	**3.17 ± 0.06**	**3.31 ± 0.09**
C18:0	0.49 ± 0.00	0.59 ± 0.02	0.60 ± 0.01	0.58 ± 0.02	0.58 ± 0.08	0.59 ± 0.02	0.57 ± 0.07	0.58 ± 0.02	0.62 ± 0.05	0.60 ± 0.05

Table 4. The effect of different combinations of ATR, MET and NH_4NO_3 on *trans/cis* fatty acid ratio in *P. putida* DSM 50026.

Tested compound (mixture)	Negative control	ATR	MET	ATR + MET	ATR + NH_4NO_3	MET + NH_4NO_3	NH_4NO_3
***trans/cis* ratio**	0.64 ± 0.04	0.63 ± 0.02	0.62 ± 0.01	0.62 ± 0.08	0.48 ± 0.07	0.56 ± 0.02	0.47 ± 0.1

Exposure of *P. putida* to ATR, MET or a combination of the two herbicides, on the other hand, did not result in any significant changes in fatty acid composition, neither growth inhibition of bacteria. However, when any of the two pesticides (or a mixture of both) was combined to NH_4NO_3, statistically significant drop in culture growth rate (growth inhibition) was observed (Figure 2).

Analysis of the fatty acid profile of cultures treated with NH_4NO_3 as well as mixtures of NH_4NO_3 and ATR or MET revealed membrane changes resulting in overall decrease in *trans/cis* fatty acid ratio (Table 4), probably due to increased *de novo* synthesis of *cis* fatty acid isomers. Obviously, the overall effect was just the opposite than in the case of exposure to PCB, HCB and FL and might be (according to previous reports) associated with increase in fluidity of cell membranes.[31]

A possible explanation for the observed results might be associated with the fact, that NH_4NO_3 represents the easiest accessible and therefore preferably used nitrogen source by *P. putida.*[31] After dissociation to NH_4^+ and NO_3^- ions in water medium, ammonium is transported across the membrane and can be directly used in anabolic metabolism. Ammonium uptake systems have been described in many bacteria, although its uncharged form, ammonia (NH_3), is supposed to be highly membrane permeable.[32] Our data suggest that the presence of high concentrations of free ammonium might also induce changes in membrane favoring its uptake. It is known that changes in cell membrane composition (fluidity) not only affect the passive transport across the membrane, but also influence the conformation and associated activity of some membrane transporters.[33,34] A possible explanation of increased ATR and MET toxicity associated with the presence of NH_4NO_3 might therefore be a consequence of a coincidental increase in ATR and MET internalization (followed by intracellular toxic action) due to NH_4NO_3–induced membrane changes. However, the mechanism should be studied further on molecular level to confirm the hypothesis. Moreover, the influence of NH_4NO_3 to toxicity of different water soluble toxicants needs to be tested on a wider spectrum of species from different trophic levels (up to mammals). In case it turns out that the phenomenon of synergistic toxic effect of these compounds is widespread, that should be acknowledged in agricultural practice. On the other hand, if the increased sensitivity for (certain) water-soluble pollutants associated with the presence of easily available nitrogen source turns out to be specific to (these) bacteria, that might be usefully applied in environmental monitoring (for example enable the detection of lower concentrations of pollutants by adjusting the composition of test medium). However, an accurate evaluation of the concentration-dependence of the observed effects first needs to be done.

Overall, the results show that (adaptive) membrane changes influence the detection of water non-soluble as well as water soluble toxicants by *P. putida* in different ways. It might therefore be useful to complement the standard growth inhibition test with FAME profile analysis. The presence of several different toxic compounds in the environmental samples might make the interpretation difficult, but the overall effect of the mixture on membrane fluidity should give additional information on the nature of prevalent compounds.

4. Conclusion

It was shown that adaptive membrane changes resulting from exposure of *P. putida* DSM 50026 to the most hydrophobic toxicants, namely HCB, PCB and FL can be detected at low concentrations that do not yet affect growth of bacteria. The changes resulted in increased *trans/cis* fatty acid ratio of membrane lipids, which is (according to the literature) associated with decrease in membrane fluidity. On the other hand, ATR and MET in concentrations up to 1000 μg/L do not induce 'defensive' changes in fatty acid profile nor inhibit the growth of bacteria. However, if combined with a commonly used fertilizer salt NH_4NO_3, their toxicity becomes apparent, which might be associated with NH_4^+ ions – induced membrane changes, coincidentally increasing its permeability for the two pesticides. These results clearly show the synergistic (toxic) action of the compounds that are commonly mixed in soil and (ground) water of agricultural areas and should be further investigated in the future as well as acknowledged in the practice.

5. References

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Povzetek

V raziskavi smo analizirali učinke petih izbranih kemijsko raznolikih okoljskih onesnaževal na celične membrane bakterije *Pseudomonas putida* DSM 50026. Bakterijske kulture smo izpostavili nizkim koncentracijam (0.01–1000 µg/L) atrazina (ATR), metolaklora (MET), pentaklorobifenila (PCB), heksaklorobenzena (HCB) in fluorantena (FL) ter analizirali njihove maščobnokislinske profile. Da bi ocenili občutljivost odzivov na membranskem nivoju, smo kot referenčno metodo uporabili spektrofotometrično sledenje vplivov izbranih spojin na rast. Rezultati so pokazali statistično značilne spremembe v maščobnokislinskem profilu kulture ob izpostavi trem najbolj hidrofobnim spojinam že pri najnižji testirani koncentraciji, ki je bila vsaj 10 000-krat nižja od inhibitorne. Učinki omenjenih treh spojin so se na membranskem profilu odražali podobno kot v primeru izpostavitev celic nekaterim drugim membransko aktivnim spojinam (z »obrambno« reakcijo povišanja razmerja *trans/cis* maščobnih kislin). V primeru izpostavitve kulture ATR ali/in MET nismo zaznali nobenih vplivov na analizirane parametre, medtem ko je kombinacija katerega koli od njiju z NH_4NO_3 vplivala tako na maščobnokislinski profil, kot tudi upočasnila rast bakterijske kulture. Sinergistični toksični učinki kombinacije herbicidov z NH_4NO_3 so verjetno posledica povečane propustnosti celičnih membran ob prisotnosti povišanih koncentracij NH_4^+.