SZENT ISTVÁN UNIVERSITY

Ph.D. THESIS

The importance of *Pseudomonas aeruginosa* on environmental safety in media under athropogenic affect

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TABLE OF CONTENTS

1. BACKGROUND 4

2. AIMS AND SCOPE 5

3. MATERIALS AND METHODS 7

3.1. Sampling 7

3.2. Isolation and identification of species *P. aeruginosa* 7

3.3. Virulence investigations 8

3.4. Antibiotic resistance investigations 8

4. RESULTS AND DISCUSSION 9

4.1. The detection rate of *P. aeruginosa* in hydrocarbon contaminated sites and compost 9

4.2. Creation of culture collection 10

4.3. Results of virulence assay 11

4.4. Results of antibiotic resistance investigations 14

5. CONCLUSIONS AND SUGGESTIONS 18

6. PUBLICATIONS 21

1. BACKGROUND

Nowadays, the widespread (industrial, agricultural, health and environmental) application of biotechnological processes creates new challenges for scientists. The increasing number of microorganisms encountered during these processes raises the issue of ensuring human-health and environmental safety of biotechnological processes. Despite this, we can still find examples of the application of unidentified microorganisms for bioremediation purposes that possibly endanger the chemical and/or biological safety of humans and the environment.

In addition to the conscious application of microorganisms in interventions such as bioaugmentation, the spontaneous growth of human-, animal-, and plant pathogen microbes can also be problematic in contaminated sites. These microorganisms are able to adapt to diverse contaminants and to reach the infectious dose to humans in polluted areas. The significance of microorganisms can be considerable in the biotransformation of various raw materials such as sewage sludge, the liquid and solid manure of animal husbandry, plant straw and nowadays several by-products of biogas and heat power plants. The key process of these biotransformations is composting. The effectiveness and environmental safety of this procedure, as in the case of bioremediation, is determined by the applied microorganisms.

Inquiries about the biological hazards of applicable microorganisms in bioremediation processes is currently based on the classification and human-health estimation of the given genus or species. However, there are several microbial factors that play role in colonization, infection and treatment. Clinical practice has a large toolbox for examining such features as antibiotic resistance and virulence; however, there are only a few examples of the application of these processes in environmental strains.

Species *Pseudomonas aeruginosa* is a typical example of double assessment; in a clinical environment, it is one of the most significant opportunistic pathogen bacteria and is responsible for the majority of nosocomial infections. However, in the field of environmental protection, the human health concerns of this organism are not recognized, and the strains of this species are commonly used for bioremediation purposes.

2. AIMS AND SCOPE

In my Ph.D. work four objectives were formulated:

- **The detection of *P. aeruginosa* and the determination of its cell count in environmental samples under direct anthropogenic effects in:**
    - **hydrocarbon-contaminated sites,**
    - **by-products of heat power plants and biogas plants and their composts.**
- **Virulence assay via molecular genetic and traditional microbiological methods of environmentally originating strains of *P. aeruginosa* for the laboratory scale verification of several direct and indirect virulence markers.**
- **Antibiotic resistance investigations for the determination of the resistance profiles of environmental strains of *P. aeruginosa*.**
- **The comparison of virulence and antibiotic resistance features of environmental strains with the available data from clinical strains.**

For the first objective, namely for the determination of detection rate of species *P. aeruginosa,* we planned to choose several contaminated sites of Hungary that are currently under active environmental investments of remediation and primarily contaminated with hydrocarbon compounds.

For the compost examination component in the first objective, the aim was to choose pilot scale composting investigations of raw materials originating from heat power plants and biogas plants where composting process can be followed, and raw materials, maturing compost and final compost product are available for the determination of *P. aeruginosa* cell counts.

For our further aims, the objective was to prepare environmental samples for the creation of a culture collection, whose members are identified not only with traditional growing methods (in our case in compliance with the procedure of the relevant Hungarian Standard) but with molecular genetic (16S rDNA based) methods as well. As a result, all the *P. aeruginosa* strains in this collection are species-level identified and well characterized.

Our second objective, namely the examination of pathogenic features, was reasonable for the evaluation of current environmental practice where negligible hazard is assigned to the application of environmentally originating strains of *P. aeruginosa*. In our research work we studied phenotypic and genetic virulence markers in order to get an overview of their frequency among environmental strains. To that purpose a traditional growing method was chosen for the detection of hemolytic activity, which is a direct virulence marker, while

molecular genetic methods were applied to the evaluation of virulence genes which play a main role in pathogenesis. The frequency of gene sequences responsible for the production of the most important exoenzymes and exotoxins (ExoA, ExoU, ExoT, ExoY, ExoS) was compared with the results of clinical investigations.

Antibiotic resistance assay, our third objective, was due to the lack of information on non-clinical strains of *P. aeruginosa* in scientific literature. To correct this deficiency, we planned antibiotic resistance investigations, based on internationally accepted, standardized methods. The examinations were carried out in two main phases:

- **Preliminary resistance investigations: a comprehensive resistance assay with 31 agents based on a semi-quantitative disc diffusion method.**
- **Quantitative investigations: determination of Minimal Inhibitory Concentration (MIC) values of 10 clinically important agents that were chosen by the use of disc diffusion tests.**

At the end of this process quantitative results were presumed regarding environmental resistance to clinically significant antibiotic agents, and our data became comparable with results of clinical investigations.

The realization of our aims made it possible to get a detailed picture about human health concerns of species *P. aeruginosa* in environmental media under anthropoenic effects.

Results and theses described in this Ph.D. work are based on our own laboratory-scale investigations. The basis for the comparisons and interpretations of my data was the referred clinically available literature.

3. MATERIALS AND METHODS

3.1. Sampling

Our sampling areas, where our environmental samples were taken, were hydrocarbon contaminated sites, and composting pilot plant experimental sites. The sampling period was between 2002 and 2009. Altogether 235 samples from 49 hydrocarbon contaminated sites (soil, groundwater and biofilter), and 101 samples of composing (raw materials of heat power plants and biogas plants, immature and mature compost, and compost treated soil) were examined.

Solid and liquid materials were sampled in accordance with the relevant Hungarian Standards (MSZ 21470-1: 1998, MSZ 21464: 1998).

3.2. Isolation and identification of species *P. aeruginosa*

Isolation of *P. aeruginosa* strains and the determination of their cell counts were performed in accordance with Hungarian Standard (MSZ 21470-77:1988). Enumeration of cell counts was based on MPN (Most Probable Number) method. Further examinations of the clean cultures isolated from environmental samples and the comparative clinical strains were investigated using a multiple-stage protocol (see Figure 1.).

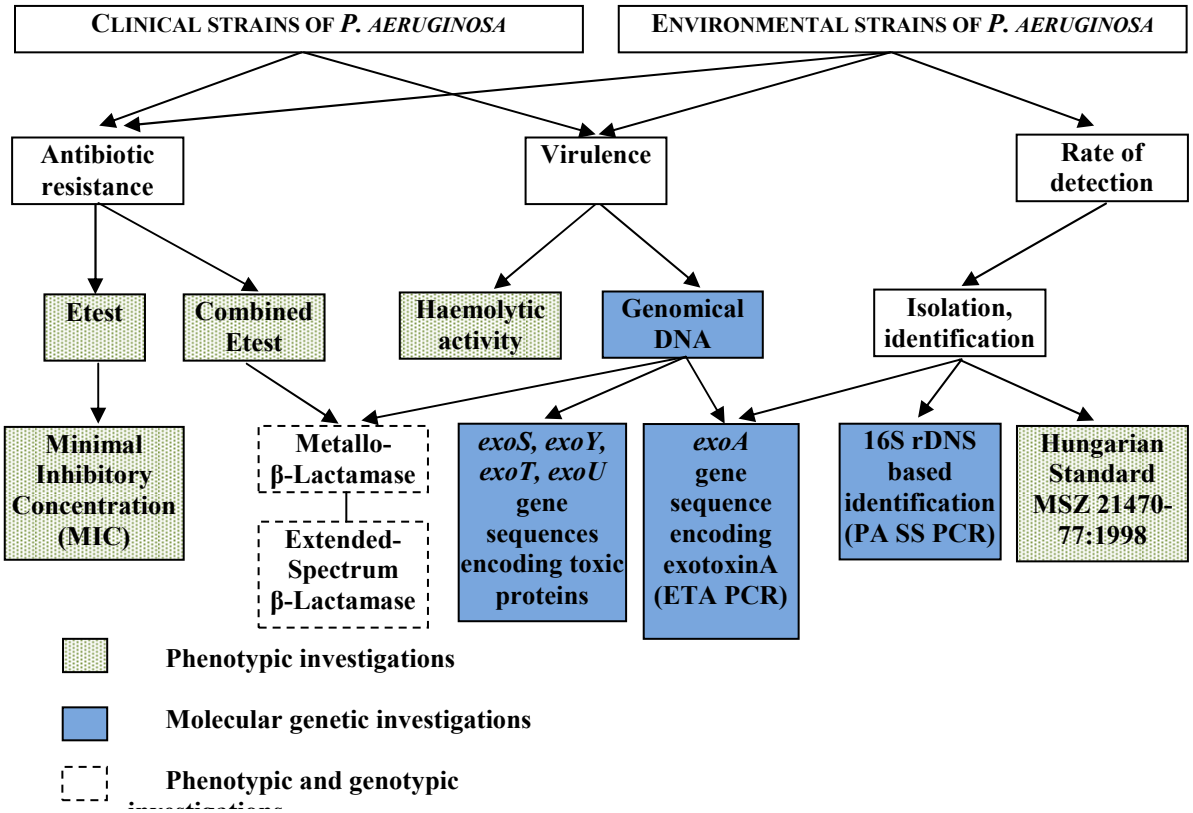


Figure 1: Identification, resistance and virulence investigations of *P. aeruginosa*

Clean cultures of environmental strains isolated by the above mentioned standardized method and after the isolation of their genomic DNA, were identified with PA-SS PCR reaction, too. During the course of this process, species specific region V2 and V8 of 16S rDNA were detected with the application of the following primers: PA-SS-F (5'-GGGGGATCTTCGGACCTCA-3') and PA-SS-R (5'-TCCTTAGAGTGCCCACCCG-3').

3.3. Virulence investigations

Determination of hemolytic activity: clean cultures of the examined strains were cultured on Columbia blutagar (incubation: 37 °C, 22 h). Hemolytic activity was characterized in a five-graded scale.

Detection of gene sequences responsible for the production of exotoxins (ExoA ExoU), and exoenzymes (ExoS, ExoT, ExoY): after the isolation of genomic DNA, *exoA, exoU, exoS, exoT* and *exoY* gene sequences were detected using a PCR method with primer pairs specified in Table 1.

Table 1. Primer sequences of PCR-based examination of *P. aeruginosa* strains

Gene	Primer sequences (5'-3')									
exoA	F:	5'	AAC CAG CTC AGC CAC ATG TC	3'	R:	5'	CGC TGG CCC ATT CGC TCC AGC GCT	3'		
exoS	F:	5'	GCG AGG TCA GCA GAG TAT CG	3'	R:	5'	TTC GGC GTC ACT GTG GAT GC	3'		
exoT	F:	5'	AAT CGC CGT CCA ACT GCA TGC G	3'	R:	5'	TGT TCG CCG AGG TAC TGC TC	3'		
exoY	F:	5'	CGG ATT CTA TGG CAG GGA GG	3'	R:	5'	GCC CTT GAT GCA CTC GAC CA	3'		
exoU	F:	5'	CCG TTG TGG TGC CGT TGA AG	3'	R:	5'	CCA GAT GTT CAC CGA CTC GC	3'		

3.4. Antibiotic resistance investigations

Disc diffusion method: comprehensive investigations of 31 agents of 9 classes of antibiotics that are applied routinely in clinical tests.

Detection of Minimal Inhibitory Concentrations: 10 agents of 5 classes of antibiotics which are the first choice for clinical therapy of *P. aeruginosa* (Penicillins, Carbapenems, Cephalosporins, Aminoglycosides, Quinolones) were investigated. Quantitative results were reached by Etest (AbBiodisk, Solna, Sweden).

ESBL (Extended-Spectrum Beta-Lactamases) and MBL (Metallo Beta-Lactamases) production: were examined with the application of combined Etest strips (AbBiodisk, Solna, Sweden).

Implementing and evaluation: were based on the recommendations of Clinical Laboratory Standards Institute and the instructions of the manufacturer (AbBiodisk). Reference strain: ATCC 27853.

4. RESULTS AND DISCUSSION

4.1. The detection rate of *P. aeruginosa* in hydrocarbon contaminated sites and compost

Results of the detection rates and living cell counts of *P. aeruginosa* are summarized in Table 2.

Table 2. The detection rate of *P. aeruginosa* in hydrocarbon contaminated sites and during composting

Origin	Number of sampling sites	Detection of *P. aeruginosa* in sites	Rate of detection (%)	Number of examined samples	Number of samples containing *P. aeruginosa*	Rate of detection (%)	Living cell count (MPN/g, ml)
Hydrocarbon contaminated media	49	34	69,3%	235	80	34,0%	10^1-10^4
Raw material of from composting	8	1	12,5%	17	1	5,8%	10^3
Compost	2	2		78	36	46,1%	
Open piles	1	1		54	29	53,7%	10^1-10^6
Aerated static piles	1	1		24	7	29,1%	10^1-10^4
Compost treated soil	1	1		6	5	83,3%	

According to our investigations of *P. aeruginosa* detection the commonly used theory of the general appearance of *P. aeruginosa* in hydrocarbon contaminated soil and groundwater was verified. By the representative number of samples reached in our present work, numerical values were determined.

NEW SCIENTIFIC RESULT (according to the results of chapter 4.1.):
(Thesis No. 1.) During the examination of 235 samples of 49 hydrocarbon contaminated sites via the introduced, standardized isolation and identification methods *P. aeruginosa* was detected at least once in the case of 69.3% of the investigated sites. According to the total number of samples the typical detection rate was 34.0%. Our results were published in an international publication (KASZAB ET AL., 2010a).

Our investigations of composting showed that the chosen composting technology significantly influences the reproduction rate of *P. aeruginosa*; an aerated static pile is much more effective in the elimination of species *P. aeruginosa* than an open pile (see Table 2.). However, mesophilic *P. aeruginosa* species was capable of surviving the thermophilic phase of composting in both kinds of technology (see Figure 2.).

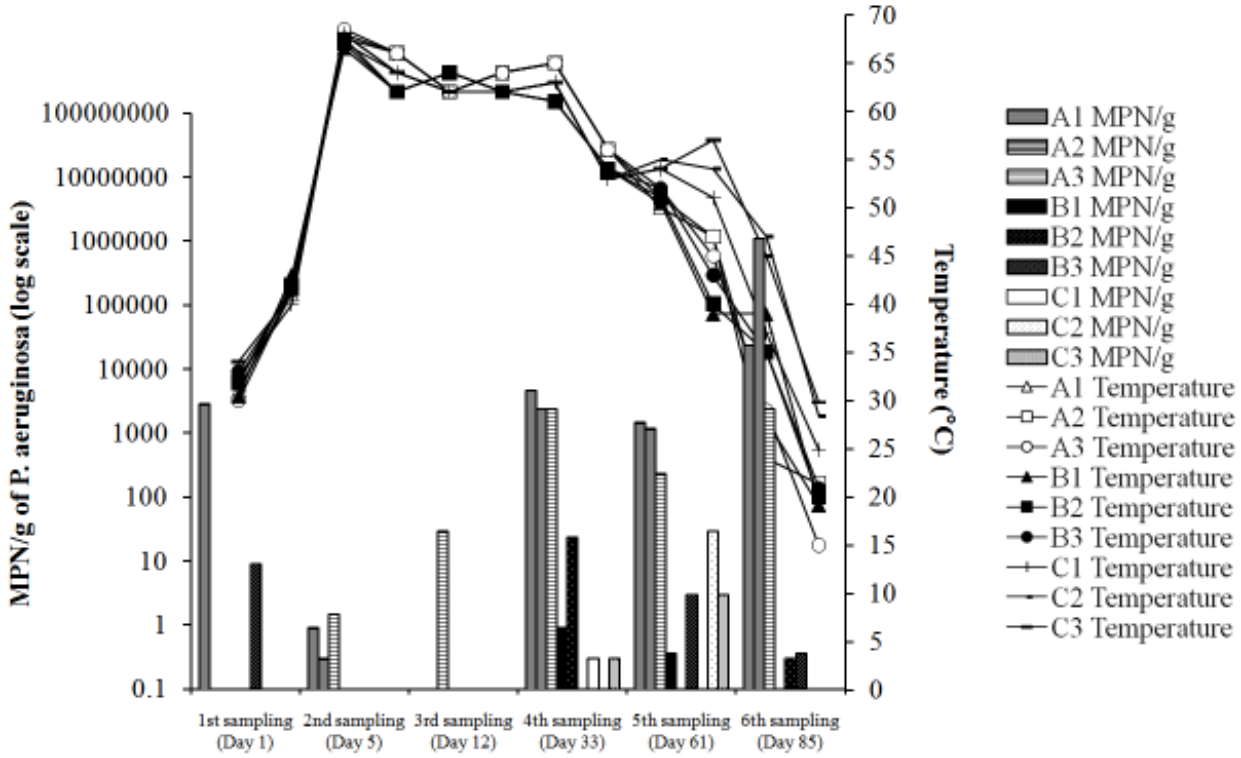


Figure 2. *P. aeruginosa* cell counts (log MPN), and changes of temperature (°C) during an open pile composting experiment (Kecskemét). Results of 3 parallel piles (A1-A3., B1-B3., C1-C3).

NEW SCIECTIFIC RESULT (according to the results of chapter 4.1.):

(Thesis No. 2.) Based on a composting experiment of previously unexamined raw materials (originated from heat power plants and biogas plants) we can conclude that species *P. aeruginosa* is capable of surviving the thermophilic phase and of reaching considerable cell counts in the final compost product even when specified temperature and time criteria of the relevant low are met. Our results were published in an international scientific journal (KASZAB ET AL., 2010a, KASZAB ET AL., 2010b).

4.2. Creation of culture collection

In our investigation period between 2002 and 2009, 36 strains of hydrocarbon contaminated sites and 25 compost related strains of *P. aeruginosa* were isolated and identified on a species level according to the Hungarian Standards and molecular genetic investigations. Isolated strains were preserved in a strain collection for further phenotypic and molecular genetic analysis.

4.3. Results of virulence assay

For the laboratory verification of virulence markers in the environmental strains of *P. aeruginosa* direct and indirect virulence factors were examined by genetic and traditional microbiological methods. Results of isolates originating from hydrocarbon contaminated sites are summarized in Table 3. Results of compost resultant strains can be seen in Table 4.

Table 3: Hemolytic activity and exoenzyme, exotoxin encoding gene frequency of *P. aeruginosa* strains isolated from hydrocarbon contaminated sites

Designation of strains	Origin	Hemolytic activity	Frequency of exoenzyme, exotoxinencoding gene sequences				
			exoS	*exoY*	*exoT*	*exoU*	*exoA*
			results of PCR				
ATCC 27853	Clin.	+	+	+	+	-	+
KPS-1	Clin.	+	-	+	+	+	-
KPS-2	Clin.	++	+	-	+	-	-
KPS-3	Clin.	+	-	+	+	+	+
KPS-4	Clin.	++	-	+	+	+	+
P2	Diósd	-	+	+	+	-	+
P9	Diósd	-	-	+	+	+	+
P10	Diósd	-	-	+	+	+	+
P11	Diósd	-	+	+	+	-	+
P14	Tököl	+/-	+	+	+	-	+
P15	Tököl	++	+	+	+	-	+
P16	Tököl	+/-	+	+	+	-	+
P17	Tököl	+	+	+	+	-	+
P18	Tököl	+/-	+	+	+	-	+
P22	Túrkeve	+	+	+	+	-	+
P28	Püspökladány	+	+	+	+	-	+
P30	Szabadszállás	-	+	+	+	-	+
P31	Komádi	+++	+	+	+	-	+
P32	Komádi	++	+	+	+	-	+
P33	Tököl	+++	+	+	+	-	+
P35	Szabadszállás	++	+	+	+	-	+
P36	Szabadszállás	+++	+	+	+	-	+
P37	Szabadszállás	+	+	+	+	-	+
P38	Ópusztaszer	+	+	+	+	-	+
P39	Algyő	+++	+	+	+	-	+
P42	Mezőtúr	+	+	+	+	-	+
P43	Ópusztaszer	+++	-	+	+	+	+
P45	Budapest I	-	+	+	+	-	+
P46	Budapest I	+	+	+	+	-	+
P49	Bátonyterenye	+++	+	+	+	-	+
P50	Bátonyterenye	+++	+	+	+	-	+
P53	Zalaegerszeg I	+	+	+	+	-	+
P62	Szarvas II	-	+	+	+	-	+
P65	Zalaegerszeg II	+	+	+	+	-	+
P66	Zalaegerszeg II	++	+	+	+	-	+
P69	Nagyszénás	++	+	+	+	-	+
P70	Nagyszénás	+++	-	+	+	+	+
P71	Debrecen	+	-	+	+	+	+
P77	Zalaegerszeg II	+	+	+	+	-	+
P78	Szarvas II	++	+	+	+	-	+
P79	Szarvas II	++	+	+	+	-	+

Clin.-clinical strains

Hemolytic activity tests: - no hemolysis; +/- doubtful hemolysis, + weak hemolysis ; ++ hemolysis; +++ intensive hemolysis;

PCR tests: + positive PCR reaction; - negative PCR reaction

Table 4. Hemolytic activity and exoenzyme, exotoxin encoding gene frequency of *P. aeruginosa* strains isolated from compost

Designation of strains	Origin	Hemolytic activity	Frequency of exoenzyme, exotoxinencoding gene sequences				
			exoS	*exoY*	*exoT*	*exoU*	*exoA*
			results of PCR tests				
ATCC 27853	Clin.	+	+	+	+	-	+
KPS-1	Clin.	+	-	+	+	+	-
KPS-2	Clin.	++	+	-	+	-	-
KPS-3	Clin.	+	-	+	+	+	+
KPS-4	Clin.	++	-	+	+	+	+
K1	Őrbottyán	+	+	+	+	-	+
K2	Őrbottyán	+++	+	+	+	-	+
K3	Őrbottyán	++	-	-	-	-	+
K4	Őrbottyán	++	-	+	+	-	+
K5	Őrbottyán	+	+	+	+	-	+
K13	Kecskemét	+++	-	-	-	-	+
K15	Kecskemét	+++	+	+	+	-	+
K16	Kecskemét	-	+	+	+	-	+
K19	Kecskemét	++	+	+	+	-	+
K20	Kecskemét	-	+	+	+	-	+
K21	Kecskemét	++	+	+	+	-	+
K22	Kecskemét	+++	+	+	+	-	+
K23	Kecskemét	+++	+	+	+	-	+
K24	Kecskemét	+++	+	+	+	-	+
K25	Kecskemét	++	-	-	+	-	+
K26	Kecskemét	+++	+	+	+	-	+
K29	Kecskemét	+++	-	-	+	-	+
K30	Kecskemét	++	+	+	+	-	+
K31	Kecskemét	++	+	+	+	-	+
K32	Kecskemét	+	+	+	+	-	+
K35	Kecskemét	++	+	+	+	-	+
K37	Kecskemét	+++	-	-	+	-	+
K38	Balatonfűzfő	+	+	+	+	-	+
K39	Balatonfűzfő	+++	+	+	+	-	+
K40	Balatonfűzfő	++	+	+	+	-	+

Clin.-clinical strains

Hemolytic activity tests: - no hemolysis; +/- doubtful hemolysis, + weak hemolysis ; ++ hemolysis; +++ intensive hemolysis;

PCR tests: + positive PCR reaction; - negative PCR reaction

Results of hemolytic activity tests

During our investigations it was established that 80.3% of environmental *P. aeruginosa* strains shows hemolytic activity with different intensity. This means that about four-fifths of the examined strains have the ability to damage erythrocytes which is a direct virulence factor. The different levels of detected hemolytic activity can be seen in Figure 3. and Figure 4.

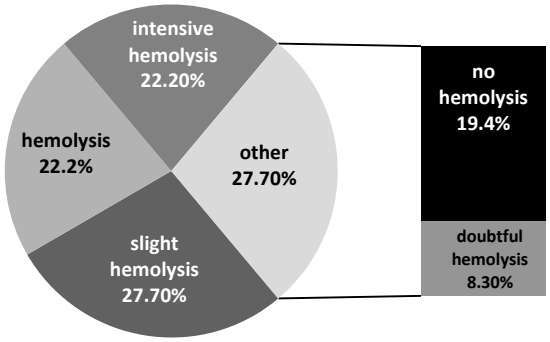


Figure 3. *Hemolytic intensity among P. aeruginosa isolated from hydrocarbon contaminated environments*

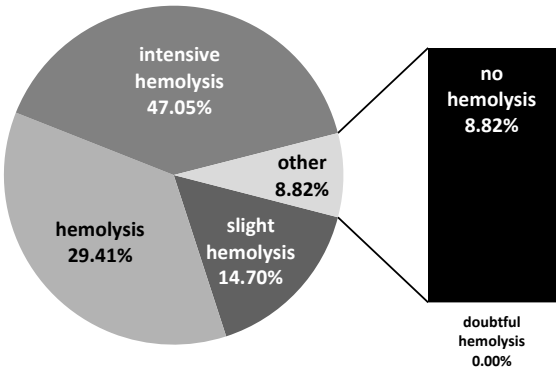


Figure 4. *Hemolytic intensity among P. aeruginosa isolated from compost*

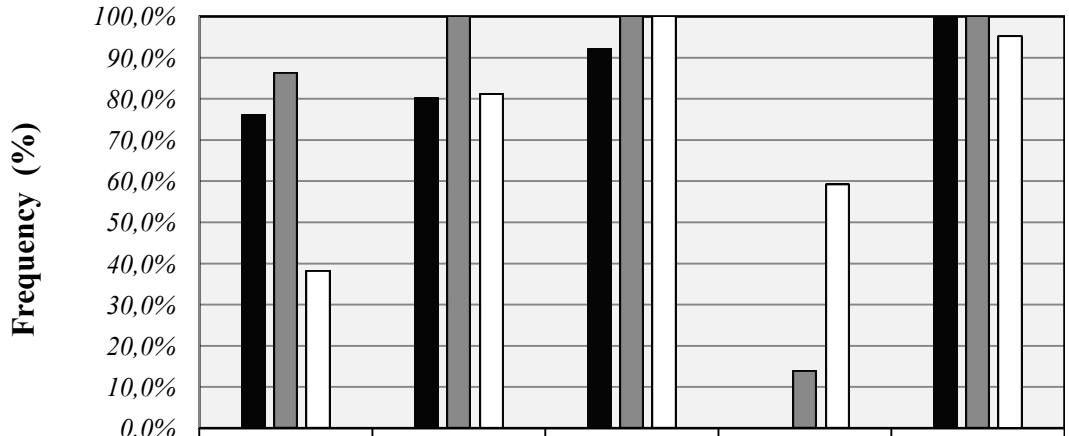
NEW SCIECTIFIC RESULT (according to results of chapter 4.3.):

(Thesis No. 3.) According to hemolytic activity tests it can be concluded that 72.1% of the 36 strains, isolated from hydrocarbon contaminated sites, and 91.1% of the 25 compost originated strains showed beta-hemolysis. Our results from the hydrocarbon contaminated site resultant strains were published in a national scientific journal (KASZAB ET AL., 2010c).

Results of exotoxin and exoenzyme encoding gene sequences

In the case of the examined indirect virulence determinants, namely gene sequences which are responsible for the production of toxins and toxic proteins of Type II and Type III Secretions System (*exoA, exoU, exoS, exoT, exoY*), we concluded that these gene sequences are commonly detectable in the genomic DNA of the examined environmental strains. In most cases at least two different gene sequences were present simultaneously.

The detection rates of the examined genes from strains, isolated from hydrocarbon contaminated sites were as follows: *exoA*: 100.0%; *exoS*: 86.2%; *exoY*: 100.0%; *exoT*: 100.0% and *exoU*: 13.8%. The detection rates for compost originating strains were: *exoA*: 100.0%; *exoS*: 76.0%; *exoY*: 80.0%; *exoT*: 92.0%; *exoU*: 0.0% (see Figure 5.).



	exoS	exoY	exoT	exoU	exoA
Strains isolated from composts	76,0%	80,0%	92,0%	0,0%	100,0%
Strains isolated from hydrocarbon contaminated sites	86,2%	100,0%	100,0%	13,8%	100,0%
Clinical strains*	38,0%	81,0%	100,0%	59,0%	95,0%

Figure 5. *The frequency of exotoxin and exoenzyme encoding gene sequences among environmental and clinical strains of P. aeruginosa*
**scientific literature sources*

NEW SCIECTIFIC RESULT (according to the results of chapter 4.3.):

(Thesis No. 4.) The majority (96.7%) of the environmental *P. aeruginosa* isolates carrying at least two different virulence genes and theoretically capable for producing toxic proteins. In the case of compost originated strains our results were published in international scientific journal (KASZAB ET AL., 2010b).

Comparison of virulence and antibiotic resistance results of environmental strains to clinical data

According to the available scientific sources *exoS* and *exoT* genes are detectable mainly among *P. aeruginosa* strains with invasive characteristics. Isolates with citotoxic effects mostly lose their *exoS* gene sequence while simultaneously preserving *exoT*. Therefore citotoxicity of the non-invasive strains is attributed to the presence and activity of *exoU*. Based on our investigations and according to their genetic properties and the literature sources, the majority of environmental isolates can be classified as invasive strains.

NEW SCIECTIFIC RESULT (according to results of chapter 4.3.):

(Thesis No. 5.) *P. aeruginosa* strains, isolated from environmental and clinical surroundings, show no major differences in the frequency of *exoA*, *exoY* and *exoT*. *ExoU* is less frequent, than in clinical environments, while *exoS* gene sequence seems to be dominant. 100.0% of the compost originated strains and 86.2% of strains isolated from hydrocarbon contaminated environments are classified as invasive. At the same time, 13.8% of strains originating from hydrocarbon contaminated sites are related to the cytotoxic group. Our results, according to the compost originated strains, were published in an international journal (KASZAB ET AL., 2010a, KASZAB ET AL., 2010b).

4.4. Results of antibiotic resistance investigations

Results of antibiotic resistance tests of environmental strains are detailed in Table 5. and Table 6. The applied 10 antibiotic agents that are important in the clinical therapy of *P. aeruginosa* varied in effectiveness. Agents that lost their effectiveness were mostly third generation Cephalosporins, Carbapenems, wide spectrum Penicillins and, in some cases, Aminoglycosides. ESBL and MBL production, which is responsible for the inefficiency of Beta-lactam antibiotics, was phenotypically verified in 4 environmental isolates.

Table 5. Minimal Inhibitory Concentration values of *P. aeruginosa* strains isolated from hydrocarbon contaminated sites

		Group	third generation Cephalosporins				fourth generation Cephalosporins	wide spectrum Penicillins	Carbapenems	Fluoroquionolones		Aminoglycosides
		Name	cefoperazone/ sulbactam	cefotaxime	ceftazidime	ceftriaxone	cefepime	piperacillin	imipenem	ciprofloxacin	ofloxacin	gentamicin
		Ranges of test strips (µg/ml)	0.016-256	0.016-256	0.016-256	0.016-256	0.016-256	0.016-256	0.002-32	0.002-32	0.002-32	0.016-256
Designation	Origin	No. of sampling sites	Minimal Inhibitory Concentration (MIC) values									
ATCC 27853	Clin.	-	6	24	1.5	>256	3	4	6	0.75	6	1.5
KPS-1	Clin.	-	12	16	0.38	24	1.5	6	>32	0.19	1.5	1.5
KPS-2	Clin.	-	4	>256	12	>256	2	16	4	0.125	1.5	1.5
KPS-3	Clin.	-	2	8	4	24	1	3	1	0.94	1	4
KPS-4	Clin.	-	12	>256	24	>256	8	>256	1.5	0.94	0.5	>256
P2	Diósd	14	1.5	8	1	6	0.75	6	4	0.032	0.5	1.5
P9	Diósd	14	2	8	0.75	6	2	4	>32	0.047	0.38	2
P10	Diósd	14	1.5	8	1	3	1.5	2	1	0.032	0.16	2
P11	Diósd	14	3	>256	32	>256	1.5	64	3	0.047	0.5	3
P14	Tököl	45	8	>256	>256	>256	4	4	0.38	0.125	2	8
P15	Tököl	45	1.5	12	1	32	2	3	1.5	0.094	0.5	3
P16	Tököl	45	3	>256	1.5	48	2	4	>32	0.125	1	2
P17	Tököl	45	2	>256	1.5	24	2	4	3	0.125	0.75	3
P18	Tököl	45	2	3	0.75	4	0.75	1.5	0.25	0.047	0.25	1
P22	Túrkeve	46	2	12	1	48	4	4	0.5	0.125	1.5	2
P28	Püspökladány	33	6	12	1	>256	6	4	>32	0.38	2	2
P30	Szabadszállás	35	3	16	1.5	>256	4	6	>32	0.125	0.75	3
P31	Komádi	25	4	24	1	>256	3	16	2	0.094	2	2
P32	Komádi	25	8	>256	8	>256	3	48	>32	0.064	4	1.5
P33	Tököl	45	2	32	8	>256	4	6	3	0.16	2	3
P35	Szabadszállás	35	12	12	2	>256	4	8	6	0.094	0.75	3
P36	Szabadszállás	35	2	12	1.5	>256	3	4	>32	0.94	3	1.5
P37	Szabadszállás	35	3	16	3	>256	3	6	6	0.094	1	2
P38	Ópusztaszer	31	3	12	1.5	12	6	8	3	0.38	4	1.5
P39	Algyő	1	3	16	1	>256	6	16	>32	0.25	6	2
P42	Mezőtúr	29	1	12	1.5	8	0.5	8	1	0.032	2	1.5
P43	Ópusztaszer	31	16	>256	64	>256	24	>256	>32	0.125	2	>256
P45	Budapest I	5	6	>256	24	>256	4	16	>32	0.045	0.75	1
P46	Budapest I	5	6	>256	8	>256	3	>256	>32	0.064	3	2
P49	Bátonyterenye	3	3	>256	2	32	3	16	1	0.25	1.5	2
P50	Bátonyterenye	3	3	24	1.5	>256	2	8	3	0.16	2	3
P53	Zalaegerszeg I	47	2	>256	1	>256	4	6	>32	0.125	3	2
P62	Szarvas II	37	2	16	2	16	0.75	8	2	0.047	1	2
P65	Zalaegerszeg II	48	2	12	1.5	16	6	1.5	3	0.094	1	1.5
P66	Zalaegerszeg II	48	1.5	8	6	48	1.5	8	3	0.125	0.75	3
P69	Nagyszénás	30	24	>256	24	>256	4	>256	>32	0.19	>32	2
P70	Nagyszénás	30	2	>256	1.5	>256	3	8	1.5	0.094	3	2
P71	Debrecen	12	2	16	2	48	3	>256	3	0.064	1.5	2
P77	Zalaegerszeg II	48	4	12	1.5	16	3	4	3	0.094	0.75	1.5
P78	Szarvas II	37	2	16	1.5	12	2	4	2	0.047	0.75	3
P79	Szarvas II	37	4	12	2	16	1.5	4	2	0.125	1	1.5

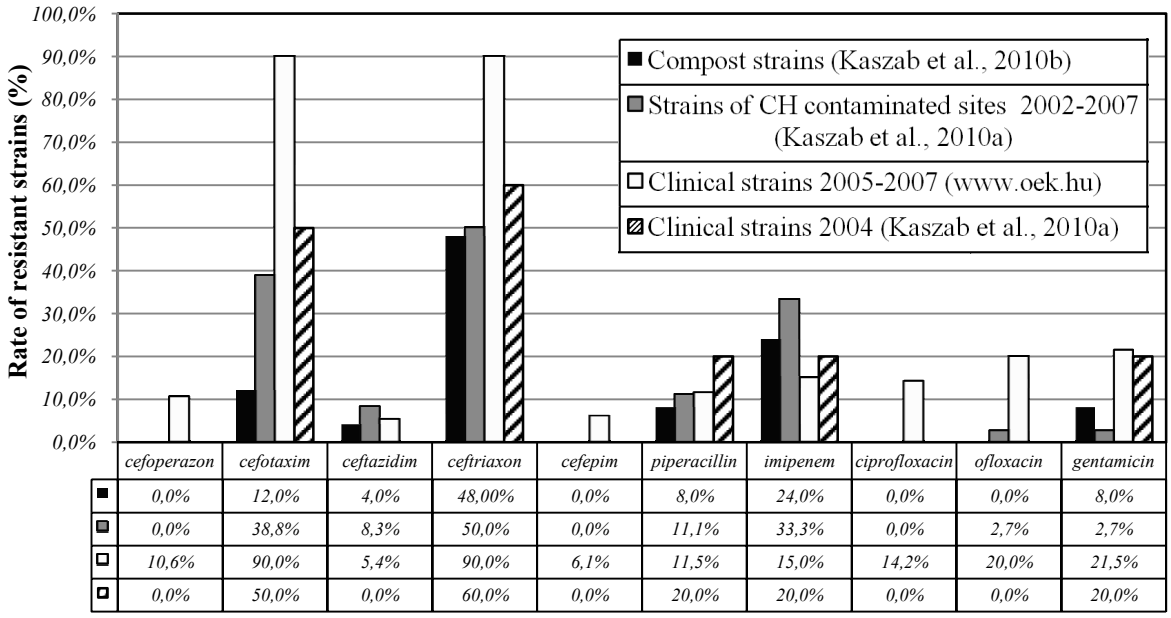
CLIN – clinical strains, grey colored – resistance (CLSI)

Table 6. Minimal Inhibitory Concentration values of *P. aeruginosa* strains isolated from composts

Group			third generation Cephalosporins				fourth generation Cephalosporins	wide spectrum Penicillins	Carbapenems	Fluoroquionolones		Aminoglycosides
Name			cefoperazone/ sulbactam	cefotaxime	ceftazidime	ceftriaxone	cefepime	piperacillin	imipenem	ciprofloxacin	ofloxacin	gentamicin
Ranges of test strips (µg/ml)			0.016-256	0.016-256	0.016-256	0.016-256	0.016-256	0.016-256	0.016-256	0.016-256	0.016-256	0.016-256
Designation	Origin	No. of sampling sites	Minimális Gátló Koncentráció (MIC) értékek									
ATCC 27853	Clin.	-	6	24	1.5	>256	3	4	6	0.75	6	1.5
K1	CTS	20	8	16	1.5	256	3	6	4	0.19	1.5	1.5
K2	CTS	20	3	8	1.5	64	2	3	32	0.094	0.75	1.5
K3	CTS	20	0,5	8	1.5	48	1.5	6	32	0.047	1.0	1.5
K4	CTS	20	1.5	256	24	256	2	256	1.5	0.094	1.5	3
K5	CTS	20	4	16	1.0	192	1.5	32	32	0.064	0.5	0.19
K13	RM	8	3	48	2	128	6	12	2	1.0	1.5	16
K15	OP	9	4	12	3	64	1.5	4	3	0.064	0.75	2
K16	OP	9	3	16	1.5	256	1.5	4	1.5	0.125	0.5	2
K19	OP	9	2	24	3	24	3	12	1.5	0.064	1.5	8
K20	OP	9	3	32	1.0	256	1.5	3	1.0	1.5	1.0	4
K21	OP	9	2	8	3	32	1.0	3	1.5	0.125	1.0	8
K22	OP	9	3	16	2	24	3	4	3	1.19	0.75	4
K23	OP	9	2	8	1.5	64	1.0	4	1.5	0.5	1.5	4
K24	OP	9	1.5	12	1.0	256	1.5	4	2	0.125	1.0	95
K25	OP	9	1.5	12	0.75	16	0.75	3	3	0.064	1.0	1.0
K26	OP	9	6	24	2	24	2	4	4	0.094	0.75	3
K29	OP	9	4	24	1.5	48	1.0	6	32	0.25	2	2
K30	OP	9	3	24	1.0	48	1.0	6	1.5	0.125	1.0	4
K31	OP	9	3	8	1.5	16	1.0	4	32	0.19	1.0	2
K32	OP	9	2	48	1.5	32	1.5	4	32	0.125	2	0.75
K35	OP	9	3	12	1.0	8	1.0	3	1.0	0.064	0.75	2
K37	OP	9	2	256	1.0	256	1.5	4	1.5	0.064	0.5	3
K38	ASP	16	3	24	1.5	8	1.0	6	2	0.125	1.0	2
K39	ASP	17	4	256	64	256	12	256	2	0.125	0.75	4
K40	ASP	19	6	16	4	24	1.5	12	2	0.064	0.5	4

CLIN – clinical strains; RM – raw material; OP – open pile, ASP – aerated static pile, CTS – compost treated soil; grey colored – resistance (CLSI)

Based on our antibiotic resistance investigations it was established that the importance of the issue is not lower in the environment than in clinical surroundings. According to the comparison of the available data from the National Epidemiological Centre, the fact of environmental resistance without direct selection pressure (antibiotic therapy) was verified (see Figure 6.).



	cefoperazon	cefotaxim	ceftazidim	ceftriaxon	cefepim	piperacillin	imipenem	ciprofloxacin	ofloxacin	gentamicin
■	0,0%	12,0%	4,0%	48,00%	0,0%	8,0%	24,0%	0,0%	0,0%	8,0%
▩	0,0%	38,8%	8,3%	50,0%	0,0%	11,1%	33,3%	0,0%	2,7%	2,7%
□	10,6%	90,0%	5,4%	90,0%	6,1%	11,5%	15,0%	14,2%	20,0%	21,5%
▨	0,0%	50,0%	0,0%	60,0%	0,0%	20,0%	20,0%	0,0%	0,0%	20,0%

Figure 6. *Rate of resistant strains of P. aeruginosa among clinical (2004; 2005-2007) and environmental (2002-2007) isolates (Kaszab et al., 2010a, Kaszab et al., 2010b)*

17 environmental strains of *P. aeruginosa* were detected that were resistant to two or more different groups of antibiotics. It was verified that multiresistance is detectable among microbial populations of geological media, subsurface water and compost was verified.

NEW SCIECTIFIC RESULT (according to results of chapter 4.4.):

(Thesis No. 6.) Antibiotic resistance against 2 or more different classes of antibiotic agents that are in first choice for treatment of *P. aeruginosa* was detected in 11 strains of hydrocarbon contaminated sites and 6, compost originated *P. aeruginosa* isolates. Our results are the first verification of Aminoglycoside (P43) and Fluoroquinolone (P69) resistant *P. aeruginosa* strains from non clinical environments. Our results were published in scientific journals (KASZAB ET AL., 2010a, KASZAB ET AL., 2010b).

5. CONCLUSIONS AND SUGGESTIONS

Based on our results species *P. aeruginosa* is a prominent member of the examined media that are under anthropoenic effect, e.g. the special microbiota of hydrocarbon contaminated sites. Moreover, during composting, strains were capable of surviving the thermophilic phase which has been suggested as being able to eliminate mesophilic pathogens. Based on the detected cell counts, it can be established that the examined bacterial species is capable of mass growth when special environmental conditions (e.g. hydrocarbon contaminated media and compost) are present and can reach the lower threshold of an infectious dose. This result, besides its human-health risk concerns, means that this pathogenic species is able to become the outstanding member of a given environmental microbiota.

Within the frame of our present work we concluded, that the majority of environmental strains (over 90%) carry direct or indirect virulence markers that are presumably playing a role in infectious diseases. Based on our results the importance of antibiotic resistance among environmentally originating *P. aeruginosa* strains is not shown on the clinically experienced level; environmental strains are capable of showing multiresistant features. In addition, we proved the wide hydrocarbon degradation activity of clinical and environmental strains in previous investigations (KASZAB ET AL., 2006). Comprehensively we can conclude that the commonly accepted theory of the 'price of adaptation' (namely the loss or decrease of properties like vitality, virulence, degradation activity or antibiotic resistance as a result of specialization in extreme environmental conditions) should be revised.

The direct human-health concerns of our results are the knowledge of the antibiotic resistance and virulence features in the environmental strains of *P. aeruginosa* or other opportunistic species which can dictate the choice of a proper agent or therapy for infections.

However, therapy for multidrug resistant strains creates an almost unsolvable problem for the medical profession. Therefore, the prevention and delay of antibiotic resisastance is of high priority. Based on our results the appearance of hyper-resistant environmental strains is not just a possibility for the distant future but outdoor multiresistance is already present.

The origins of multiple drug resistance have not yet been determined but can be attributed to various sources. One source could be the selection pressure of antibiotics

excreted by the human body and appear in the environment via several routes including sewage. In the environment these excreted agents can increase the antibiotic resistance of bacterial species. The application of antibiotics for agricultural purposes and the antibiotic content of agricultural wastes, manure or compost can lead to similar consequences.

It is also presumable that clinical strains, which are under a higher pressure of antibiotics, therefore usually have a higher rate of resistance, can emerge in the environment via patients, health workers or visitors.

The effects of spontaneous mutations and the spread of resistance genes encoded in transferable genetic elements are also probable. The later opportunity is demonstrated by the increased resistance of environmental strains to azlocillin and piperacillin agents that are mainly attributable to acquired Beta-lactamases. Horizontal gene transfer (such as plasmid mediated processes) is also possible, but this potential between environmental and clinical strains of *P. aeruginosa* is still not proven and needs further investigations. The possible ways of spreading antibiotics and resistant strains of *P. aeruginosa* are summarized in Figure 7.

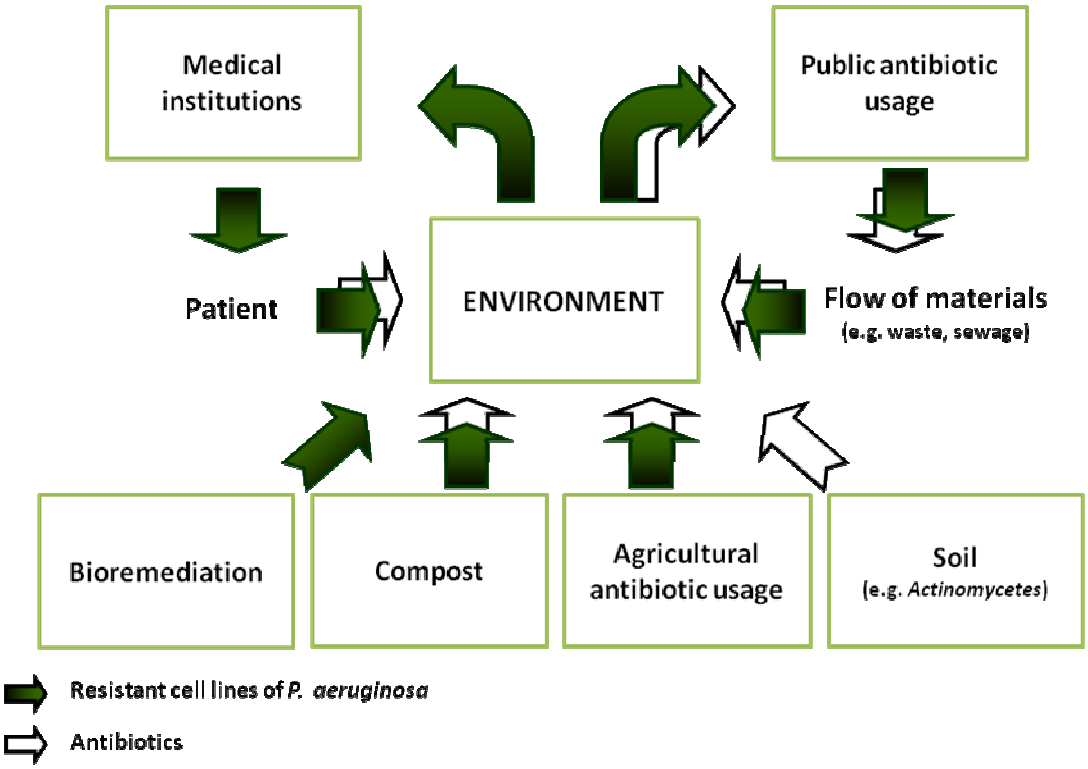


Figure 7. The possible ways for development and spread of multiresistance in the environment (KASZAB ET AL., 2010A)

In Hungary, multiresistance isolates have been detected only in the last few years, therefore, we can expect increasing rates of antibiotic resistant strains of *P. aeruginosa* in both clinical and non-clinical surroundings. In the future, changes in antibiotic resistance must be monitored continuously, in hospitals and the environment as well. The isolation of further strains from environmental samples can also help us draw a more reliable picture about the spread of antibiotic resistance features.

From a practical aspect, our results support the evidence to revise commonly applied biodegradation procedures where intervention causes the mass growth of unidentified members of natural microbiota or uses unidentified inocula. Via these procedures *P. aeruginosa* and other pathogens, that are possibly resistant to antibiotics, are able to proliferate and reach the infectious dose.

Based on the above mentioned results the strict national regulation for bioremediation processes [16/2002 (IV.10.) statute of Ministry of Health] is necessary. The recognition and verification of the influence of pathogenic microorganisms on environmental safety will hopefully encourage the development of international law. Meanwhile, pathogen microorganisms ought to be eliminated from working processes as efficiently as possible for the protection of workers of remediation, especially in the case of inocula with high microbial cell counts.

Regarding possible effects on natural microbiota it can be concluded that virulence determinants of species *P. aeruginosa* (such as hemolytic activity and the presence of several virulence genes encoding exotoxins and exoenzymes) and antibiotic resistance gene sequences are commonly encoded on mobile genetic elements. These sequences usually have different G+C compositions than the other parts of the bacterial genome that emphasize the importance of horizontal gene transfer. It suggesting that virulent and multiple drug resistant strains of *P. aeruginosa* can create a reservoir of genes encoding these properties and can spread these mechanisms among other members of the natural microbial ecosystem. If this hypothesis is sound, the virulent and antibiotic resistant environmental isolates can directly or indirectly endanger human health and the present form of the microbial ecosystem which can lead to adverse changes.

To eliminate these harmful effects the above mentioned continuous monitoring of hydrocarbon contaminated sites, the control of pathogenic microorganisms and the conformation to provisions of law is strongly recommended. In the case of composting the suggested steps are the strict application of technical specifications, and in any given situation, the choice of processes ensuring higher level of hygienization. The relevant

laws for the quality of composts, in particular the 23/2003. (XII.29.) statute of the Ministry of Rural Development, do not have a limit value for *P. aeruginosa* in final compost products. Based on our results and in light of the international advisory opinions this possibility should be examined.

6. PUBLICATIONS

Journal articles:

Kaszab, E., Kriszt, B., Atzél, B., Szabó, G., Szabó, I., Harkai, P., Szoboszlay, S. (2010a): The occurrence of multidrug resistant *Pseudomonas aeruginosa* on hydrocarbon contaminated sites. *Microbial Ecology*, 59 (1): 37-45. [IF(2009): 3.251]

Kaszab, E., Szoboszlay, S., Dobolyi, Cs., Háhn, J., Pék, N. & Kriszt, B. (2010b): Antibiotic resistance profiles and virulence markers of *Pseudomonas aeruginosa* strains isolated from composts. *Bioresource Technology*, doi: 10.1016/j.biortech.2010.08.027 [IF(2008): 4.253]

Kaszab E., Pék N., Farkas M., Kriszt B. & Szoboszlay S. (2010c): Környezeti eredetű *Pseudomonas aeruginosa* törzsek virulenciájának vizsgálata. *Tájökológiai Lapok*, 8 (1) 135-146.

Kaszab, E., Bedros, J. R., Szoboszlay, S., Atzél, B., Szabó, I., Cserháti, M., Kriszt, B. (2006): Problems with environmental safety on bioremediated sites. *AARMS, (Academic and Applied Research in Military Science)*, 5 (3) 383-397.

Proceedings:

Szabó, I., Háhn, J., Harkai, P., Kaszab, E., Szoboszlay, S. (2009): Effects of heavy metal components on hydrocarbon degrading bacteria as abiotic stress on hydrocarbon contaminated sites. Cereal Research Communications 37 (Suppl 4): 561-564.