”BABEŞ-BOLYAI” UNIVERSITY
OF CLUJ-NAPOCA

Faculty of Environmental Sciences and Engineering

CONTRIBUTIONS REGARDING THE INFLUENCE OF ANTIBIOTICS ON SOME ENVIRONMENTAL FACTORS

- PhD THESIS SUMMARY -

PhD coordinator:
Prof. Univ. Dr. Dumitru Ristoiu

PhD student:
Ocsana-Ileana Axuc (Opriș)

CLUJ-NAPOCA - 2012

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Fondul Social European POSDRU 2007-2013
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November, 2012

CONTENT

The summary of the thesis presents some of the results of the experimental research, conclusions and the selective bibliography. The same notations for chapters, figures, tables used in the text of the thesis were kept in drawing of the summary.

***KEYWORDS**: antibiotics, solid phase extraction, liquid chromatography, Pseudokirchneriella subcapitata L., cell density, Triticum aestivum L., photosynthetic parameters, assimilating pigments, volatile organic compounds, flavonoids.*

INTRODUCTION

THE IMPORTANCE AND THE ACTUALITY OF THE APPROACHED TOPIC

In the present study, the compounds of interest are antibiotics, a class of drugs widely used in medicine and related fields. Chemically, antibiotics are a wide variety of organic substances that are produced by microorganisms or obtained by synthesis and semisynthesis. In very small doses, antibiotics inhibit growth of pathogenic microorganisms or even can lead to their destruction (Choma, 2004; Oniscu, 1988).

Antibiotics were detected in various environmental compartments such as surface water, groundwater, drinking water, soil and wastewater (Seifrtová et al., 2009). Because of the inefficiency of the treatment processes, wastewater treatment plants and wastewaters generated by hospitals are major contributors for the presence of pharmaceuticals in the environment. Further, these types of wastewaters can reach groundwater, surface water and may lead to potential risks to aquatic organisms. The interest regarding the persistence and evolution of antibiotics in the environment is caused by the fact that these drugs can increase the resistance of pathogenic bacteria. The use of antibiotics as animal growth promoters was associated with the increasing of antibiotic resistance, which can lead to potential impact on human health. Use of antibiotics for such purposes leads to their adsorption on sediments, and soil (Seifrtová et al., 2009).

For preventig the antibiotic resistance it is necessary to control their consumption, to monitor them, to folow their evolution and to eliminate them from the environment. Due to the increasing resistance of human body to antibiotic treatments, and many side effects that can be caused by antibiotics, and because at national level are a few studies regarding this topic, the issue of antibiotics into environment is very important. Thus, the importance of the theme *"Contributions regarding the influence of antibiotics on some environmental factors"* is obvious, because in small amounts and prolonged intake, antibiotics can have negative effects on the environment and humans.

The present study is aimed to bring contributions regarding the development of procedures for determination of antibiotics from surface water and wastewater samples and highlight their negative influences on green algae (*Pseudokirchneriella subcapitata* L.) and wheat plants (*Triticum aestivum* L.). Nine antibiotics belonging to five different classes: penicillins (amoxicillin, ampicillin and penicillin G), cephalosporins (ceftazidime and ceftriaxone), tetracyclines (tetracycline and doxycycline), fluoroquinolones (ciprofloxacin) and macrolides (erythromycin) were selected for the proposed research.

This PhD thesis contains introduction, five chapters divided in two parts (theoretical notions and original contributions), followed by conclusions and references.

Thus, in the first three chapters are presented general aspects regarding to antibiotics, the main sources of environmental pollution with antibiotics, preventive measures of environmental contamination with antibiotics, and aspects regarding their ecotoxicology. Also, the first part of the thesis provides an overview on the most appropriate and used techniques for isolation/concentration, identification and quantification of antibiotics from wastewater samples and environmental samples.

Original contributions (working procedure and interpretation of experimental results) are presented in the second part of the thesis. Chapter 4 includes the presentation of two procedures that were developed for determination of antibiotics from surface water samples (Someşul Mic River, Cluj-Napoca) and wastewater samples (collected from a clinical hospital and from a wastewater treatment plant). Isolation/concentration of the investigated antibiotics from different samples was performed using solid

phase extraction technique (SPE), and their identification and quantification were performed using two techniques corresponding to each procedure developed: high performance thin layer chromatography (HPTLC) and high performance liquid chromatography coupled with diode array detector (DAD) and mass spectrometry (MS). For the procedures developed SPE-HPTLC and SPE-HPLC-DAD/MS, studies of selectivity, linearity, limits of detection and of quantification, precision and accuracy were performed. Also, in Chapter 4 physicochemical characteristics of the investigated wastewater samples were determined for comparing the two types of wastewater in terms of organic pollutants.

In the last chapter of the thesis the results obtained from the experimental investigations regarding the influence of antibiotics on cell density of green algae (*Pseudokirchneriella subcapitata* L.) and wheat plants (*Triticum aestivum* L.) are presented. Because algae are considered to be the basis of the food chain and light decreases in the population of algae can affect the balance into an aquatic system, in this research, the influence of antibiotics on algae could not be excluded.

The tests on wheat plants (*Triticum aestivum* L.) was performed in order to obtain a perspective regarding the negative effects of antibiotics and to identify the most suitable characteristics for a rapid assessment of their toxicity. Thus, are presented the effects of antibiotics on the photosynthetic parameters (electron transport rate, net assimilation rate, stomatal conductance to water vapor), volatile organic compounds (lipoxygenase pathway products and monoterpenes), assimilating pigments (chlorophylls and carotenoids) and total flavonoid content in wheat plants.

The results obtained were presented at scientific manifestations and also published/accepted/submitted for publication in different journals (ANNEX II).

Because antibiotic residues from the environment are suspected to induce resistant bacterial strains, causing a serious threat to public health (human body, animal respectively, creates resistance to certain types of antibiotics), developing procedures for determination of antibiotics from environmental samples and assessing the impact of antibiotics and their metabolites on the environment is a necessity.

PART II. ORIGINAL CONTRIBUTIONS

Chapter 4. Researches regarding the development of procedures for the determination of antibiotics from surface water and wastewater samples

According to the literature, the extraction of antibiotics from different samples is performed using solid phase extraction technique (Ašperger et al., 2006; Feitosa-Felizzola et al., 2007; Kasprzyk-Hordern et al., 2008; Mutavdžić et al., 2006) followed by their identification and quantification using different techniques such as capillary electrophoresis (Ašperger et al., 2006; García-Campaña et al., 2009), thin layer chromatography (Ašperger et al., 2006; Joshi et al., 2009; Mutavdžić et al., 2006) and high performance liquid chromatography (Benito-Peña et al., 2006; Feitosa-Felizzola et al., 2007; García-Galán et al., 2010).

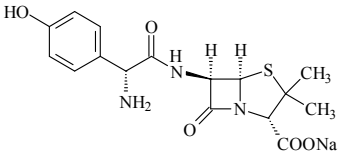
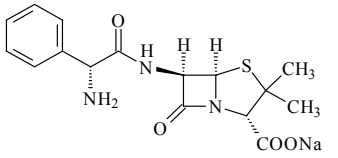
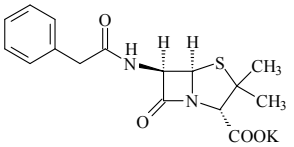
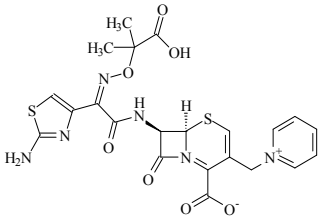
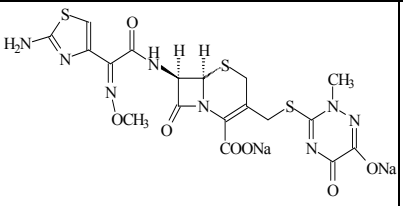
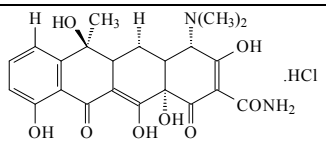
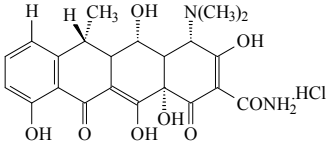
In the present doctoral study, two procedures were developed in order to determine the antibiotics from surface water and wastewater samples using liquid chromatography technique.

4.1. Selected antibiotics and their main characteristics

According to the classes of antibiotics that were determined in environmental samples and reported in the literature, in our case were selected most common seven antibiotics used to treat or

prevent various diseases. These antibiotics were purchased from different manufacturers in different presentation modes (powder for injection, capsules or tablets). In all experiments that were conducted during the doctoral studies, these antibiotics were used as references. Table 4.2 contains the main characteristics (chemical formula, molecular weight, structural formula and mode of action) of the studied antibiotics.

Table 4.2. Chemical formula, molecular weight, structural formula and mode of action of studied antibiotics.

Antibiotic	Chemical formula	Molecular weight (g mol^{-1})	Structural formula	Mode of action[1)]
AMOX	$C_{16}H_{18}N_3O_5SNa$	387.39		Bactericidal
AMP	$C_{16}H_{18}N_3O_4SNa$	371.39		Bactericidal
PENG	$C_{16}H_{17}N_2O_4SK$	372.48		Bactericidal
CFZ	$C_{22}H_{22}N_6O_7S_2$	546.58		Bactericidal
CFX	$C_{18}H_{16}N_8O_7S_3Na_2$	598.54		Bactericidal
TET	$C_{22}H_{24}N_2O_8$.HCl	480.89		Bacteriostatic
DOXY	$C_{22}H_{24}N_2O_8$.HCl	480.89		Bacteriostatic

[1)]Bactericidal antibiotics such as β-lactams (penicillins and cephalosporins) acts on the bacterial cell wall, inhibiting the peptidoglycan synthesis, an essential constituent of the cell wall. Bacteriostatic antibiotics such as tetracyclines inhibit protein synthesis, blocking the introduction of new amino acids in the polypeptide chain (Dahl et al., 2006; Matinca, 2002).

4.2. Analysis of antibiotics from surface water samples using high performance thin layer chromatography (HPTLC) technique

When in a matrix is present more than one antibiotic, compounds which can interact with each other, their separation prior to their determination is required. Thin layer chromatography is an ideal technique for the determination of different antibiotics because it is inexpensive, rapid, easy to use and maintenance, and has the possibility of simultaneously analysis of many samples (Hussain et al., 2004). Therefore, in our case this technique was used for determination of antibiotics (Table 4.2) from surface water samples.

4.2.1. Isolation/concentration of antibiotics from liquid matrices using solid phase extraction technique (SPE)

Working procedure

A stock solution of studied antibiotics (1 mg mL^{-1} of CFZ, CFX, TET and DOXY; 5 mg mL^{-1} of AMOX, AMP and PENG) was prepared by dissolving with methanol (MeOH), in a volumetric flask of 5 mL. The solutions of antibiotics were prepared in MeOH and stored in dark flasks. For the calibration curves the work solutions were prepared by dilutions of the stock solution with MeOH.

For the preparation of samples and mobile phase we used MeOH (≥99.9%, HPLC) and ethyl acetate purchased from Merck (Germany), and acetone from Microchim (Romania). Ethylenediamine tetraacetic acid disodium salt dihydrate ($Na_2EDTA{\cdot}2H_2O$, *noted* EDTA) was purchased from Fluka (Germany). Hydrochloric acid (HCl) from Poch (Poland) and sodium hydroxide (NaOH) from Merck (Germany) were used for the pH adjustement of the sample, and of the EDTA solution, respectively. Water of high purity used in all experiments was prepared using a Milli-Q Ultrapure water purification system (Millipore, USA).

The extraction of antibiotics from water matrices was carried out on SPE cartridges Oasis HLB (hydrophilic lipophilic balance, 6 mL, 200 mg) purchased from Waters (USA). The stationary phase is based on two monomers, one hydrophilic N-vinylpyrrolidone and one lipophilic divinylbenzene. This type of cartridge is suitable for the extraction of a wide range of antibiotics, because doesn't contain free silanol groups that compounds of interest can directly interact or form metal complexes (Feitosa-Felizzola et al., 2007). Another advantage of this stationary phase is that has excellent wetting properties due to the hydrophilic N-vinylpyrrolidone monomer (Gómez et al., 2006). High recoveries of a wide range of analytes are obtained even if it happens, in some cases, the stationary phase cartridge to remain dry during SPE extraction.

Working protocol followed for the extraction of studied antibiotics from liquid matrices consisted in several steps:

- stationary phase ***conditioning*** with 10 mL MeOH and 10 mL ultrapure water;
- ***passing a volume*** of 100 mL ***of sample*** through the extraction cartridge with a flow rate of 1.2 mL min^{-1};
- the ***elution*** of the antibiotics retained on the stationary phase was performed with 6 mL MeOH.

Before passing the sample through the cartridges, the samples were processed. This step consisted in adding 1 mL of aqueous solution of 5% EDTA in 100 mL sample volume to chelate the residual metals from water (Feitosa-Felizzola et al., 2007). The pH of the samples was adjusted at 3 with 0.5 N HCl.

The eluate was evaporated to dryness at 40ºC by a rotaryevaporator (Laborata 4000, Heidolph, Germany) and then solubilised in 0.5 mL MeOH. All extracts were performed three times and were evaluated by HPTLC.

Results and discussions

The recoveries (average of three extractions) of the target antibiotics obtained with Oasis HLB cartridges are presented in Figure 4.3 with the RSD values (%, relative standard deviation). The percent recoveries are over 80%, excepting AMP with a recovery of 64.5% (Opriș et al., 2012a). The addition of EDTA in water matrices was done because improves the recovery of tetracyclines (Granados et al., 2005). In our case for TET and DOXY were obtained recoveries of 90.9% and 99.5%, respectively (Figure 4.3).

The pH of the water samples adjusted below the acidity constant (*pKa*) value of antibiotics increases their retention on this type of SPE cartridge, Oasis HLB (Yang et al., 2005). The pKa values of the studied antibiotics according to the literature data are as follow: penicillins $pKa_1 \approx$ 2-3 and $pKa_2 \approx 7$ (Benito-Peña et al., 2006); CFZ $pKa_1 = 1{,}9$; $pKa_2 = 2{,}7$ and $pKa_3 \approx 4{,}1$ (Abounassif et al., 1990); CFX $pKa_1 = 1{,}7$; 3,1 and $pKa_2 = pKa_3 \approx 4{,}3$ (Nakai et al., 2010); tetracyclines $pKa_1 \approx 3$, $pKa_2 \approx 7$, $pKa_3 \approx 9$) (Batt and Aga, 2005; Seifrtová et al., 2009). Thus, to improve the extraction of the studied antibiotics from water samples, the pH was adjusted at value of 3.

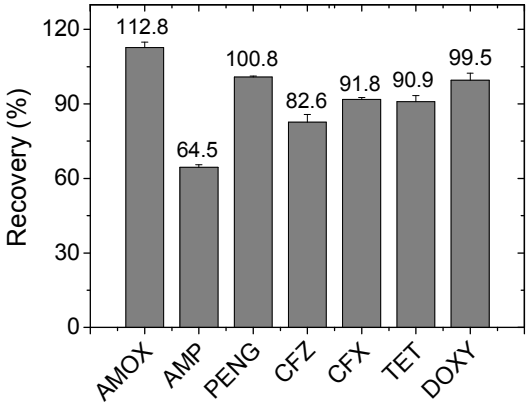


Figure 4.3. Mean recovery (± RSD, %, three determinations) of the selected antibiotics using Oasis HLB cartridges, pH 3.

4.2.2. Qualitative and quantitative analysis of antibiotics using the HPTLC technique

Working procedure

Chromatographic separation was performed on Alugram SIL G/UV_{254} (10 × 20 cm) plates coated with 0.20 mm of silica gel 60 with fluorescent indicator (Macherey-Nagel, Germany). Before applying the bands, the SIL G/UV_{254} plate was immersed in 10% EDTA solution adjusted at pH 7.5 value with 1N NaOH solution in order to avoid the formation of metal ion/tetracycline complexes (Meisen et al., 2010) and then dryed in a Memmert oven (Germany) for 30 min at 125ºC. By means of a Desaga AS 30 Applicator (Germany), 2 µL per band of each antibiotic solution and also water extracts were applied on the HPTLC plate as 5 mm bands. Further, the plate was developed 25 min by ascending technique to a distance of 8.5 cm in a Desaga twin-trough glass chamber. The glass chamber was previously saturated for 30 min with a quaternary mixture of ethyl acetate - methanol - acetone - water 5 : 2.5 : 2.5 : 1.5 (*v/v*) as mobile phase at 23ºC room temperature. After development, the plate was dried at room temperature under a hood and then scanned in absorbance mode under UV light at 254 nm using a Desaga CD-60 densitometer (Germany) (Opriș et al., 2012a).

Results and discussions

The good separation of the studied antibiotics can be observed in the densitogram illustrated in Figure 4.6, and is given by the chromatographic resolution (*Rs*) calculated from the densitometry scan. The *Rs* of two neighbouring bands was determined by the calculation of the ratio of the distance between the maximum of the bands and the average of the band widths at base (Fried and Sherma, 2005). The values of separation resolution obtained for the target antibiotics are in the range 2-8.59 ($Rs_{CFZ-CFX}$ 4.10; $Rs_{CFX-TET}$ 3.79; $Rs_{TET-AMOX}$ 2.56; $Rs_{AMOX-DOXY}$ 2.84; $Rs_{DOXY-AMP}$ 2.00; $Rs_{AMP-PENG}$ 8.59). According to theoretical concepts, to analyze a component in good condition, it must be separated from the components with a resolution $Rs \geq 2$ (Gocan, 2002).

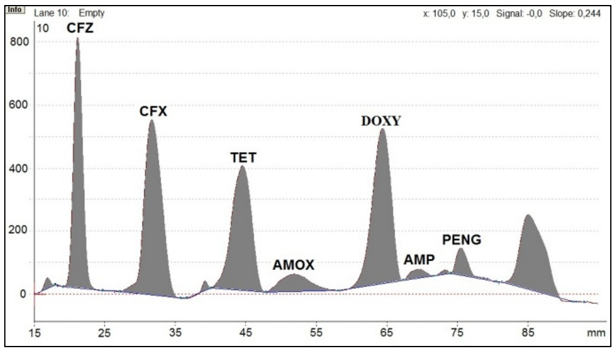


Figure 4.6. The densitogram of studied antibiotics.

4.2.3. Studies regarding the validation of SPE-HPTLC procedure

The antibiotics considered as references in the present study were medicines containing active substance (antibiotic) and excipients as admixture (Table 4.1). Therefore, the mass spectrum of each antibiotic was performed by HPLC-DAD/MS Shimadzu (Japan) in positive electrospray ionization mode, with capillary voltage of 1.5 kV, curved desolvation line temperature of 250°C and interface temperature of 250°C.

The results of HPLC-DAD/MS experiments in terms of molecular ion (*m*/*z*) were in agreement with those previously reported in literature: 366 for AMOX (Lindberg et al., 2004), 350 for AMP (Li et al., 2009; Lindberg et al., 2004; Pozo et al., 2006), 335 for PENG (Xu et al., 2010), 547 for CFZ (Abounassif et al., 1990), 555 for CFX (Kato et al., 2008), 445 for TET (Feitosa-Felizzola et al., 2007; Hu et al., 2010; Li et al., 2009), 445 for DOXY (Grujić et al., 2009). The mass spectra are presented in Section 4.3.2 where is described another procedure developed for the determination of antibiotics, but from wastewater samples.

In order to determine the studied antibiotics from surface water samples, the proposed SPE-HPTLC procedure was validated. The experimental data showed that the proposed SPE-HPTLC procedure is selective and presents good linearity, precision and accuracy. Also in this study were determined limits of detection and of quantification.

4.2.4. Determination of antibiotics from surface water samples using the SPE-HPTLC procedure

Working procedure

The surface water samples were collected in clean polyethylene bottles from Someşul Mic River that crosses Cluj-Napoca city. This river receives a variety of organic wastes from different industries, households and municipal wastewater effluents. Two samples of river water were dailly collected for a

period of one week, stored at 4°C in refrigerator and further the target antibiotics were extracted and analyzed in less than 24 hours from sampling.

Results and discussions

In Figure 4.12 is presented a HPTLC plate which was spotted with some river water extracts in order to determine the content of antibiotics. The plate presents the separation of the seven antibiotic references spotted individually (positions 1-7) and in mixture (positions 8 and 17), and also the river water samples (positions 9-12) and the spiked river water samples (positions 13-16). (Opriş et al., 2012a).

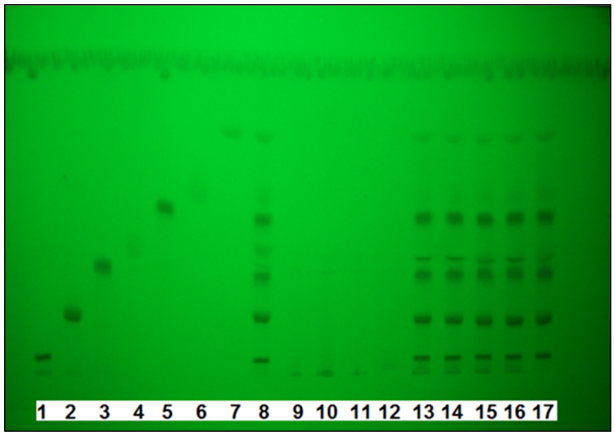


Figure 4.12. HPTLC chromatoplate of the studied antibiotics. Positions 1-7, individual references: 1-CFZ, 2-CFX, 3-TET, 4-AMOX, 5-DOXY, 6-AMP, 7-PENG; Positions 8 and 17, reference antibiotic mixture; Positions 9-12, river water samples; and Positions 13-16, spiked river water samples.

In Figure 4.13 are presented the overlapped densitograms of the reference mixture of studied antibiotics (17) and of the spiked river water sample (14).

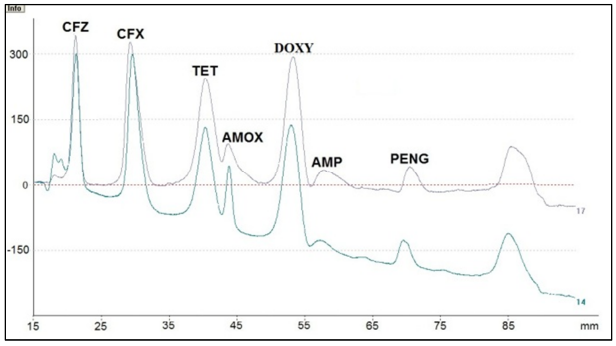


Figure 4.13. The overlapped densitograms of the reference mixture of studied antibiotics (17) and of the spiked river water sample (14).

In Table 4.7 are given the antibiotic concentrations which were determined in river water samples. In the analyzed samples only two antibiotics were detected at different concentration levels: 2115-2249 μg L^{-1} AMOX and 55-78 μg L^{-1} TET (Opriş et al., 2012a).

Table 4.7. Amounts (μg L^{-1}) of studied antibiotics in surface water samples collected from Someşul Mic River, Cluj-Napoca.

Sample no.	Amount (μg L^{-1})						
	AMOX	**AMP**	**PENG**	**CFZ**	**CFX**	**TET**	**DOXY**
1	2201	nd	nd	nd	nd	nd	nd
2	nd[1)]	nd	nd	nd	nd	59	nd
3	nd	nd	nd	nd	nd	68	nd
4	nd	nd	nd	nd	nd	nd	nd
5	2115	nd	nd	nd	nd	55	nd
6	2176	nd	nd	nd	nd	78	nd
7	nd	nd	nd	nd	nd	nd	nd
8	2249	nd	nd	nd	nd	64	nd
9	nd	nd	nd	nd	nd	nd	nd
10	nd	nd	nd	nd	nd	nd	nd

[1)]nd - not detected.

4.3. Physicochemical characterization of some wastewater samples. Analysis of antibiotics using high performance liquid chromatography (HPLC) technique

In Section 4.3.1 is presented the physicochemical characterization of wastewater samples produced by a clinical hospital and the wastewater quality (influent and effluent) from a wastewater treatment plant. Usually, the physicochemical parameters of these two types of wastewater are not different (Abd El-Gawad and Aly, 2011). Wastewaters generated by hospitals contain pollutants in high concentration as those from the treatment plants. For this reason quality indicators were performed in order to compare the two types of wastewater. Also, in our case, the quality indicators analyzed did not vary depending on the nature of the wastewater samples (collected from a clinical hospital and from a treatment plant). This was observed in the case of chemical and biochemical oxygen demand, and total suspended solids.

4.3.2. Analysis of antibiotics from wastewater samples using high performance liquid chromatography technique coupled with diode array detector and mass spectrometry (HPLC-DAD/MS)

In Section 4.3.2 is presented SPE-HPLC-DAD/MS procedure that was developed for the simultaneous determination of seven antibiotics from wastewater samples collected from a clinical hospital and from a wastewater treatment plant. The study was focused on the determination of antibiotics in wastewater samples generated by a clinical hospital becasuse hospitals are considered to be the main source of environmental pollution with antibiotics (Duong et al., 2008). Because the wastewater samples collected from a wastewater treatment plant may contain small or large quantities of antibiotics depending on the sources, the presence of antibiotics in influent and effluent was also investigated. For this study, the selected antibiotics were AMOX, AMP, CFZ, PENG, CFX, TET and DOXY (Section 4.1).

4.3.2.1. Isolation/concentration of antibiotics from liquid matrices using SPE

Working procedure

Stock solutions of all compounds were prepared in ultrapure water. Acetonitrile of HPLC grade was purchased from Sigma-Aldrich (Germany) and formic acid from Cristal R Chim (Romania). For the SPE method, methanol (MeOH) was bought from Merck (Germany), the ammonia from Primexchim (Romania) and the hydrochloric acid (HCl) from Poch (Poland). Ethylenediamine tetraacetic acid disodium salt dihydrate ($Na_2EDTA \cdot 2H_2O$ *noted* EDTA) was purchased from Fluka (Germany). Water of high purity used in all experiments was prepared using a Milli-Q Ultrapure water purification system (Millipore, USA). The extraction of antibiotics from spiked and wastewater samples was carried out on SPE cartridges Oasis HLB (6 mL, 200 mg, Waters, USA) using a SPE device model Supelco vacuum manifold from Sigma-Aldrich (Germany).

The main steps of the SPE were consisted in:

- Oasis HLB cartridge was ***conditionated*** with 10 mL MeOH and 10 mL ultrapure water;
- ***a volume of*** 100 mL ***wastewater sample*** previously prepared by adding EDTA 5% for residual metal complexation (Feitosa-Felizzola et al., 2007), was passed through the Oasis HLB cartridge;
- the ***elution*** of the antibiotics was performed with 6 mL MeOH.

In order to find the optimal pH for the extraction of antibiotics from real samples, the selected antibiotics were extracted from 100 mL ultrapure water spiked with 120 μg of each antibiotic, at different pH values (2, 3, 7 and 9). The pH of the samples was adjusted with 0.5 N HCl, 5% NH_4OH respectively.

The wastewater samples were passed through the SPE cartridges with a flow rate of 1.2 mL min^{-1}. The obtained extracts were concentrated to dryness with a rotaryevaporator at 40°C and then solubilised in 2 mL ultrapure water. All the procedures were performed three times and the extracts were analysed with the HPLC-DAD/MS method described in Section 4.3.2.2.

Results and discussions

From the pH values tested, the best recoveries were obtained at pH 3 for CFZ, CFX, TET, and at pH 7 for the other antibiotics studied AMP, PENG, and DOXY. AMOX showed a recovery less than 50% for both pH values (3 and 7). In Figure 4.23 is presented the effect of pH on the SPE extraction efficiency of the studied antibiotics.

At pH 3 the recoveries were higher for CFZ 68.7%, CFX 110.1% and TET 102.6%, and at pH 7 for AMP 88.8%, PENG 98.7%, 114,6% TET and DOXY 84.4%, excepting AMOX 42.9% at pH 3, and 31.7% at pH 7. Because the best recoveries of antibiotics extracted from ultrapurewater were obtained at pH 3 and 7, these pH values were applied to all wastewater samples that were analyzed in this study.

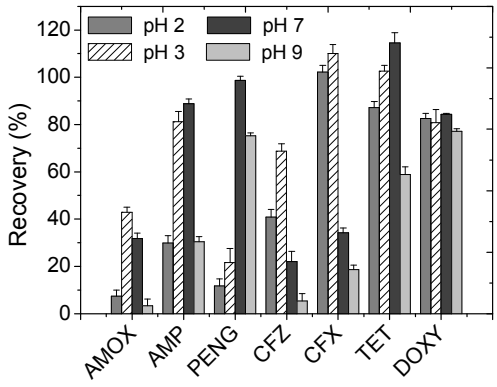


Figure 4.23. Recoveries of studied antibiotics extracted from ultrapure water at pH values of 2, 3, 7, and 9, obtained with Oasis HLB cartridges.

4.3.2.2. Qualitative and quantitative analysis of antibiotics using the HPLC-DAD/MS technique

Working procedure

The analyses of studied antibiotics were performed using a high performance liquid chromatograph Shimadzu HPLC equipped with two detectors: DAD and MS.

The chromatographic separation was performed using a Grace Alltima C18 column (100 × 3 mm, 3 μm) thermostated at 26°C, and with a gradient elution (Table 4.10). The used eluents were ultrapure water with formic acid at pH 3 (A) and acetonitrile (B). The injection volume was 20 μL, the flow rate was set at 0.5 mL min^{-1}, and the column equilibration was 5 min.

Table 4.10. The gradient program used for the chromatographic separation of the studied antibiotics. Mobile phase: ultrapure water with formic acid at pH 3 (A) and acetonitrile (B).

Time (min)	A (%)	B (%)
0:00	90	10
6:00	30	70
10:00	20	20
10:01	90	10
15:00	Stop	Stop

Results and discussions

All antibiotics studied were separated and eluted in a very short time of 7.5 min. Corresponding peaks of studied antibiotics presented maximum absorption as follow: AMOX, AMP, PENG, CFZ and CFX at 197 nm, TET and DOXY at 272 nm.

The parameters used for MS detection in positive electrospray ionization (ESI) mode were: capillary voltage of 1.5 kV, curved desolvation line temperature of 250°C and interface temperature of 250°C. All analytes were confirmed based on the retention time from chromatogram and the molecular ion (*m/z*) from MS spectrum obtained with ESI in positive mode. The results obtained in terms of the molecular ion *(m/z*) were generally in agreement with those previously reported in the literature (see Section 4.2.3).

The good separation of studied antibiotics can be observed from the diference between the retention times (Table 4.11). Also in Table 4.11 are presented the ratio *m/z* and relative intensity of each antibiotic studied according to mass spectra recorded.

Table 4.11. HPLC-MS parameters corresponding to the studied antibiotics.

Antibiotic	Retention time (min)	Molecular ion (*m/z*)	Relative intensity (%)
AMOX	1.78	366	28
AMP	4.34	350	100
PENG	7.11	335	<10
CFZ	3.96	547	81
CFX	4.76	555	100
TET	5.39	445	100
DOXY	6.08	445	100

4.3.2.3. Studies regarding the linearity, limits of detection and of quantification, precision and accuracy of the SPE-HPLC-DAD/MS procedure

To allow the quantification of the selected antibiotics in wastewater samples using the proposed SPE-HPLC-DAD/MS procedure, studies of linearity, limits of detection, of quantification respectively, precision and accuracy were performed.

4.3.2.4. Determination of antibiotics from wastewater samples using SPE-HPLC-DAD/MS procedure

Working procedure

The samples were filtered through Macherey-Nagel paper filters (MN 640 d·ø 125 mm) and stored at 4°C in refrigerator. For minimizing the microbial degradation the antibiotics were extracted, in less than 24 hours from sampling. Wastewater samples were collected from February to September 2011: sample no. 1 was collected in February, no. 2 in March 2011, no. 3 in May 2011, no. 4 in June, no. 5 in August 2011, and no. 6 and 7 in September 2011.

Results and discussions

a). The content of antibiotics from wastewater samples collected from a clinical hospital

In the wastewater samples collected from the clinical hospital three antibiotics were detected CFZ, TET and DOXY in ranges between 83-164 mg L^{-1}, 152-789 mg L^{-1}, and 285-544 mg L^{-1} respectively (Table 4.15).

Table 4.15. Amounts (µg L^{-1}) of studied antibiotics in wastewater samples collected from a clinical hospital.

Sample no.	Amount (µg L^{-1})						
	AMOX	**AMP**	**PENG**	**CFZ**	**CFX**	**TET**	**DOXY**
1	nd[1)]	nd	nd	nd	nd	nd	nd
2	nd	nd	nd	nd	nd	152	nd
3	nd	nd	nd	83	nd	nd	nd
4	nd	nd	nd	nd	nd	546	285
5	nd	nd	nd	nd	nd	nd	nd
6	nd	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	164	nd	789	544

[1)]nd - not detected.

One of the simplest methods to identify the compounds is to compare the chromatogram of the standard with the chromatogram of the sample (Gocan, 2002). An example is shown in Figure 4.28 where are presented the overlapped chromatograms of the wastewater sample in which were detected three antibiotics (CFZ, TET and DOXY) and of the studied antibiotics reference. Also, the mass spectra of antibiotics reference was compared with those from wastewater samples, confirming once again their presence in the wastewater samples collected from the clinical hospital.

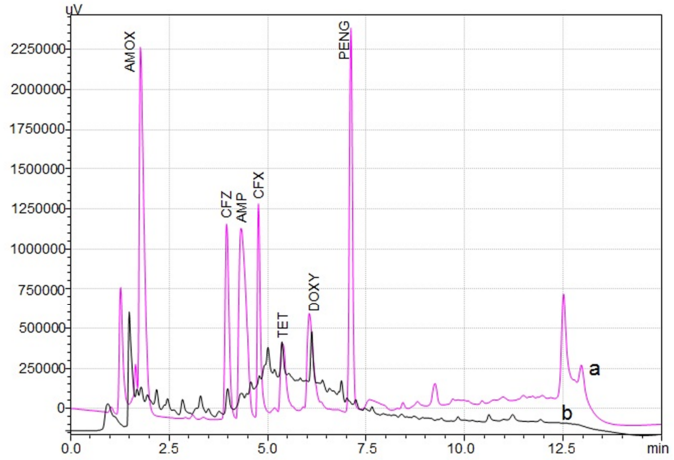


Figure 4.28. The HPLC-DAD overlapped chromatograms of the reference mixture of studied antibiotics (a) and of the wastewater sample collected from a clinical hospital (b).

b). The content of antibiotics from wastewater samples collected from a wastewater treatment plant

In the wastewater samples collected from a wastewater treatment plant were determined antibiotics that belong to the same antibiotic classes (cephalosporins and tetracyclines), as in the case of those determined in wastewater samples collected from a clinical hospital. From the tetracycline class were determined both TET and DOXY. Their persistence may be due to other wastewaters which were discharged in this treatment plant, from other hospitals, and also because are the most important and used antibiotics (Hernández et al., 2003; Kim et al., 2005).

The amounts of antibiotics determined in the samples collected from a wastewater treatment plant were: 245 μg L^{-1} CFX, 263-388 μg L^{-1} TET, and 83 μg L^{-1} DOXY.

Also, were collected samples from the wastewater treatment plant during one day, at different times. Thus, three samples were collected from influent at 9, 12, 15 and their corresponding effluent at 16 o'clock, 19 and 22. In influent samples were detected only three of the seven antibiotics studied, in concentrations of: 334 μg L^{-1} CFX, 146 μg L^{-1} and TET 110 μg L^{-1} DOXY (Table 4.17).

In the corresponding effluents was not detected any antibiotic of the seven studied in this research or were in low concentration, under the limit of detection of the proposed procedure. The absence of the antibiotics in effluents can be due to the efficiency of the treatment process for wastewaters.

Table 4.16. Amounts (μg L^{-1}) of studied antibiotics in wastewater samples collected from a wastewater treatment plant.

Sample no.	Amount (μg L^{-1})													
	AMOX		AMP		PENG		CFZ		CFX		TET		DOXY	
	influent	effluent	influent	effluent	influent	effluent	influent	effluent	influent	effluent	influent	effluent	influent	effluent
1	nd[1)]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3	nd	nd	nd	nd	nd	nd	nd	nd	245	nd	nd	nd	nd	nd
4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	263	nd	nd	nd
5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	388	nd	83	nd

[1)]nd - not detected.

Table 4.17. Amounts (μg L^{-1}) of studied antibiotics in wastewater samples collected from a wastewater treatment plant, in the same day at different hours.

Sampling time	Wastewater	Amount (μg L^{-1})						
		AMOX	AMP	PENG	CFZ	CFX	TET	DOXY
9	Influent	nd[1)]	nd	nd	nd	nd	146	110
12	Influent	nd	nd	nd	nd	nd	nd	nd
15	Influent	nd	nd	nd	nd	334	nd	nd
16	Effluent	nd	nd	nd	nd	nd	nd	nd
19	Effluent	nd	nd	nd	nd	nd	nd	nd
22	Effluent	nd	nd	nd	nd	nd	nd	nd

[1)]nd - not detected.

Chapter 5. Researches regarding the influence of antibiotics on green algae (*Pseudokirchneriella subcapitata* L.) and wheat plants (*Triticum aestivum* L.)

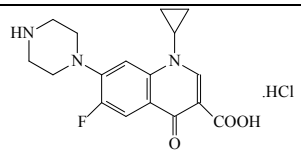
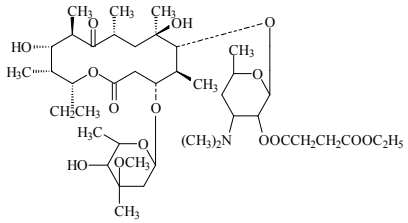
Monitoring and toxicity studies of pharmaceuticals are required to assess their impact on the environment (Choma, 2004). In the present thesis, studies regarding the influence of antibiotics on freshwater green algae (*Pseudokirchneriella subcapitata* L.) (Section 5.2) and wheat plants (*Triticum aestivum* L.) (Section 5.3) were performed. In Section 5.1 the antibiotics selected for this research are presented.

5.1. Selected antibiotics and their main characteristics

In experiments that were aimed to evaluate the influence of antibiotics on algae (*Pseudokirchneriella subcapitata* L.) (Section 5.2) were selected three antibiotics belonging to two different classes: penicillins (AMP, PENG) and tetracycline (DOXY) (see Section 4.1).

In the study regarding the influence of antibiotics on wheat plants (*Triticum aestivum* L.) (Section 5.2) nine antibiotics (AMOX, AMP, PENG, CFZ, CFX, TET, DOXY, ciprofloxacin - CIP and erythromycin - ERY) were selected. The characteristics of all antibiotics mentioned above are presented in Section 4.1, except CIP and ERY. In Table 5.2 the characteristics of CIP and ERY are presented.

Table 5.2. Chemical formula, molecular weight, structural formula and mode of action of ciprofloxacin and erythromycin.

Antibiotic	Chemical formula	Molecular weight (g mol^{-1})	Structural formula	Mode of action[1)]
CIP	$C_{17}H_{18}FN_3O_3.HCl$	367.80		Bactericidal
ERY	$C_{43}H_{75}NO_{16}$	862.05		Bacteriostatic

[1)]Bactericidal antibiotics such as fluoroquinolones inhibit DNA and protein synthesis. Bacteriostatic antibiotics such as macrolides inhibit protein synthesis by preventing the elongation of the polypeptide chain (Dahl et al., 2006; Matinca, 2002).

5.2. Researches regarding the influence of antibiotics on cell density of green algae (*Pseudokirchneriella subcapitata* L.)

Usually, growth inhibition toxicity tests on living organisms are applied to determine the presence of toxic substances found in the environment. Green algae are successfully used to determine the effects of some toxic or mutagenic chemicals, heavy metals, pesticides or wastewater generated by different industries. Algae have a very important role in the structure and function of the aquatic ecosystem (Yang et al., 2008).

5.2.1. Green algae (*Pseudokirchneriella subcapitata* L.), growth conditions and antibiotic treatments. Working procedure

To motivate the following researches regarding the influence of antibiotics on wheat plants (*Triticum aestivum* L.), the influence of antibiotics on green algae was also studied. Green algae selected for these tests were freshwater algae *Pseudokirchneriella subcapitata* L. These are considered to be bioindicators in assessing the toxicity of chemicals found in the environment.

Thus, rapid assessment of the negative influences of three antibiotics (AMP, PENG and DOXY, see Section 4.1) on algae by determining the cell density was performed. Algal growth nutrient media used in this study were: nutrient medium for algal growth in normal conditions (reference or control algae), nutrient medium with antibiotic solutions (AMP, PENG and DOXY) of concentration 1.5 mg L^{-1}, and nutrient medium with potassium dichromate ($K_2Cr_2O_7$) of concentration 1.5 mg L^{-1} used also as reference for detecting the possible unsatisfactory test conditions (Halling-Sørensen, 2000). Antibiotics concentration selected for this study is an intermediate one comparing to those reported in the literature (Qin et al., 2012, van der Grinten et al., 2010; Yang et al, 2008).

Algal cell density was determined using an optical microscope (CETI, England) and Neubauer counting chambers (Weber Scientific International, England). Algal cell counting was performed using the enlarge objective of 40 times and the measuring Neubauer chambers had the following characteristics: depth 0.1 mm, size 1 × 4000 mm^2. 20 μL of algal suspension of each test included in this experiment were introduced in the Neubauer chambers. Counting network of Neubauer chamber was composed of 9 fields of cell counting (large squares, 1 × 1 mm) each square was divided in 16 small squares. To avoid counting cells twice or several times, cell counting was performed in three different fields arranged diagonally. All measurements were performed three times and then the average was calculated. The measurements of algal cell density were performed at three time intervals: 24 h, 48 h and 72 h (EN ISO 8692:2004).

5.2.2. Results and discussions regarding the influence of antibiotics on algal cell density

At 24 h after starting the experiment, the number of cells mL^{-1} decreased in all three tests with antibiotics (AMP, PENG and DOXY). Compared to control, these decreases ranged between 38.6-50.8%. The largest amount of cells mL^{-1} was inhibited by DOXY (50.8%).

In this study, the second set of measurements was performed at 48 h after starting the experiment. Compared to control, the algal cell density in nutrient medium containing 1.5 mg L^{-1} DOXY decreased with 74.2%. PENG also changed the cell density of algae by increasing significantly with 31.8% compared to control algae. It was observed a decrease in algal cell density in the presence of AMP, but this decrease was not statistically significant.

The influence of DOXY on cell density of algae *Pseudokirchneriella subcapitata* L. was observed at 72 h after starting the experiment. Compared to control, cell density decreased with 66.9%. The decrease of cell density in the case of the AMP test was not as significant as in the case of the DOXY test. Compared to control algae this decrease was 24.3%. The algae grown in the presence of PENG kept the same increase in the number of algal cells, as in the case of measurements performed at 48 h after starting the experiment. At 72 h, the number of algal cells grown in the presence PENG increased (50%) compared to control.

The test with $K_2Cr_2O_7$ was not different from the control in terms of the number of algal cells mL^{-1} (24 h, 48 h, and 72 h).

In this study, growth rates (μi) of the algal cells and the percentages of inhibition cell growth (Iμi) for each test also were calculated (EN ISO 8692:2004). Growth rates ranged between 0.054-0.085 cells mL^{-1} h^{-1}, the lowest value was obtained in the case of DOXY test. The percentage of inhibition cell growth ranged between 24.1-35.1%.

5.3. Researches regarding the influence of antibiotics on wheat plants (*Triticum aestivum* L.)

So far, understanding the effects of many important antibiotics on plant physiological activity is still limited. Thus, to evaluate these effects and to identify the best characteristics for a rapid assessment of antibiotics toxicity, in this section are presented the influence of nine antibiotics (AMOX, AMP, PENG, CFZ, CFX, TET, DOXY, CIP and ERY, Section 4.1 and 5.1) of concentration 0.5 mg L^{-1} and 1.5 mg L^{-1} on the photosynthetic parameters, volatile organic compounds, assimilating pigments and the total flavonoid content in wheat plants (*Triticum aestivum* L. cv. "Lovrin"). Photosynthesis is the most fundamental biological process supporting growth, nutrient uptake, and affecting resistance to biotic and abiotic stresses. All plants are known to emit volatile organic compounds in response to various biotic and abiotic stresses (Loreto and Schnitzler, 2010; Niinemets, 2010). In this study we hypothesized that emissions of volatiles are also enhanced in response to stress resulting from antibiotics.

5.3.1. Wheat plants (*Triticum aestivum* L.), growth conditions and abiotic stress induced by antibiotics. Working procedure

Wheat (*Triticum aestivum* L. cv. "Lovrin", Fundulea, Romania) seeds were sown in 1 L (10 × 10 × 10 cm) plastic pots filled with commercial garden soil with slow release NPK fertilizer with microelements (Biolan, Finland). The plants were grown in a growth chamber (Percival LT36VL model, IA, USA) under a light intensity of 1000 μmol m^{-2} s^{-1} 130 at plant level provided for 12 h light period and day/night temperatures of 25/18ºC.

The abiotic stress consisted in watering the plants every day with 71 mL aqueous solution of given antibiotic with either 0.50 ± 0.03 mg L^{-1} 136 or 1.50 ± 0.08 mg L^{-1} concentration. The control plants were given equal amounts of distilled water. The effects of treatment with antibiotics were analyzed after the plants had received 0.5 L of the antibiotic solution (one week old plants) and after that had received 1 L of the solution (two weeks old plants) (Opriș et al., 2012b).

The concentrations applied in our study were lower, but exposure times longer than in some other studies (Pan et al., 2008; Xie et al., 2011). We suggest that these treatments more realistically reflect the plant response to diffuse dispersal of antibiotics residues in environment. In fact, past studies do demonstrate that for prolonged exposures, a number of antibiotics at these concentrations significantly inhibit plant growth (Brain et al., 2004, 2005, 2008; Hillis et al., 2011; Liu et al., 2009; Pomati et al., 2004).

5.3.2. The influence of antibiotics on photosynthetic parameters of wheat plants

Photosynthesis is considered to be the most complex process of synthesis from the Earth's surface. Through this process, in the presence of light, green plants take CO_2 from the atmosphere and convert it into organic matter, releasing O_2 (Dobrotă, 2010). Thus, under stress conditions, this process

can be affected, leading to an inhibition of plant development. Regarding the effects of antibiotics on photosynthesis, we analyzed three photosynthetic parameters: electron transport rate (J_{ETR}), stomatal conductance to water vapor (g_s) and net assimilation rate (A). These three parameters are particularly important in photosynthesis. Electron transport rate acts as a reducing agent in the formation of NADPH (nicotinamide adenine dinucleotide phosphate oxidase) and energy molecules of ATP (adenosine triphosphate) which are used in fixation and reduction of CO_2 (Dobrotă, 2010). In plants, assimilation occurs simultaneous with the stomatal water elimination.

5.3.2.1. Determination of photosynthetic parameters of wheat plants. Working procedure

A Portable Gas Exchange System, GFS-3000 (Waltz, Germany), was used for measurements of leaf gas exchange characteristics. The system has an environmental-controlle cuvette with 8 cm^2 window area and is equipped with full window leaf chamber fluorimeter for sample illumination and chlorophyll fluorescence measurements. The measurements were made at an ambient CO_2 concentration of 385 μmol mol^{-1} and light intensity of 1000 μmol m^{-2} s^{-1}. The leaf temperature was kept at 25ºC, and the relative humidity was 70% in all the experiments. Multiple leaves were enclosed side by side in the cuvette to fill the entire cuvette window. After the enclosure of the leaves in the cuvette, light was switched on and the leaf was stabilized until stomata opened and steady-state values of net assimilation rate (A) and stomatal conductance to water vapor (g_s) were obtained, typically 20-30 min. after foliage enclosure. Thereafter, steadystate fluorescence yield (F) was recorded, and a 1 s flash of saturating white light of 4500 μmol m^{-2} s^{-1} was given to measure the maximum fluorescence yield (Fm).

All gas-exchange characteristics were calculated according to von Caemmerer and Farquhar (1981). The electron transport rate (J_{ETR}) was calculated from the chlorophyll fluorescence measurements according to Genty et al. (1989), using a constant leaf absorptance of 0.85 and assuming that light is distributed equally between the two photosystems (Niinemets et al., 2010a) PS I and PS II. In the photosynthesis is involved the interaction between two light reactions PS I and PS II, acting in series to produce molecular oxygen for NADPH and ATP synthesis (Bercea, 2008).

5.3.2.2. Results and discussions regarding the influence of antibiotics on photosynthetic parameters of wheat plants

In Figure 5.12.a and b the photosynthetic electron transport was reduced by antibiotic treatments, especially at the highest concentration of antibiotic solutions used (1.5 mg L^{-1}) for watering the plants. The exceptions were the treatments with CIP and ERY (Figure 5.12.b). The plants treated with AMP showed a significant decrease in electron transport rate at both concentrations (0.5 mg L^{-1} and 1.5 mg L^{-1}) of antibiotics used for watering the plants (Figure 5.12.a and b) (Opriş et al., 2012b). Across all treatments, the biggest decrease in electron transport rate, 16% relative to control, was observed for the treatment with 1.5 mg L^{-1} CFX.

We observed significant effects of penicillins and tetracyclines on photosynthetic electron transport rate (Figure 5.12.a and b), and these effects were also associated with reduced chlorophyll content (Figure 5.20). The effect of AMOX at higher concentration on electron transport rate is in agreement with previous observations on PS II inhibition by this antibiotic (Opriş et al., 2012b). This aspect was observed by other researchers, but at relatively high concentrations (Pan et al., 2008).

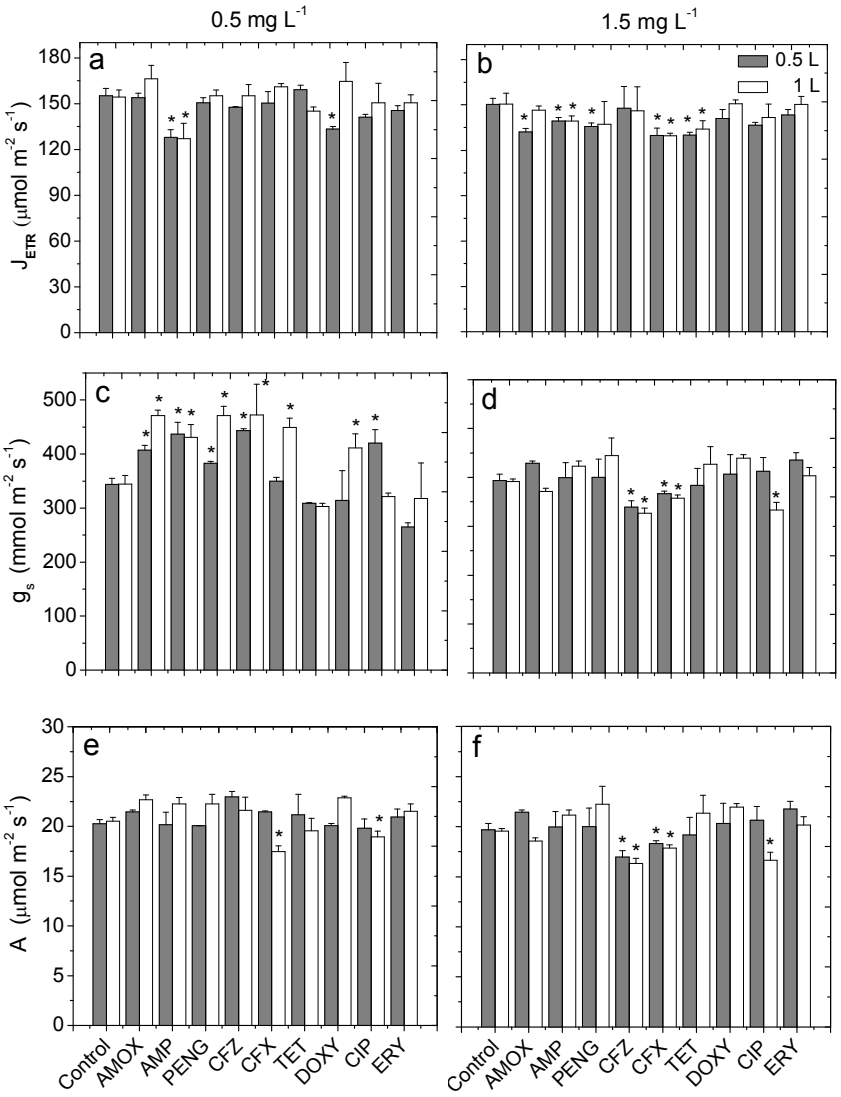


Figure 5.12. Changes in electron transport rate (J_{ETR}, µmol m^{-2} s^{-1}) (a and b), stomatal conductance to water vapor (g_s, mmol m^{-2} s^{-1}) (c and d), and net assimilation rate (A, µmol m^{-2} s^{-1}) (e and f) in *Triticum aestivum* L. cv. "Lovrin" plants treated with nine antibiotic solutions in two concentrations 0.5 mg L^{-1} (a, c and e) and 1.5 mg L^{-1} (b, d and f). The measurements were performed after the plants were watered with a total volume of antibiotic solutions of 0.5 L (one week old plants) and 1 L (two week old plants). Each data point is the mean (± SE) of three independent replicate experiments with a different plant. "*" demonstrates statistically significant differences between the given antibiotic treatment and control ($P < 0.05$).

In the treatments with the antibiotics concentration of 0.5 mg L^{-1}, stomatal conductance to water vapor generally increased (Figure 5.12.c), except for TET and ERY treatments where stomatal conductance to water vapor was not affected by application of both 0.5 L and 1 L antibiotic solutions. In contrast, stomatal conductance to water vapor decreased, by 10-30%, under the higher concentration (1.5 mg L^{-1}), in treatments with cephalosporins CFZ and CFX, and with tetracycline TET (0.5 L and 1 L antibiotic solutions) (Figure 5.12.d). Decreases in stomatal conductance to water vapour were also observed in plants treated with tetracycline DOXY and fluoroquinolone CIP, but only after application of 1 L of antibiotic solution (Figure 5.12.d) (Opriş et al., 2012b).

Enhanced stomatal conductance for some antibiotics applied at 0.5 mg L^{-1} is initially puzzling. However, in AMP treatment, this effect was associated with reduced zeaxanthin content (Figure 5.21) that is the presumable precursor for the synthesis of abscisic acid (ABA), the hormone responsible for stomatal closure (Milborrow, 2001). ABA is a basic regulator of plant responses to stress factors, growth and development (Delian, 2006). Thus, the reduced stomatal control might reflect altered hormonal interactions. Yet, only a small part of carotenoids goes into ABA synthesis, and changes in overall carotenoid pool size clearly were not associated with reduced stomatal conductance to water vapor at higher antibiotic concentration in our study.

The treatment with 0.5 mg L^{-1} concentration of given antibiotic solutions resulted in reduced net assimilation rate for the treatments with cephalosporin antibiotic CFX and fluoroquinolone antibiotic CIP, but only after application of 1 L antibiotic solutions (Figue 5.12.e). The higher concentration used (1.5 mg L^{-1}) led to more pronounced declines in net assimilation rate with significant effects of cephalosporins CFZ and CFX being already observed after application of 0.5 L or the antibiotic solution (Figue 5.12.e). Still, with fluoroquinolone antibiotic CIP the effect became significant only after application of 1 L of antibiotic solution (Figure 5.12.f) (Opriș et al., 2012b). The effects of CIP agree with past observations (Aristilde et al., 2010). Aristilde et al. (2010) suggested that the structure of CIP is similar to the known inhibitors of the oxidizing side of PS II.

The decrease of net assimilation rate for plants treated with antibiotics from cephalosporins class could be explained by the same mechanism, where the inhibition of the catalytic activity of PS II could be associated with the NH_2 group of cephalosporins (Opriș et al., 2012b). In our study, the reduction in net assimilation rate for 1.5 mg L^{-1} treatment was actually associated with reduced stomatal conductance (Figure 5.12.d) and no modification of photosynthetic electron transport rate for CIP and CFZ treatments (Figure 5.12.b). Thus, at least for these two treatments, reduction in photosynthesis rate was not associated with inhibition of PS II activity, but rather reflected reduced CO_2 entry into the leaf. The same behavior was observed for a sensitive wheat variety in response to 80 μM oxytetracycline (Li et al., 2011).

Overall, the effects of antibiotics on photosynthesis, ca. 10-17%, were moderate, and in most cases, the effects were indirect, modulated by changes in stomatal conductance, with some evidence of inhibition of light harvesting reactions for tetracyclines, penicillins and cephalosporin CFX (Opriș et al., 2012b).

5.3.3. The emissions of volatile organic compounds from wheat plants treated with antibiotics

A wide wide range of volatile organic compounds (VOC) is emitted by plants that are subject to a variety of biotic or abiotic stress (Copolovici et al., 2012). Therefore, to get a more complete perspective on the effects of antibiotics on plants, VOC emissions were analyzed. In our study, the VOC emitted by wheat plants that were analyzed were the lipoxygenase pathway products (LOX) and monoterpenes.

LOX are originate from free fatty acids released by phospholipases from membranes in response to a variety of stresses, and constitute one of the initial stress responses (Liavonchanka and Feussner, 2006).

Monoterpenes are plant secondary metabolism compounds are insoluble in water and are derived from acetyl coenzyme A and other intermediaries of glycolysis. Many of monoterpenes and their derivatives are very important toxic agents against insects (Dobrotă, 2005). Monoterpenes, plant

secondary metabolism compounds, are insoluble in water and are derived from acetyl coenzyme A or other intermediates of glycolysis. Many of monoterpenes and their derivatives are very important toxic agents against insects (Dobrotă, 2005). Wheat is not a constitutive monoterpene emitter under non-stressed conditions. However, monoterpenes are typically induced in response to biotic or abiotic stresses similarly to LOX in many plant species, although these emissions are typically elicited somewhat later (Copolovici et al., 2012; Niinemets, 2010; Niinemets et al., 2010b, 2010c).

5.3.3.1. The capture and analysis of volatile organic compounds emitted by wheat plants. Working procedure

VOC sampling was performed using the same system as for the gas exchange measurements. After steady-state values of gas exchange characteristics were achieved, an air volume of 3 L exiting the cuvette was sampled in a multibed stainless steel cartridge (10.5 × 3 cm, Supelco, USA) filled with Carbopack adsorbents (C 20/40 mesh, C 40/60 mesh, and X 20/40 mesh) optimized for the quantitative analyses of the LOX and monoterpenes. The chamber air was sampled at a flow rate of 200 mL min^{-1} for 15 min using a 1003-SKC constant flow sampling pump (SKC Inc., USA) at room temperature. Background air samples were taken from the empty leaf cuvette before and after the measurements.

The device used for VOC analysis was a combined automated cartridge desorber (Shimadzu TD20, Japan) and a gas chromatograph-mass spectrometer instrument (Shimadzu QP2010 Plus GC-MS, Japan). The volatiles were analyzed according to the method described in detail by Copolovici et al. (2009) and Toome et al. (2010). The volatiles were identified by comparing the mass spectrum of individual compounds with the spectra of external standards (GC purity, Sigma-Aldrich, USA) and with the spectra in the NIST Library.

The flux of total lipoxygenase pathway products (Φ_{LOX}) is the sum of emissions of 1-hexanol, (Z)-3-hexenol, (Z)-2-hexenal, and (Z)-3-hexenyl acetate and the total monoterpene emission flux ($\Phi_{MONOTERPENE}$) is the sum of emissions of α-pinene, *β*-pinene, camphene, limonene, 3-carene, *p*-cymene, and *β*-phellandrene (Opriș et al., 2012b).

5.3.3.2. Results and discussions regarding the emissions of volatile organic compounds from wheat plants treated with antibiotics

Both total Φ_{LOX} and $\Phi_{MONOTERPENE}$ were low in control treatments (Figure 5.16). Enhanced Φ_{LOX} were observed at the lowest concentration (0.5 mg L^{-1}) after application of 1 L of antibiotic solution for AMP, PENG, CFX, DOXY, and CIP (Figure 5.16.a). At the higher application concentration of 1.5 mg L^{-1}, Φ_{LOX} increased after adding 1 L antibiotic solution for all antibiotics, except for AMP (Figure 5.16.b). In this treatment, Φ_{LOX} was enhanced up to four times relative to the control. In general, ΦLOX was greater for the application concentration of 1.5 mg L^{-1} than for the concentration of 0.5 mg L^{-1}, on average of over 100%. However, AMP and CIP constituted exceptions in the general trend (Opriș et al., 2012b).

In most of the cases Φ_{LOX} increased with increasing the concentration and the volume of antibiotic solutions used for watering the wheat plants (Figure 5.16.a and b). Quantitative relationships between Φ_{LOX} and stress severity have been previously shown for other abiotic stresses such as ozone (Beauchamp et al., 2005), temperature (Copolovici et al., 2012), and flooding (Copolovici and Niinemets, 2010), our study highlights that LOX emissions constitute a highly sensitive indicator of plant antibiotic response.

At both concentrations of antibiotics used, monoterpene emissions increased for all antibiotics used (Figure 5.16.c and d). The effects were generally more pronounced after application of 1 L of given antibiotic solution than after application of 0.5 L, but the differences among different antibiotic concentrations were non-significant with the exception of the plants treated with ERY (Figure 5.16.c and d) (Opriș et al., 2012b).

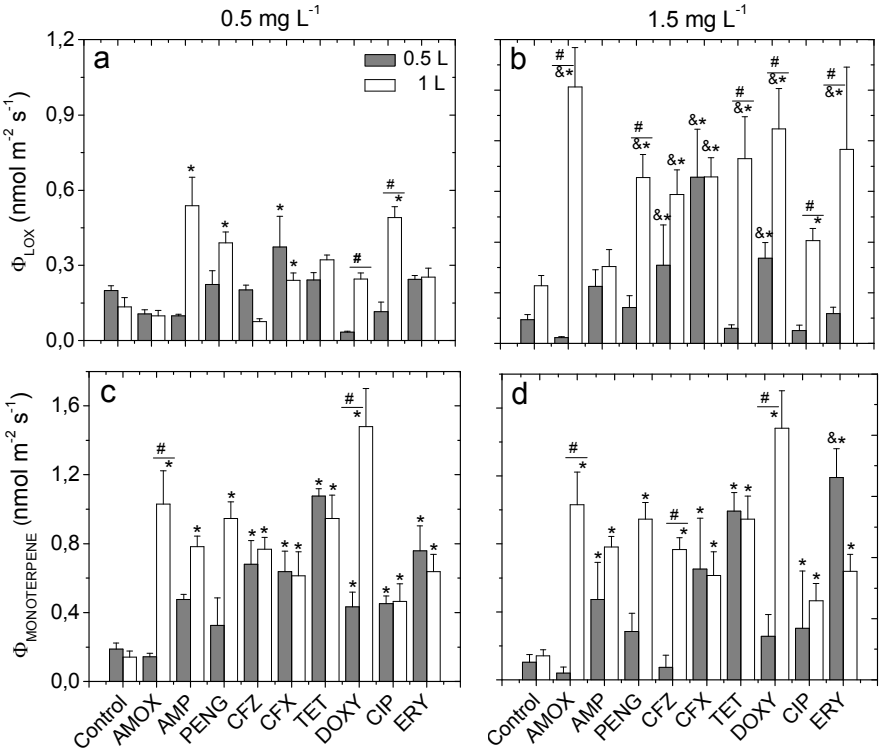


Figure 5.16. Emissions of lipoxygenase pathway products (Φ_{LOX}, nmol m^{-2} s^{-1}) (a and b) and monoterpenes ($\Phi_{MONOTERPENE}$, nmol m^{-2} s^{-1}) (c and d) from *Triticum aestivum* cv. "Lovrin" plants in response to antibiotic treatments. The measurements were performed after the plants had been watered with a total volume of antibiotic solutions of 0.5 L and 1 L in concentration of 0.5 mg L^{-1} (a and c) and 1.5 mg L^{-1} (b and d). The sum Φ_{LOX} consist of emissions of 1-hexanol, (*Z*)-3-hexenol, (*Z*)-2-hexenal, and (*Z*)-3-hexenyl acetate and the $\Phi_{MONOTERPENE}$ emissions consist of *α*-pinene, *β*-pinene, camphene, limonene, 3-carene, *p*-cymene, and *β*-phellandrene. Each data point is the mean (± SE) of three independent replicate experiments with a different plant. The symbols above the columns represent statistically significant differences ($P < 0.05$). "*" - statistical difference between the given antibiotic treatment and control; "#" - statistical difference between the treatments with different volumes of applied antibiotic solutions (0.5 L and 1 L) at given concentration (0.5 mg L^{-1} or 1.5 mg L^{-1}); "&" - statistical difference between the two concentrations of given antibiotic (0.5 mg L^{-1} and 1.5 mg L^{-1}) at the same volume of antibiotic solution applied (0.5 L or 1 L).

The evidence that several antibiotic treatments resulted in reduced carotenoid contents (Figures 5.21 and 5.22) that are formed by the same plastidic deoxyxylulose pathway, suggests that under higher treatment concentration of these antibiotics, a greater faction of the overall pathway flux was shifted towards monoterpene emissions. In fact, the treatments with TET and DOXY induced the highest $\Phi_{MONOTERPENE}$ emissions (Figure 5.16.c and d), and this was associated with strongest carotenoid

reductions (Figures 5.21.c and 5.22.d) (Opriş et al., 2012b). It has been demonstrated that TET treatments can switch genes on and off at any given time during pathogen plants interaction (Zarnack et al., 2006).

5.3.4. The influence of antibiotics on assimilating pigments from wheat plants

In our study, the assimilating pigments that were determined in wheat plants are chlorophyll pigments (chlorophyll *a* and chlorophyll *b*) and carotenoid pigments (*β*-carotene, zeaxanthin, lutein, neoxanthin and violaxanthin). Pigments zeaxanthin, lutein, neoxanthin and violaxanthin are xanthophylls carotenoids, pigments in which the free radicals of the carbon chain are represented by hydroxyl -OH groups (Dobrotă, 2010).

Chlorophylls are particularly important in light absorption in photosynthesis. Chlorophyll *a* is found in all green plants, and through photosynthesis removes O_2, and chlorophyll *b* accompanies chlorophyll *a* in all higher plants, green algae and flagellates (Dobrotă, 2010).

Carotenoid pigments from plants are fatty liposoluble antioxidants (Havaux, 1998; Havaux et al., 1998) and their photoprotective role can be considered as a safety valve that allows excess energy dissipation before the ocurrence of damage at the cellular level (Dobrotă, 2010). In plants subjected to severe stress conditions, carotenoids, can be rapidly destroyed and therefore are no longer available to protect against oxidative damage and protect from photoinhibition (Munné-Bosch and Alegre, 2000).

5.3.4.1. The extraction and analysis of assimilating pigments from wheat plants.

Working procedure

For the determination of chholophylls and carotenoid pigments leaf samples of 4 cm^2 were taken after gas exchange measurements and immediately frozen in liquid nitrogen. The pigments were extracted in ice-cold 100% acetone with calcium carbonate (both purchased from Sigma-Aldrich, Germany) with mortar and pestle, and centrifuged with a Hettich 320 R Universal centrifuge (Hettich GmbH, Germany) at 0ºC and 9500 g for 3 min and the supernatant was decanted. The extraction was repeated at least three times with small amounts of acetone until the supernatant remained colorless. The extracts were pooled and brought to a final volume of 1 mL acetone and then filtered through a 0.45 μm PTFE membrane filter (VWR International, USA) (Opriş et al., 2012b).

The obtained extracts were analyzed by a high pressure liquid chromatograph (HPLC, Agilent Technologies 1200 Series, USA) equipped with a diode array detector (DAD) according to a modified method described by Niinemets et al. (1998) using a Zorbax Eclipse XDB-C18 reversed-phase column (4.6 mm i.d. × 150 mm column length, 5 μm particle size, Agilent Technologies, USA). The column temperature was maintained at 10ºC and the flow rate at 1.5 ml min^{-1}. The solvents used for the chromatographic elution consisted in ultrapure water with Hepes 0.1 M, pH 8 (A) and acetone HPLC grade (B), both purchased from Sigma-Aldrich (Germany). The chromatographic elution program used was as follows: 25% A and 75% B was run for the first 7.5 min, followed by a 9.5 min 100% B, which was run for 3 min. Further, the eluent was changed to the initial composition of 25% A and 75% B 2 min.

The HPLC was calibrated using commercially available pigments: chlorophyll *a*, chlorophyll *b*, and *β*-carotene, purchased from Sigma-Aldrich (Germany) and zeaxanthin and lutein from Fluka (Germany).

Some researchers (Niinemtes et al., 1998, Pocock et al., 2004; Polle et al., 2001) obtained references (standards) pigments from plants or algae by their prior extraction and then purified by TLC

plates. After separation, the compounds of interest from the TLC plates are resumed in different solvents, and their concentrations are determined from the calculation of the extinction coefficient from spectrophotometric measurements.

In our case, carotenoid pigments, violaxanthin and neoxanthin were extracted from fresh leaves of *Primula vulgaris* L. with liquid nitrogen. These two pigments were extracted in 100% ethanol (Sigma-Aldrich, Germany). Their prior separation was performed by TLC using silicagel preparative plates (20 × 20, 500 μm, Analtech, USA). The extracts were applied on TLC plate as bands using a microsyringe Hamilton model 705 RN SYR (50 mL, Germany). The TLC plates were developed using a solvent mixture of 400 mL petroleum eter (Sigma-Aldrich, Germany), 44 mL propane-2-ol (Romil Ltd., UK) and 20 μL of ultrapure water (same components used for the mobile phase as in the study conducted by Pocock et al., 2004, but in different proportions). Further, the plate was developed 15 min by ascending technique to a distance of 10 cm in a developing chamber. After development, the plate was dried at room temperature. TLC plate obtained after the chromatographic separation of studied pigments is shown in Figure 5.18.

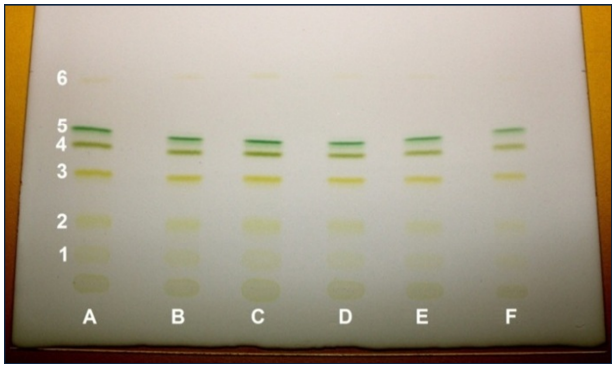


Figure 5.18. TLC chromatoplate of the studied assimilating pigments extracted from leaves of *Primula vulgaris* L. Positions A-F are the extracts obtained, and position 1-6 the pigments: 1-neoxanthin, 2-violaxanthin, 3-lutein, 4-chlorophyll *b*, 5-chlorophyll *a*, 6- *β*-caroten.

With the TLC method outlined above, besides violaxanthin and neoxanthin pigments, lutein, chlorophyll *b*, chlorophyll *a*, and *β*-carotene were also separated. The last ones were not purified and quantified in such way because in these cases commercial standards were used.

Resumption of neoxanthin and violaxanthin from the TLC plates was performed in acetone, and their concentrations were calculated from extinction measurements using appropriate wavelengths and extinction coefficients for carotenoids (Davies, 1976). For subsequent quantitative determinations of the neoxanthin and violaxanthin from wheat plants treated with antibiotics, the purified standards obtained were analyzed by HPLC-DAD system obtaining a signal corresponding to the extinction coefficients obtained from calculations of the spectrophotometric measurements.

Pigment peaks presented maximum intensity as follows: 430 nm for chlorophyll *a*; 450 nm for neoxanthin and violaxanthin; 455 nm for the rest of the pigments chlorophyll *b*, *β*-carotene, zeaxanthin, and lutein (Opriş et al., 2012b).

5.3.4.2. Results and discussions regarding the influence of antibiotics on assimilating pigments from wheat plants

Separation of all assimilating pigments was performed in a maximum time of 19 min. At the concentration of 0.5 mg L^{-1}, total chlorophyll content decreased only for CFX after application of 0.5 L and 1 L of solutions, and for CIP after application of 1 L of solution (Figure 5.20.a). Treatment with 1.5

mg L^{-1} antibiotic solutions reduced total chlorophyll content for AMOX, tetracyclines, CIP, and ERY (Figure 5.20.b). The effect of antibiotic treatments was similar for chlorophylls *a* and *b*, especially for 1.5 mg L^{-1} treatments (Figure 5.20.c and d). For 0.5 mg L^{-1} treatments, chlorophyll *a/b* ratio was elevated for AMOX, CFX (0.5 L application) and CFX (1 L application), but reduced for CIP (1 L application) (Figure 5.20.c) (Opriş et al., 2012b).

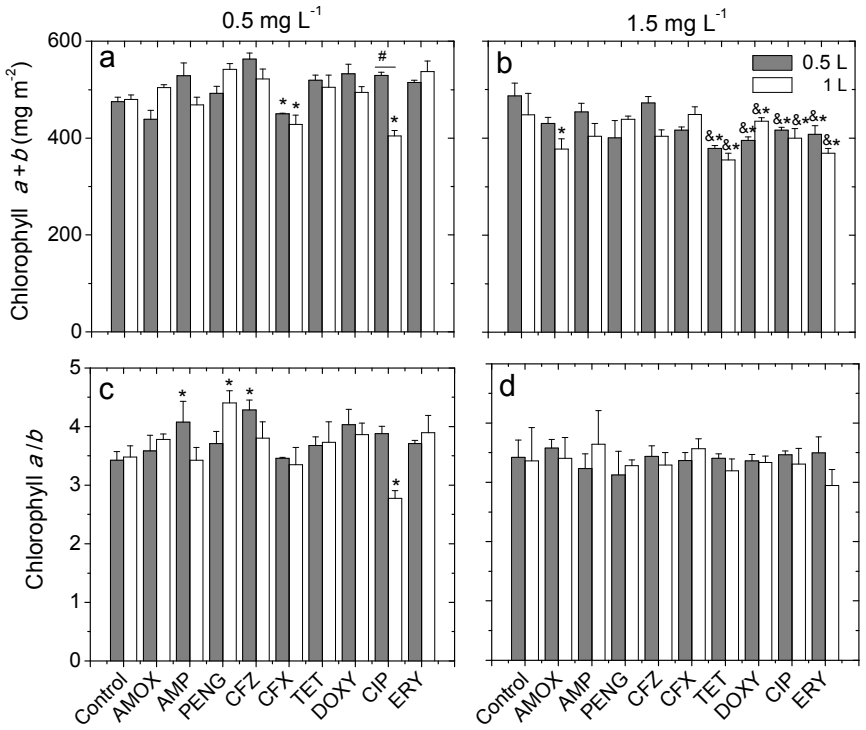


Figure 5.20. Changes in total chlorophyll *a+b* content (mg m^{-2}) (a and b) and chlorophyll *a*/*b* ratio (c and d) in *Triticum aestivum* L. cv. "Lovrin" leaves trated with nine antibiotic solutions. The measurements were performed after the plants were provided with a total volume of antibiotic solutions of 0.5 L and 1 L in concentration of 0.5 mg L^{-1} (a and c) and 1.5 mg L^{-1} (b and d). The symbols above the columns stand for statistical differences as in Figure 5.16.

Decreases in chlorophyll content are a typical stress response that results in reduced light interception (Munné-Bosch and Alegre, 2000). In our study, the effects were particularly pronounced for TET, CIP and ERY at higher treatment concentration. This agrees with an analogous reduction in chlorophyll content in Li et al. (2011) for wheat plants treated with oxytetracycline, and inhibition of chlorophyll synthesis by TET (Bradel et al., 2000). Also, out of 25 antibiotics tested by Brain et al. (2004), tetracyclines were among the most influential pharmaceuticals affecting chlorophyll content of the aquatic plant *Lemna gibba*.

However, occasionally some penicillins and cephalosporins also significantly reduced chlorophyll content in our study (Figure 5.20) (Opriş et al., 2012b). This may be associated with a certain effect on chloroplast multiplication. A more likely explanation is the effect of these antibiotics on the inhibition of light reactions of photosynthesis (see Section 5.3.2.) and resulting photoinhibitory conditions leading to reduced cross-section of PS II (Osmond et al., 1999).

In fact, chlorophyll *a*/*b* ratio that characterizes the pigment distribution between the reaction centers and light harvesting complexes (Bassi and Caffarri, 2000) importantly increases for some penicillins and cephalosporins (Figure 5.20.c), suggesting reduction of light-harvesting complexes of PS

II relative to reaction centers. Normal values for the ratio chlorophyll *a/b* are between 2.5 to 3.5 (Bercea, 2008). In contrast, a reduction in chlorophyll *a*/*b* ratio was observed for CIP treatment (Figure 5.20.c), suggesting stronger effect on the content of reaction centers. In general, reduction in carotenoids in response to antibiotic treatment mirrored changes in chlorophyll contents, with particularly strong effects for tetracyclines, CIP and ERY at concentration of 1.5 mg L^{-1} (Figures 5.20.b and 5.21.c) (Opriș et al., 2012b).

The treatment with 0.5 L solution of antibiotics at concentration 0.5 mg L^{-1} did not affect carotenoid (lutein, zeaxanthin and *β*-caroten) contents (Figure 5.21.a). However, after application of 1 L solution, this concentration resulted in reduced zeaxanthin and *β* -carotene contents for CIP treatment and reduced zeaxanthin content for AMP treatment (Figure 5.21.b) (Opriș et al., 2012b).

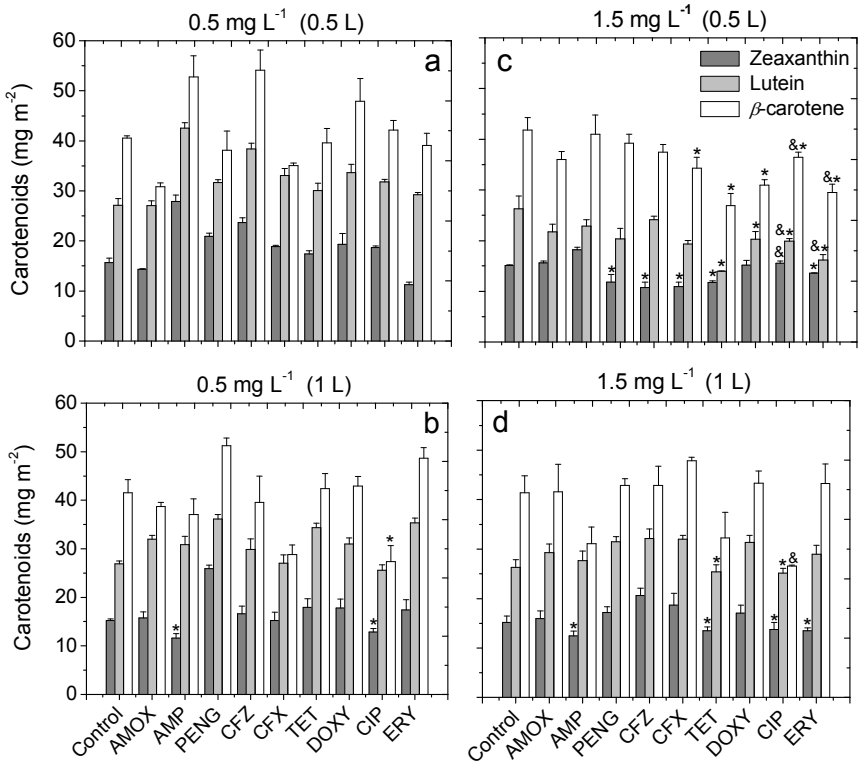


Figure 5.21. Changes in carotenoids (zeaxanthin, lutein and *β*-carotene, mg m^{-2}) content in *Triticum aestivum* L. cv. "Lovrin" plants in response to treatments with nine antibiotics after the plants had been watered with a total volume of antibiotic solutions of 0.5 L and 1 L in concentration of 0.5 mg L^{-1} (a and b) and 1.5 mg L^{-1} (c and d). The symbols denoting statistical significance as in Figure 5.16.

Treatments with the antibiotic concentration of 1.5 mg L^{-1} resulted in reduced zeaxanthin contents for most antibiotics, either after application of 0.5 L or 1 L solution or for both applications, except for AMOX (Figure 5.21.c and d). *β*-carotene and lutein were affected by fewer treatments, with no effect observed for penicillins and cephalosporins (Opriș et al., 2012b).

However, important reductions in only zeaxanthin were observed for treatments with AMP (Figure 5.21.b and d), PENG, CFX and CFZ (Figure 5.21.c) (Opriș et al., 2012). Differently from lutein and *β*-carotene, a certain free pool of zeaxanthin can be present in thylakoid membranes and possibly also in non photosynthetic membranes (Havaux and Niyogi, 1999; Müller-Moulé et al., 2003). Zeaxanthin formation is correlated with the dissipation of excess absorbed energy as heat, and reflects the de epoxidation state of the xanthophyll cycle and the non-photochemical quenching of chlorophyll

fluorescence (Demmig-Adams and Adams, 2006; Munné-Bosch and Alegre, 2000). Thus, reduced zeaxanthin content can reflect reduced capacity for safe dissipation of excess light energy, and this may be the mechanistic explanation for the reduction of photosynthetic electron transport rate in these treatments (Figure 5.12.a and b) (Opriș et al., 2012b).

In Figure 5.22 are presented the amouts of violaxanthin and neoxanthin from wheat plants which were determined in this study. In the case of the violaxanthin, significant decreases were observed even at the concentration of 0.5 mg L^{-1}, 0.5 L antibiotic solutions.

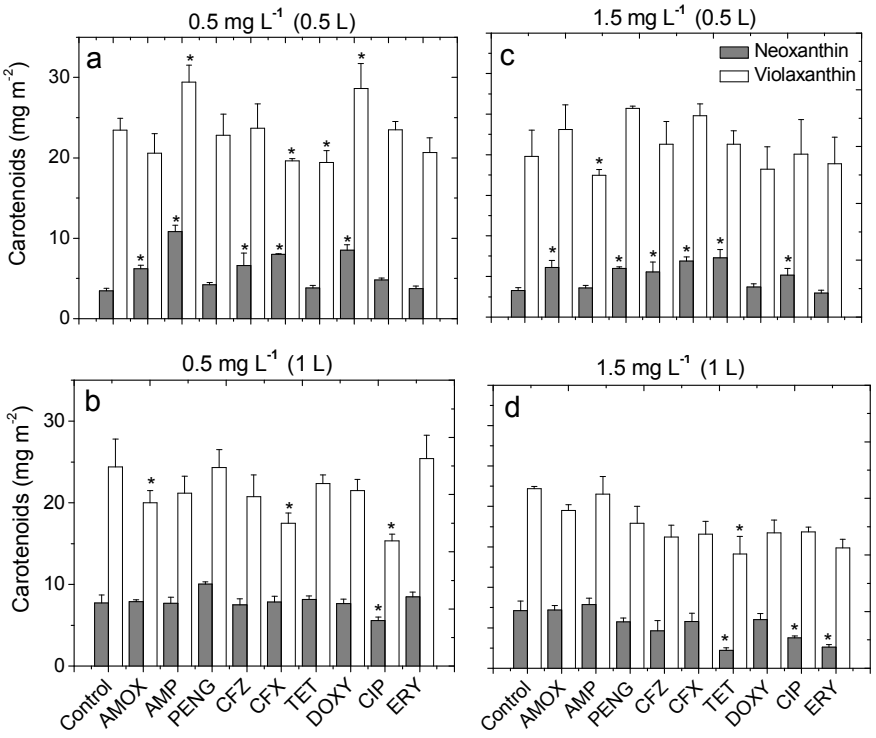


Figure 5.22. Changes in carotenoids (neoxanthin and violaxanthin, mg m^{-2}) content in *Triticum aestivum* L. cv. "Lovrin" plants in response to treatments with nine antibiotics after the plants had been watered with a total volume of antibiotic solutions of 0.5 L and 1 L in concentration of 0.5 mg L^{-1} (a and b) and 1.5 mg L^{-1} (c and d). The symbols denoting statistical significance as in Figure 5.16.

These decreases were observed in wheat plants treated with cephalosporin antibiotics (CFX) and tetracycline (TET) (Figure 5.22.a). In the case of CFX treatment, the decrease of violaxanthin content was observed after the plants were watered with a higher volume of antibiotic solution (1L, Figure 5.22.b). The lowest content of violaxanthin was obtained at the concentration of 0.5 mg L^{-1}, 1 L CIP (15.32 mg m^{-2}). At the second concentration of antibiotic solutions used for watering the plants (1.5 mg L^{-1}), it was observed that with increasing the volume of antibiotic solutions, the violaxanthin content decreased (Figure 5.22.c and d). Thus, compared to control the lowest content of violaxanthin was obtained in the case of the wheat plants treated with TET (14.11 mg m^{-2}, Figure 5.22.d).

In most cases, the content of neoxanthin varied, but significant decreases were obtained for the treatments with TET, CIP and ERY (Figure 5.22.d). The neoxanthin and violaxanthin content decreased with increasing the concentrations and with the volumes of antibiotic solutions used for watering the wheat plants.

5.3.5. The influence of antibiotics on the total flavonoid content from wheat plants

Flavonoids, the phenolic compounds formed through shikimate pathway in chloroplasts, protect plants against various biotic and abiotic stresses (Samanta et al., 2011). Therefore, to assess the effects of antibiotics on plants, in addition to analysis of photosynthetic parameters, volatile organic compounds and assimilating pigments, the total flavonoid content from wheat plants (*Triticum aestivum* L.) also was analyzed.

5.3.5.1. The optimization of extraction method and analysis of total flavonoid content from wheat plants. Working procedure

The first step made in the determination of the total flavonoid content from wheat plants was to optimize the extraction method. Before applying the extraction method on wheat plants treated with nine antibiotics, extraction tests only on control wheat plants were carried. The plants were grown at room temperature (22°C) and at natural light. Thus, for the extraction of total flavonoids from control wheat plants were tested three techniques of extraction: maceration, sonication and microwave assisted solvent (Soran et al., 2012). For each extraction technique tested, several proportion of extraction solvent ethanol: water (v/v) were used: A (100 : 0), B (80 : 20), C (70 : 30), D (60 : 40), E (50 : 50) and F (40 : 60).

The amount of total flavonoids from the extracts obtained was determined spectrophotometrically (UV-Vis spectrophotometer 1800, Shimadzu, Japan) with aluminum chloride ($AlCl_3$) at the wavelength of 420 nm and expressed as amount of rutin (Romanian Pharmacopoeia, 1993). Comparing the three tested techniques for the extraction of the total flavonoid content from wheat plants (Figure 5.26) it was observed that the biggest amount of flavonoids extracted from wheat plants was obtained using the sonication technique (Soran et al., 2012; Opriş et al., 2011).

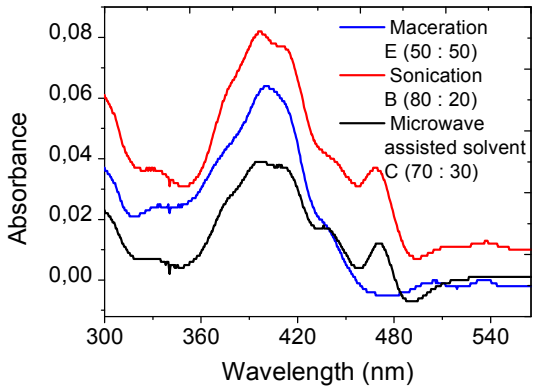


Figure 5.26. UV-Vis spectra of total flavonoid extracts obtained from wheat plants with the best proportion of solvents ethanol : water, v/v, corresponding to the three extraction techniques tested.

This technique, unlike maceration and microwave assisted solvent techniques, is much faster, relatively simply and is not expensive. After setting the best techniques and the best proportion of solvents used for the extraction of total flavonoids from wheat plants (sonication extraction technique, extraction solvent ethanol: water, 80 : 20, v/v) studies regarding the influence of nine antibiotics on wheat plants were performed.

5.3.5.2. Results and discussions regarding the influence of antibiotics on the total flavonoid content from wheat plants

Enhanced flavonoids content is a ubiquitous stress outcome observed in response to several stresses such as chilling (Havaux and Kloppstech, 2001), enhanced UV (Lavola, 1998) and visible (Havaux and Kloppstech, 2001) radiation. The role of enhanced flavonoids in stressed plants is not fully understood, but flavonoids have been suggested to serve both as pigments screening excess visible and ultraviolet radiation and as antioxidants (Havaux and Kloppstech, 2001; Neill et al., 2002). Thus, the increase after application of 0.5 L antibiotic solution for PENG, CFX, TET and DOXY treatments (Figure 5.27) may indicate enhanced antioxidative capacity under these treatments. In contrast, reduction after 1 L antibiotic solution applications at 1.5 mg L^{-1} (Figure 5.27) suggests that sustained influence of antibiotics reduces foliage antioxidative capacity.

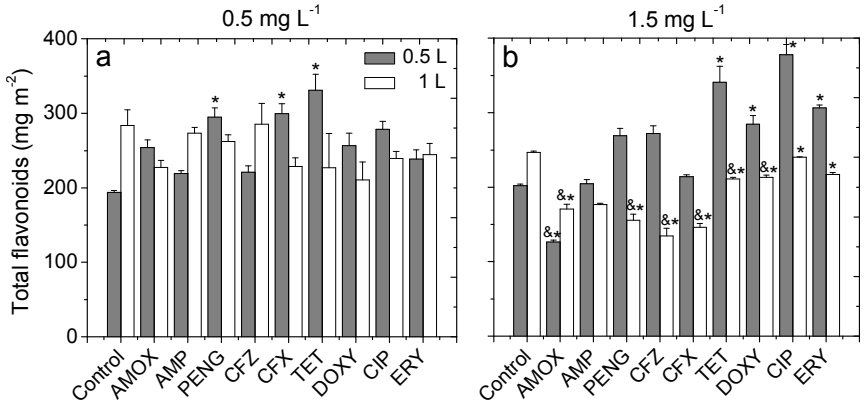


Figure 5.27. Modification in total flavonoid content in *Triticum aestivum* L. cv. "Lovrin" foliage by treatments with nine antibiotics. The measurements were conducted after the plants had been given a total volume of antibiotic solutions of 0.5 L and 1 L in concentration of 0.5 mg L^{-1} (a) and 1.5 mg L^{-1} (b). The symbols for statistical significance as in Figure 5.16.

CONCLUSIONS

Excessive use of antibiotics leads to their occurrence into the environment. In spite of their benefit properties and desired effects during the therapeutic applications, the same properties can be disadvantageous for the environment having negative influences on plants and microorganisms, and potential risks for human health.

Due to these issues, the present study was aimed to bring contributions regarding the development of procedures for determination of antibiotics from surface water and wastewater samples and highlight their negative influences on green algae (*Pseudokirchneriella subcapitata* L.) and wheat plants (*Triticum aestivum* L.). Nine antibiotics belonging to five different classes: penicillins (amoxicillin, ampicillin and penicillin G), cephalosporins (ceftazidime and ceftriaxone), tetracyclines (tetracycline and doxycycline), fluoroquinolones (ciprofloxacin) and macrolides (erythromycin) were selected for the proposed researches.

Two procedures for the determination of seven antibiotics (amoxicillin, ampicillin, penicillin G, ceftazidime, ceftriaxone, doxycycline and tetracycline) from surface water and wastewater samples were developed. For both procedures isolation/concentration of antibiotics from different samples was

performed using SPE technique. SPE methods were performed using hydrophilic-lipophilic balanced cartridges, Oasis HLB, which conducted to good recoveries.

The first procedure, SPE-HPTLC, was developed for the determination of antibiotics from surface water samples (Someşul Mic River, Cluj-Napoca).

- The experimental data obtained from the validation of proposed SPE-HPTLC procedure is selective and presents very good linearity, precision and accuracy.
- In the investigated surface water samples were determined two antibiotics (amoxicillin and tetracycline) in relatively high concentrations (55-2249 mg L^{-1}).

In the case of the wastewater samples (collected from a clinical hyospital and from a wastewater treatment plant: influent and effluent), in addition to determination of antibiotics, also their physicochemical characterization was performed. Determination of antibiotics was performed using the second procedure developed (SPE-HPLC-DAD/MS) in the present study. The conclusions drawn from this research are:

- The physicochemical parameters analyzed did not vary depending on the nature of the wastewater samples (collected from a clinical hospital and from a wastewater treatment plant). This was observed for the following indicators of water quality: chemical oxygen demand, biochemical oxygen demand and total suspended solids.
- The ratio chemical/biochemical oxygen demand taken as biodegradability index for the wastewater collected from the clinical hospital showed that this type of wastewaters may be considered to be at the limit of biodegradability due to the high consumption of drugs and cleaning products used in the hospital.
- Most physicochemical parameters determined in wastewater samples (effluent) collected from the wastewater treatment plant were within the maximum limits provided in Annex no. 1 NTPA 011/2005 of the H.G. 352/11.05.2005, except for the total nitrogen content.
- The SPE-HPLC-DAD/MS procedure used for the determination of antibiotics from wastewater samples showed good linearity, limits of detection and of quantification, precision and accuracy.
- Using the SPE-HPLC-DAD/MS procedure, in the wastewater samples collected from a clinical hospital were determined three antibiotics (ceftazidime, tetracycline and doxycycline).
- In the wastewater samples (influent) collected from the wastewater treatment plant antibiotics ceftriaxone, tetracycline and doxycycline were determined using the same procedure SPE-HPLC-DAD/MS.
- Antibiotics determined in the investigated wastewater samples belong to the most commonly classes of antibiotics (cephalosporins and tetracyclines) used in medicine and related fields.
- In the effluents was not detected any antibiotic of the seven studied in this research or were in very low concentration, under limit of detection of the proposed procedure. The absence of the antibiotics in effluents can be due to the efficiency of the treatment process for wastewaters.

The presence of antibiotics in wastewater samples (influent and effluent) is a growing environmental problem, although in this case specifically, the presence of antibiotics in effluent samples was not detected. Thus, wastewater investigated in this study does not present a danger to the aquatic environment in terms of the amount of antibiotics.

The research regarding the influence of three antibiotics (ampicillin, penicillin G and doxycycline) on cell density of algae *Pseudokirchneriella subcapitata* L. led to the following conclusions:

- In the case of the three tests with antibiotics was observed a decrease (up to 35.1% related to control) in algal cell density, at 24 h after starting the experiments.
- Doxycycline had the most pronounced effect of inhibition on algal cell density.
- In the case of ampicillin test the algal cell density decreased, but not statistically significant.

A more ccomplex study regarding the toxicity of antibiotics was performed on wheat plants (*Triticum aestivum* L.). In this research was evaluated the effects of nine selected antibiotics on photosynthetic parameters (electron transport, net assimilation rate and stomatal conductance to water vapor), assimilating pigments (chlorophylls and carotenoids), volatile organic compounds (lipoxygenase pathway products and monoterpenes), and total flavonoid content from plants. Analysis of the experimental data conducted to:

- Relative to control, the biggest decrease in electron transport rate (16%) was observed for the treatment with ceftriaxone.
- The effects of antibiotics on photosynthesis were moderate and indirect, modulated by changes in stomatal conductance, with some evidence of inhibition of light harvesting reactions for tetracyclines, penicillins and cephalosporin CFX.
- The negative effects of antibiotics on photosynthetic parameters increased with increasing the dose of antibiotics administered to wheat plants.
- The monoterpene emissions increased at both concentrations of antibiotic solution used in this study.
- Overal, it was observed an increase in emissions of lipoxygenase pathway products, which was dependent of the concentrations and volumes of antibiotic solutions used for watering the wheat plants.
- Volatile emissions appeared to be most sensitive indicators of antibiotics treatment.
- The overall effect of antibiotics on chlorophylls pigments was moderate, up to 10%, compared to control. This decrease may be associated with plant response to stress conditions and with reducing light absorption.
- Compared to control plants, the content of carotenoids (zeaxanthin, lutein, β-carotene) decreased up to 3-4%.
- The most significant decrease (up to 69%) in neoxanthin and violaxanthin content was obtained in the case of plants treated with tetracycline. These decreases may be associated with the reducing of the photoprotective capacity of plants.
- In case of the plants treated with the selected antibiotics, the total flavonoid content significantly decreased, effect associated with the decrease of the antioxidant capacity of plants.

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ANNEX I. ABBREVIATIONS

A - net asimilation rate
ABA - abscisic acid
ADN - deoxyribonucleic acid
AMOX - amoxicillin
AMP - ampicillin
ATP - adenosine triphosphate
CBO - biochemical oxygen demand
CCO - chemical oxygen demand
CFX - ceftriaxone
CFZ - ceftazidime
CIP - ciprofloxacin
DAD - diode array detector
DCI - international common name
DOXY - doxycycline
EC_{50} - effective concentration
EDTA - ethylenediamine tetraacetic acid
ERY - erythromycin
ESI - electrospray ionization
ETR - electron transport rate
g_s - stomatal conductance to water vapor
HLB - hydrophilic lipophilic balanced
HPLC - high performance liquid chromatography
HPTLC - high performance thin layer chromatography
LC - liquid chromatography
LOD - limit of detection
LOQ - limit of quantification
LOX - lipoxygenase pathway products
MS - mass spectrometry
NADPH - nicotinamide adenine dinucleotide phosphate oxidase
PENG - penicillin G
PS I - photosystem I
PS II - photosystem II
RSD - relative standard deviation
SD - standard deviation
SE - standard error
SPE - solid phase extraction
TET - tetracycline
TLC - thin layer chromatography
TMS - total suspended solids
UV-Vis - ultraviolet visible
VOC - volatile organic compounds

ANNEX II. LIST OF PUBLICATIONS RESULTED FROM THE THESIS

1. **Ocsana Opriş**, Florina Copaciu, Maria Loredana Soran, Dumitru Ristoiu, Ülo Niinemets, Lucian Copolovici, **2012**. Influence of nine antibiotics on key secondary metabolites and physiological characteristics in *Triticum aestivum*: leaf volatiles as a promising tool to assess toxicity. *Ecotoxicology and Environmental Safety*. In press - DOI: 10.1016/j.ecoenv.2012.09.019. *Impact factor (2011): 2.294*.
2. **Ocsana Opriş**, Virginia Coman, Florina Copaciu, Mihaela Vlassa, **2012**. Solid phase extraction and high performance thin layer chromatography quantification of some antibiotics from surface waters. *Journal of Planar Chromatography - Modern TLC* 25 (6), 516-522. In press - DOI: 10.1556/JPC.25.2012.6.0. *Impact factor (2011): 0.767*.
3. Loredana Maria Soran, **Ocsana Opriş**, Florina Copaciu, Codruța Varodi, **2012**. Determination of flavonoids in *Triticum aestivum* L. treated with ampicillin. Proceeding of "Processes in isotopes and molecules (PIM 2011)", edited by Lazar M.D. American Institute of Physics, Melville, New York. *AIP Conference Proceedings* 1425, 47-49. *ISI databases*.
4. **Ocsana Opriş**, Florina Copaciu, Virginia Coman, Dumitru Ristoiu, **2011**. UV-VIS study regarding the influence of two potential environmental pollutants on the total flavonoid content in *Triticum aestivum* L. and *Secale cereale* L. *Studia Universitatis Babeş-Bolyai Chemia* 56 (4), 17-25. *Impact factor (2011): 0.129*.
5. **Ocsana Opriş,** Carmen Roba, Florina Copaciu. Quality assessment of waste waters generated by some important hospitals from Cluj County. *Environmental Engineering and Management Journal*. Under revision. *Impact factor (2011): 1.004*.
6. **Ocsana Opriş,** Maria-Loredana Soran, Virginia Coman, Florina Copaciu, Dumitru Ristoiu. Determination of some frequently used antibiotics in waste waters using solid phase extraction followed by high performance liquid chromatography with diode array and mass spectrometry detection. *Environmental Science: Processes & Impacts*. Under revision. *Impact factor (2011): 1.991.*