European Commission

JRC TECHNICAL REPORTS

# Residues of antimicrobial agents and related compounds of emerging concern in manure, water and soil

*Part 1 – Pilot-sampling campaign in Slovakia and first findings*

Tavazzi S, Mariani G, Skejø H, Comero S, Głowacka N, Gaduš J, Gawlik BM

2018

Joint Research Centre

EUR 29065 EN

This publication is a Technical report by the Joint Research Centre (JRC), the European Commission's science and knowledge service. It aims to provide evidence-based scientific support to the European policymaking process. The scientific output expressed does not imply a policy position of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication.

**Contact information**
Name: Bernd Manfred Gawlik
Address: European Commission, Joint Research Centre, Via Enrico Fermi 2749, 21027 Ispra (Va), Italy
Email: bernd.gawlik@ec.europa.eu
Tel.: +39 0332 78 9487

**JRC Science Hub**
https://ec.europa.eu/jrc

JRC110565

EUR 29065 EN

| | | | |
|---|---|---|---|
| PDF | ISBN 978-92-79-77754-7 | ISSN 1831-9424 | doi:10.2760/835417 |
| Print | ISBN 978-92-79-77755-4 | ISSN 1018-5593 | doi:10.2760/36607 |

Luxembourg: Publications Office of the European Union, 2018



How to cite this report: Tavazzi S, Mariani G, Skejø H, Comero S, Głowacka N, Gaduš J, Gawlik BM, *Antimicrobial agents and other compounds of emerging concern in manure-exposed waters: Part 1 – Pilot-sampling campaign in Slovakia and first findings*, EUR 29065 EN, Publications Office of the European Union, Luxembourg, 2018, ISBN 978-92-79-77754-7, doi:10.2760/835417, JRC110565.

# Contents


**1 SETTING THE SCENE 5**
1.1 General introduction and remarks 5
1.2 The EU Water *Acquis* to be considered 6
1.3 Manure processing technologies 8
1.4 Medicinal veterinary products in manure 10
1.5 Compound classes and compounds used most frequently and the situation in the EU 12
1.6 Manure storage in the EU 18

**2 TOWARDS AN EU-WIDE PILOT-CAMPAIGN 20**
2.1 Objectives of the exploratory pilot sampling 20
2.2 Sampling location 21
*2.2.1 Site characteristics and information 21*
*2.2.2 Impressions from the landscape 23*
*2.2.3 Supplementary information on veterinary drugs employed in the area 26*
2.1 Sampling 30
2.2 Samples registry 32

**3 TOWARDS A MULTI-COMPOUND ANALYTICAL METHODOLOGY 34**
3.1 Preliminary compound selection and initial choice 34
3.2 Sample preparation 36
*3.2.1 Non-processed manure (cattle urine) 36*
*3.2.2 Processed manure 36*
3.3 Instrumental methods 37
*3.3.1 LC-MS/MS method 37*
*3.3.2 Inorganic analytical determinations 46*
3.4 First experimental and analytical results 47
*3.4.1 Non-processed manure 47*
*3.4.2 Processed manure 48*

**4 PRELIMINARY CONCLUSIONS AND NEXT STEPS 52**

**REFERENCES 53**

**LIST OF ABBREVIATIONS AND DEFINITIONS 55**

**LIST OF FIGURES 56**

**LIST OF TABLES 57**

**ANNEXES 58**
Annex 1. MARIANI Box - Instructions for use 58

## Foreword

The Laboratory of the Water and Marine Resources Unit investigates the occurrence and fate of chemical pollutants entering and travelling with the natural and urban water cycles. In doing so, the laboratory also characterises the possible treatment and removal options for such compounds. In particular the so-called Compounds of Emerging Concern (CECs) as well as their degradation and metabolisation products are of interest in its investigation.

The issue of veterinary medicinal products and in particular those of an antimicrobial effect have attracted interest while trying to understand the development and propagation of antimicrobial resistances.

In order to improve the knowledge base the laboratory prepares an EU-wide assessment on waters exposed directly or indirectly to manure and derived fertilising products. Particular attention is given to the investigation of agricultural runoff, but also to the question to which extend such chemicals will enter either the food chain or other supply chains in case of reuse of the manure.

The findings will be published in a series of technical reports in which this one is the first stepping-stone in building an enhanced knowledge base for the making and implementation of improved EU policies.

## Acknowledgements

The support of the technical services and staff of the Slovak University of Agriculture in Nitra, Faculty of European Studies and Regional Development, Department of Regional Bioenergy is gratefully acknowledged.

***Authors***

**Simona Tavazzi** (corresponding author)

European Commission, Joint Research Centre, Directorate Sustainable Resources, Water and Marine Resources Unit, Via Enrico Fermi 2749, I-21027 Ispra (Va), Italy, Email: simona.tavazzi@ec.europa.eu.

**Giulio Mariani**

European Commission, Joint Research Centre, Directorate Sustainable Resources, Water and Marine Resources Unit, Via Enrico Fermi 2749, I-21027 Ispra (Va), Italy, Email: giulio.mariani@ec.europa.eu.

**Helle Skejø**

European Commission, Joint Research Centre, Directorate Sustainable Resources, Water and Marine Resources Unit, Via Enrico Fermi 2749, I-21027 Ispra (Va), Italy, Email: helle.skejo@ec.europa.eu.

**Sara Comero**

European Commission, Joint Research Centre, Directorate Sustainable Resources, Water and Marine Resources Unit, Via Enrico Fermi 2749, I-21027 Ispra (Va), Italy, Email: sara.comero@ec.europa.eu.

**Natalia Głowacka**

Slovak University of Agriculture in Nitra, Faculty of European Studies and Regional Development, Department of Regional Bioenergy, Tr. A. Hlinku 2, SK-94976 Nitra, Slovak Republic: Email: glowacka@ei.sk.

Environmental Institute, Okruzna 784/42, SK-97241 Kos, Slovak Republic

**Ján Gaduš**

Slovak University of Agriculture in Nitra, Faculty of European Studies and Regional Development, Department of Regional Bioenergy, Tr. A. Hlinku 2, SK-94976 Nitra, Slovak Republic: Email: jan.gadus@uniag.sk.

**Bernd Manfred Gawlik**

European Commission, Joint Research Centre, Directorate Sustainable Resources, Water and Marine Resources Unit, Via Enrico Fermi 2749, I-21027 Ispra (Va), Italy, Email: bernd.gawlik@ec.europa.eu.

## Abstract

In a thinking of circular economy, the understanding of how problematic chemical substances may migrate and travel across the various boundaries of a life-cycle is of pivotal importance to ensure that the philosophy of reuse and recycle is not jeopardized by new risks of contamination.

In this framework, veterinary medicinal products (VMP) and in particular the anti-microbial agents (AMAs) are a growing source of concern in the context of the reuse of processed manure as a fertilizer. This is mainly due to lack of understanding of their role in the development of anti-microbial resistances and their propagation. While the mechanistic study on how the propagation takes place at molecular genetic level receives much attention, the actual data situation on occurrence of VMPs and AMAs in agricultural land remains opaque and poor.

In order to prepare a larger and EU-wide monitoring exercise on the waters exposed directly or indirectly to the (processed) manure a first pilot exercise was organised to develop an appropriate protocol. This first report compiles a series of background information collected. It describes the execution of first pilot sampling and presents the first elements in the development of validated analytical methods.

# 1 Setting the scene

## 1.1 General introduction and remarks

It cannot be denied that the targeted use of chemistry has played an important role in the development of an efficient and productive modern agriculture. Until the end of the last century the main environmental concern on the adverse effects of chemical use was focussed on the impact of traditional chemicals such as heavy metals and persistent organic pollutants, but in the past two decades focus has turned to other compound classes, commonly re-grouped under the heading "*compounds of emerging concern (CECs)*" or more dramatically "*emerging pollutants*". The reasons for this development are fundamentally two, i.e. on one side a significantly improved analytical capability to detect and quantify even at extreme low concentrations those chemical structures, which were invisible until then, as well as a growing understanding about how substances alone or in combination affect biological processes. Likewise the investigation of the various transfer pathways have become the subject in a steadily increasing number of scientific publications.

Figure 1 visualises the possible entry pathways to the agricultural environment of such compounds, traditionally stemming from a targeted application in crop and plant protection and management, the reuse of (treated) bio-waste, sewage sludge or reclaimed wastewater in agriculture as well as the processes involved in livestock use and meat production (Boxall, 2012). In particular, the use of untreated manure or in more or less strongly processed form has not been sufficiently investigated with regard to their potential release of pharmaceuticals and agents of an anti-microbial activity into the aquatic environment (Thanner *et al.*, 2016).

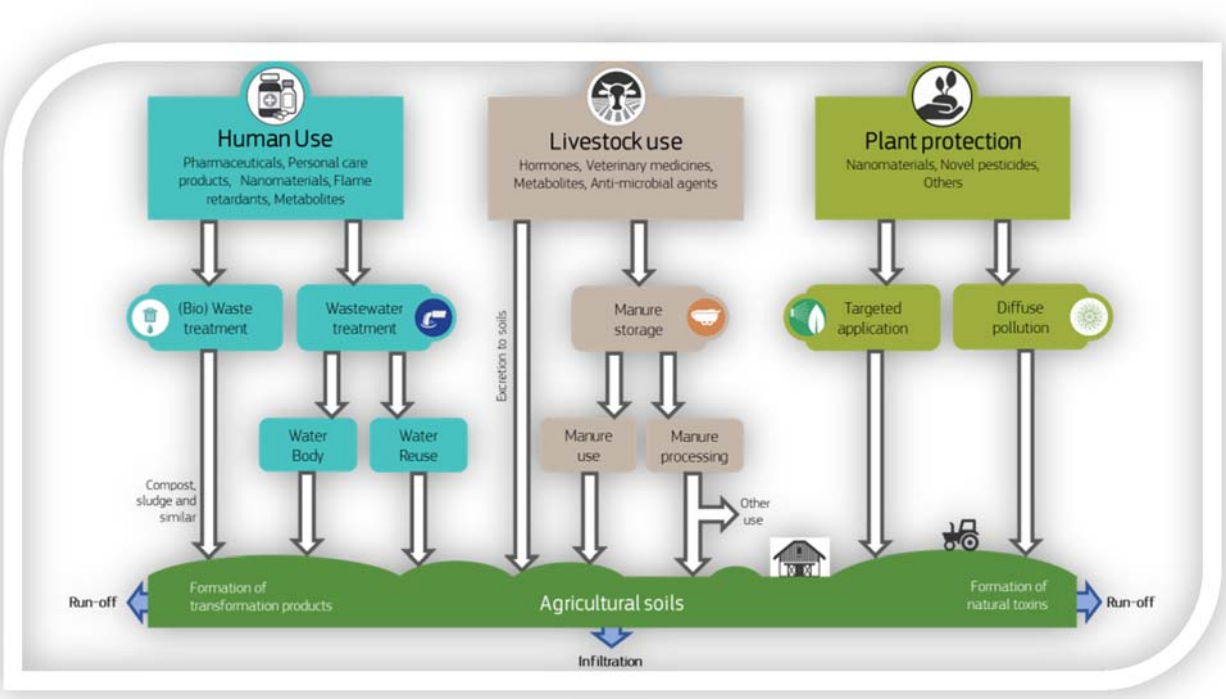



**Figure 1 - Entry pathways of CECs into the agricultural environment**

In this framework, veterinary medicinal products (VMP) and in particular the anti-microbial agents are a growing source of concern, mainly due to their role in the development of anti-microbial resistances and their propagation. While the mechanistic study on how the propagation takes place at molecular genetic level receives much attention, the actual data situation on occurrence of VMPs and AMAs in agricultural land

remains opaque and poor. Laboratory studies are usually referring to unnatural conditions, extreme scenarios or do not reflect typical European conditions. Likewise, the use of (treated) biosolids such as sewage sludge, compost or digestates as well as the employment of animal manure as mineral fertilizer substitute, experiences a renaissance with the strong commitment of the EU towards a circular economy approach. In addition, manure-derived digestates after a first energy recovery are investigated as nutrient integrated resource recovery employing e.g. algae, thus enhancing and improving the recovery and reuse aspects by unprecedented technologies (Głowacka *et al.*, 2017)

Establishing the agronomic value of such fertilizer alternatives requires to assess the contamination risk for surface and groundwater. This equally applies to untreated or processed manure, sewage sludge or other biowastes. Physico-chemical characteristics of VMPs/AMA and of the matrix (manure) of interest must be taken into consideration, too. For instance, adverse effects of polluted runoff needs to be understood to mitigate the risk that those substances cross the ground barrier and reach the connected water bodies.

Boxall (2012) reviewed carefully the fate and transport processes for CECs in the agricultural soil environment, stating that "*once released an CEC will experience the same fate and transport process that occur of other classes of agricultural contaminants*" (Fig. 2).

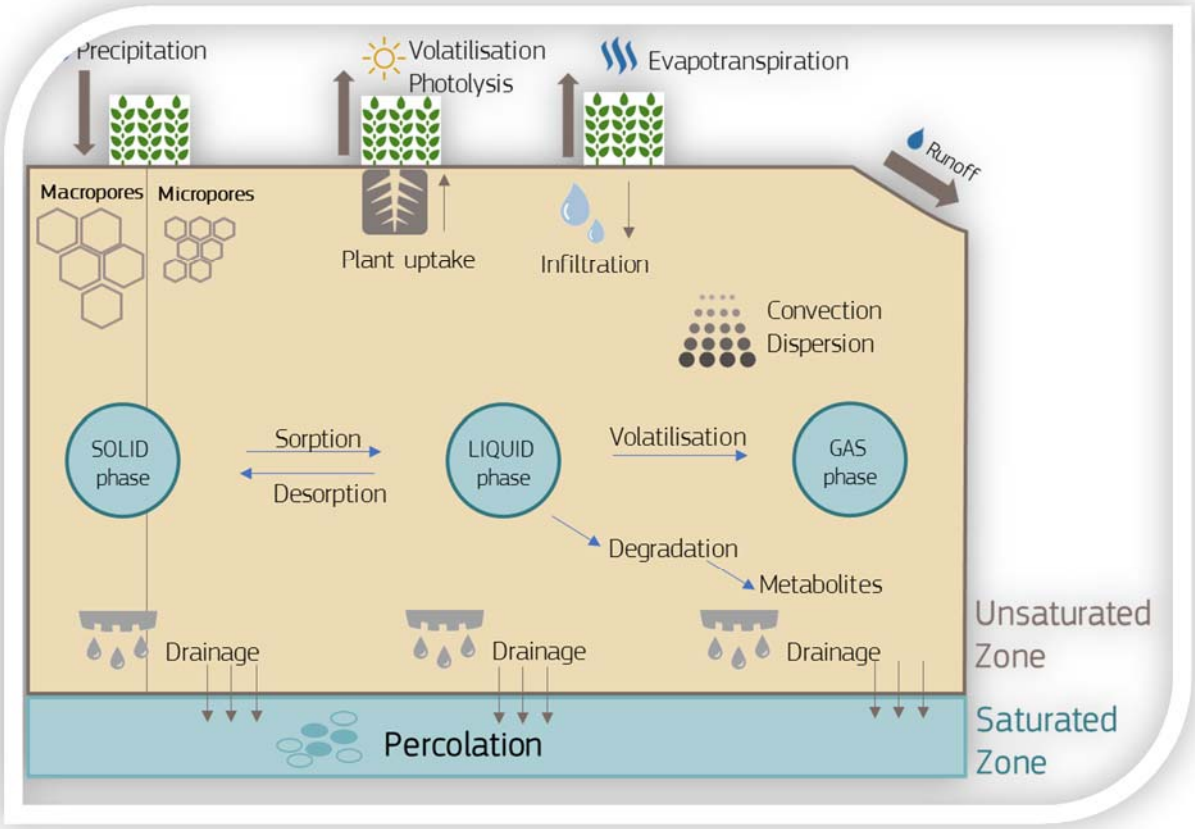



**Figure 2 – Overview on the transformation processes to be considered for CECs in agricultural soils (Modified from Boxall, 2012)**

## 1.2 The EU Water *Acquis* to be considered

The European Water Framework Directive and its daughter directives span the legal framework when it comes to the chemical and ecological status assessment of European water bodies. To this end, the WFD introduced a general requirement for ecological

protection, and a general minimum chemical standard, to cover all surface waters. The targeted "*good chemical status*" has in this context been defined in terms of compliance with all the quality standards established for chemical substances at European level. The Directive also provides a mechanism for renewing these standards and establishing new ones by means of a prioritisation mechanism for hazardous chemicals. Obviously, the process of such identifying substances as well as the sheer number of substances for which the so-called environmental quality standards (EQS) can be defined is limited.

The case of groundwater is somewhat different. The presumption in relation to groundwater should broadly be that it should not be polluted at all. For this reason, setting chemical quality standards may not be the best approach, as it gives the impression of an allowed level of pollution to which Member States can fill up. A very few such standards have been established at European level for particular issues (nitrates, pesticides and biocides), and these must always be adhered to. But for general protection, a precautionary approach has been chosen.

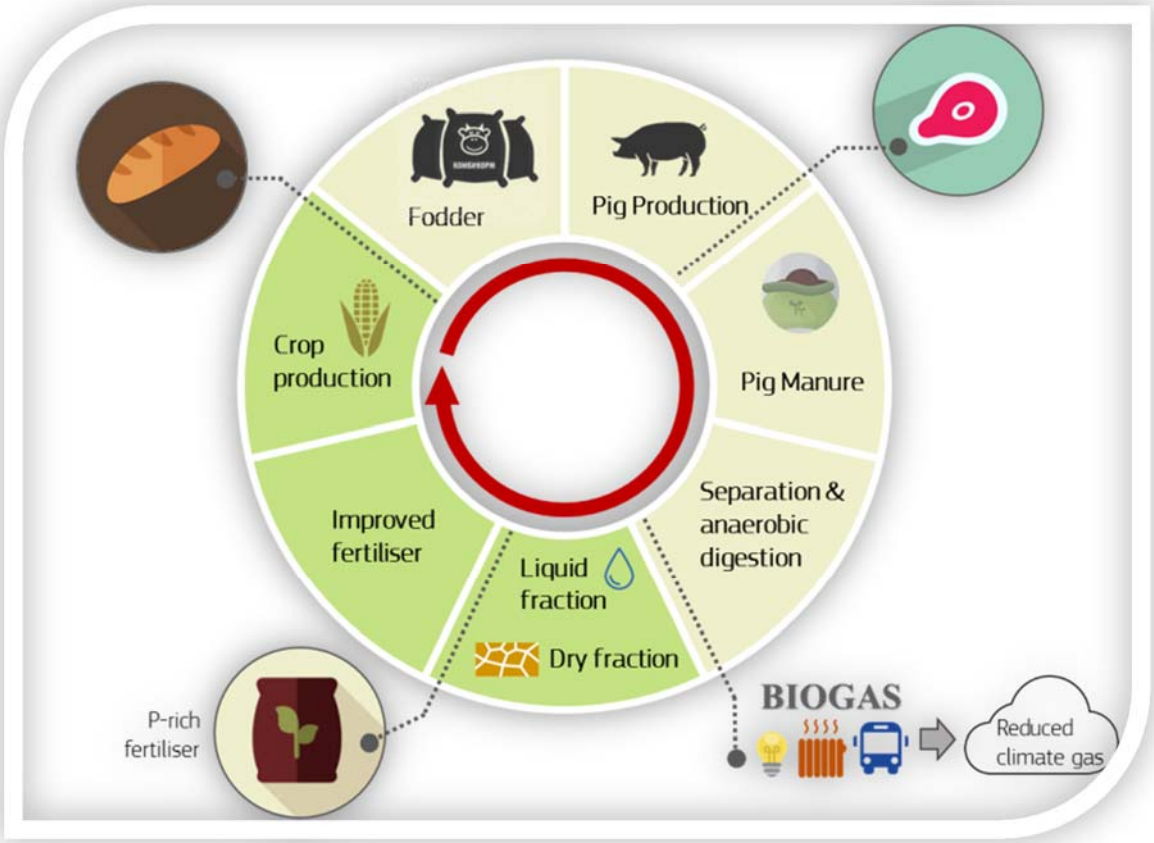



**Figure 3 – Components of an optimal processing of pig slurry (modified from EC 2010)**

In the context of manure management and the assessment of its environmental impact versus agronomic benefit, the most relevant legal instrument is presumably the Nitrates Directive. The Nitrates Directive[1] (ND) aims at protecting water from diffuse pollution (nitrates and eutrophication) from agricultural activity. To this end, the directive establishes restrictions on use of nitrogen containing fertilising materials[2] in areas with

[1] Council Directive of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources (91/676/EEC)

[2] It is to be noted that the Nitrates Directive and Fertilisers Regulation (EC 2003/2003) use a different definition and spelling for a similar word. Under the Nitrates Directive, a *fertilizer*, spelled with a Z, is defined as any nitrogen containing substance utilized on land to enhance growth of vegetation. Under the Fertilisers

nitrates pollution in waters (Nitrates Vulnerable Zones-NVZ). Manure and manure-based fertilisers are subject to more stringent restrictions than nitrogen containing mineral/chemical fertilisers. More specifically, in NVZ the ND restricts the use of manure, including processed manure, to 170 kg of N/hectare per year. This maximum limit for manure based fertilising materials in polluted areas is based on the consideration that the associated environmental risk, especially nitrogen leaching risk, is higher for manure than for other fertilisers.

In line with the objectives of the Circular Economy Action Plan, there is an opportunity to encourage recycled nutrients that can replace nutrients from primary raw materials. As shown in Fig. 3 for the example of pig production and the related generation of pig manure, the main challenge is to obtain recycled nutrient resources that have an equal or better environmental performance than the primary nutrient resources they potentially could replace.

Efforts are on-going across the EU to develop manure processing technologies that allow turning manure into a safe and agronomical valuable resource that could be more widely used in NVZ. The challenge remains on how to apply scientifically sound criteria to ensure the agronomic and environmental performance of these new materials.

It is clear that these criteria can only be developed by gaining a sound knowledge on the specific chemical compounds, which are closely related to animal husbandry in agricultural context, namely veterinary medicinal and anti-microbial agents or accompanying products.

## 1.3 Manure processing technologies

The proposed revision of the Regulation (EC) No 2003/2003[3] under the Circular Economy Action Plan, has seen a scope extension from purely mineral fertilisers to organic fertilisers. This could include materials partially or entirely processed from manure, as well as fertiliser blends with varying amounts of mineral and organic nutrient forms. This means that the definition in the original Nitrates Directive of a "*chemical fertilizer*" ("*any fertilizer which is manufactured by an industrial process*") and that of a "*livestock manure*" ("*waste products excreted by livestock or a mixture of litter and waste products excreted by livestock, even in processed form*") and their differences are in some cases becoming more and more blurred.

Therefore, action is needed to ensure that the on-going technological and market developments for the recycling of nutrients can be reconciled with the continued objective of protecting water bodies against pollution originating from manure.

The agronomic but also environmental value of such technology can only be understood if expected environmental risks are assessed properly and set against the economic potential. To do so, it is essential that the usual various technologies of processing manure are modelled/tried in various combinations and thus delivering a variety of output materials of differing properties (Fig. 4). While the technologies are fairly well described regarding technological aspects and regarding the fertilizing characteristics of the derived processed manure, it is largely unknown what happens to the VMP/AMAs stemming from animal husbandry and the related manure.

A first inventory of manure-processing technology, as well as an economic and environmental feasibility assessment were commissioned by DG ENV in 2010 (Foged *et al.*, 2011a, 2011b; Flotats *et al.*, 2011). The different processing technologies can be re-grouped in three main categories, i.e. separation techniques, anaerobic digestion and

---

Regulation, *fertiliser*, spelled with an S, has a wider definition of a material, the main function of which is to provide nutrients to plants. These nutrients can be N but also P, K, Ca, Mg, Na, S, B, Co, Cu, Fe, Mn, Mo or Zn. For clarity purposes, this document applies by default the spelling and definition from the Fertilisers Regulation and explicitly states when fertilisers are assumed to contain nitrogen. The spelling with z is only maintained for direct references to definitions from the Nitrates Directive.

[3] Regulation (EC) No 2003/2003 of the European Parliament and of the Council of 13 October 2003 relating to fertilisers

alteration by additives and further physicochemical treatment (Fig. 4). According to the study findings the overwhelming part of treatment takes place at farm level and only at significantly larger scale in small/medium[4] size installation or large-scale[5] plants. The same investigation concluded that in total 7.8% of the livestock manure production in the EU is being processed, equal to 108 million tons, containing 556 000 tons of nitrogen and 139 000 tons phosphorus. 168 million tons livestock manure and other products are processed, whereof around 60 million tons (168 minus 108 million tons) are end and by-products from other processes and non-livestock manure biomasses. The largest share of the livestock manure production is processed in Italy, Greece and Germany, with 36.8, 34.6 and 14.8% respectively (Table 1).

**Table 1 – Overview on technologies used for livestock manure treatment in the Member States** (data from Foged *et al.*, 2011a)

| Technology | Farm size installations | Small/medium size installation | Large-scale installation | Amount in 1000 tons | % of livestock manure treated in observed member states |
|---|---|---|---|---|---|
| **Separation** | 10 935 | 120 | 75 | 43 383 | 3,1 |
| **Additives and other pre/1st treatment** | 606 | 44 | 18 | 5 877 | 0,4 |
| **Anaerobic treatment** | 4 692 | 459 | 105 | 49 033 | 3,5 |
| **Solid fraction treatment** | 1 254 | 169 | 63 | 7 422 | 0,5 |
| **Liquid fraction Treatment** | 407 | 121 | 59 | 2 149 | 0,2 |
| **Air cleaning in manure processing plant*** | 0 | 30 | 39 | 0 | 0 |
| **TOTAL** | **17 894** | **943** | **359** | **107 864** | **7,8** |

Flotats *et al.* (2011) identified 45 processing technologies as standalone technologies or belonging to combined treatment systems, which have been categorised as follows:

- **Separation techniques**: System with the objective of separating manure into two flows: a concentrate (solid fibre fraction) and a diluted fraction (liquid fraction).
- **Additives and other pre/1st treatments**: Set of processes which have the objective to prepare the material for a further purpose or treatment.
- **Anaerobic treatment**: Series of biological processes in which microorganisms break down organic molecules in absence of oxygen, resulting in the production of a mixture of gases, named biogas, mainly composed of methane and carbon dioxide.
- **Treatment of the fibre/solid fraction**: Processing methods especially suitable for solid manures or solid fractions obtained after separation.
- **Treatment of the liquid fraction**: Processing methods especially suitable for much diluted manures or liquid fractions obtained after separation.

[4] treating up to 50 000 tons of manure per year
[5] treating more than 50 000 tons of manure per year

- **Air cleaning** (as part of manure processing plant): Methods applied to clean process air used during some manure treatment (i.e. exhaust air from composting).

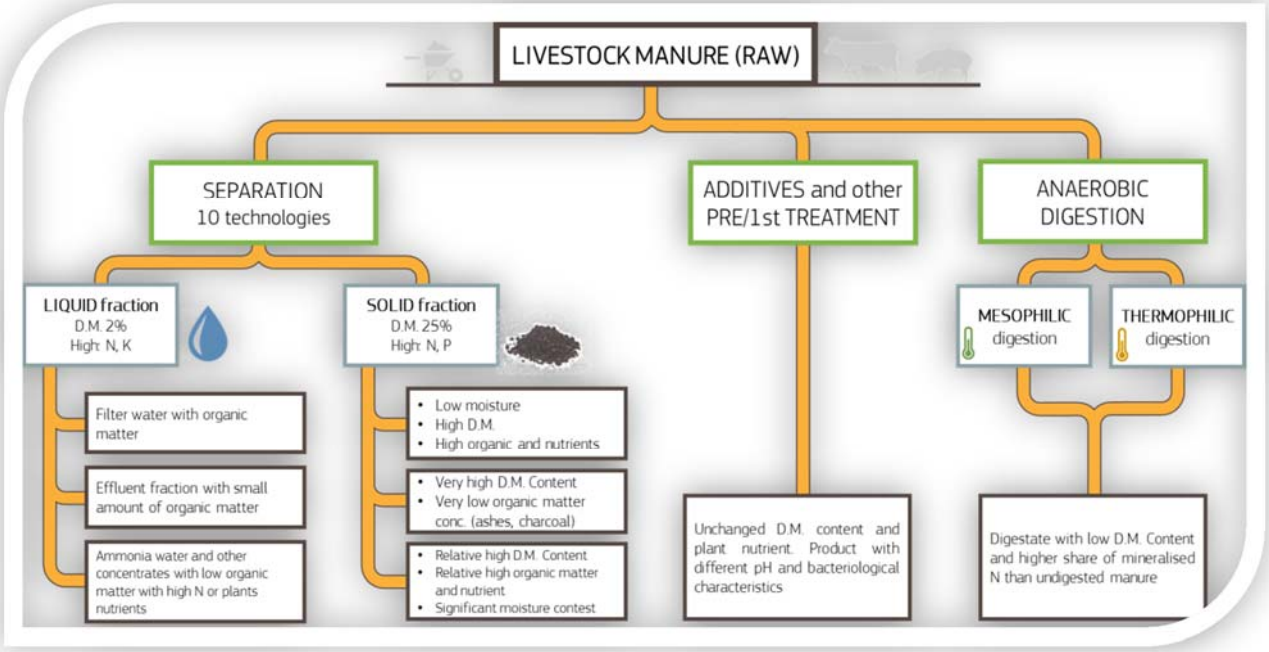



**Figure 4 – Overview of manure processing technologies**

Although the 45 unitary processes identified could be theoretically combined and integrated in different ways in a given facility, the fact is that only few combinations or groups of combinations are possible and interesting for building a ***technological strategy*** fitting a given objective.

## 1.4 Medicinal veterinary products in manure

"*Veterinary medicinal products (VMPs) are excreted by the treated animals in the form of unchanged parent substances and metabolized compounds. The excrements from stabled animals are usually collected and stored mainly as liquid or solid manure before they are used as fertilizers on arable land and grassland. Biocides, which are used for the disinfection of stables, end up in the stored animal excrements. Via manure application in agriculture, veterinary medicines and biocides are hence released into the environment and consequently affect soil and water quality*" (Wohde *et al.*, 2016) (Fig. 5). When considering the risk of contamination of surface and groundwater adjacent to lands fertilised with animal manure or biosolids, both physico-chemical characteristics of VMP/AMA and of the matrix (manure) of interest must be considered. Polluted runoff, caused by rainfalls or snowmelt or even irrigation, moves over and through the ground and carries natural and human-made pollutants that can potentially reach surface water and the underground source of drinking water.

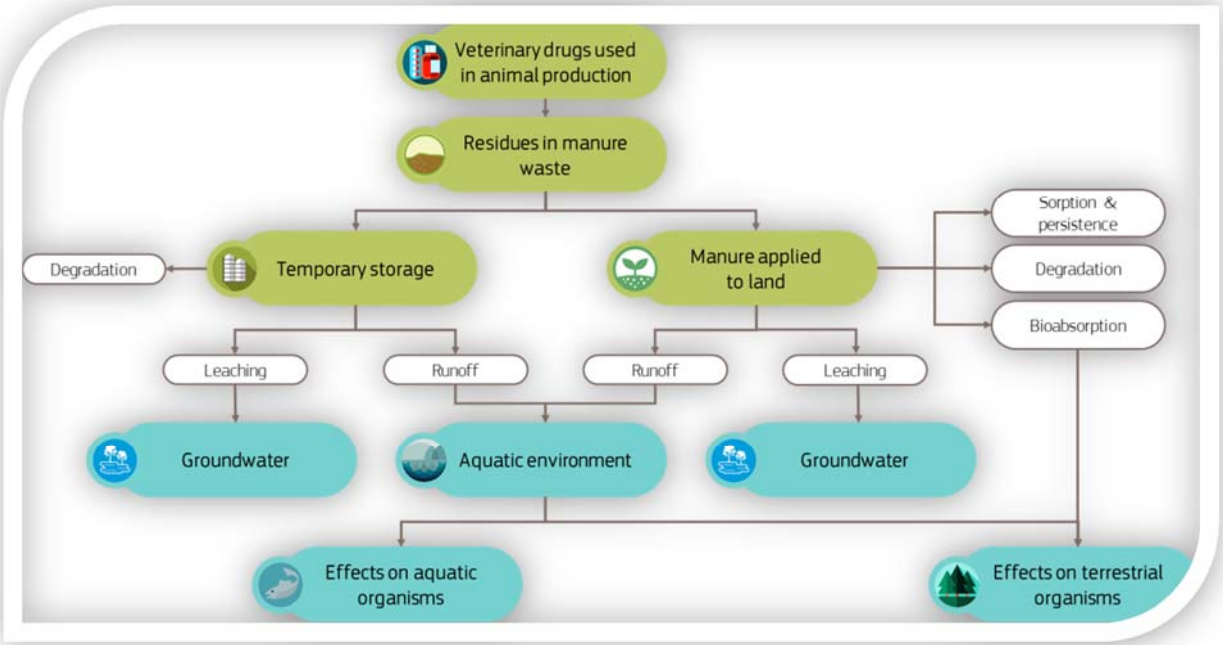



**Figure 5 –Relevant entry pathways of VMP via manure into the environment**

In a study report prepared for the Executive Agency for Health and Consumers a comparison was made between medicinal products used for humans and veterinary applications (BIO Intelligence Service, 2013). In general, veterinary medicinal products were reported to be used in smaller quantities than human medicinal products. Veterinary medicinal products are extensively used in farming for therapeutic and metaphylactic purposes, which represent more than 95% of the use of medicines in the rearing of piglets and turkey, more than 70% of the use of medicines for pigs and poultry and 30% of the use of medicines for bovine (Kools *et al.*, 2008). Some commonly used treatment practices, such as campaign treatment of all animals in the farm, need very high quantities of veterinary medicinal products. For example, if 1 000 cows or 10 000 pigs or 100 000 poultry are treated through feed, the quantities of the used preparation of veterinary medicinal products in the campaign may be remarkably high. The types of medicinal products used and prescribing patterns (dosage, length of treatment periods and formulation) may vary significantly for the various species in different countries, as for antimicrobials (EMA, 2010).

The same study also quotes that in Germany for instance, 98% of the antibiotic APIs (Active Pharmaceutical Ingredient) in veterinary medicines are used for treating pigs and poultry, while the remaining 2% are spread among other species. In Belgium on the other hand, cattle farming is a major consumer of antimicrobial agents. According to our knowledge still today, there is no overall EU picture regarding these figures. EMA however, reviewed again in 2014 reported sales figures for veterinary antimicrobial agents in 29 European countries (EMA, 2014). The EMA report concluded that in EU-27 plus Switzerland and Iceland, the total amount of active ingredients used primarily for food producing animals passed 9 000 tons, of which more than 60 per cent where consumed in three countries (Spain, Italy and Germany).

## 1.5 Compound classes and compounds used most frequently and the situation in the EU

Medicinal products for veterinary use, have to be authorised either at Member State or Community level before they can be placed on the Medicinal products for veterinary use, just like medicinal products for human use, have to be authorised either at Member State or Community level before they can be employed in the European Union.

The following section is retrieved of the Commission´s homepage informing about the state of play (https://ec.europa.eu/food/animals/health/regulation_en):

*"The first objective of the European legislation is to protect public and animal health. The second objective is the completion of the internal market for pharmaceutical products. Particular special rules are applied to ensure consumer protection from residue limits from pharmacologically active substances used in food-producing animals.*

*The Commission is considering a revision of the legal framework for veterinary medicinal products. On 10 September 2014 the European Commission has adopted a pair of proposals on veterinary medicinal products and medicated feed. The proposal on veterinary medicinal products aims to:*

- *Increase the availability of veterinary medicinal products;*
- *Reduce administrative burden;*
- *Stimulate competitiveness and innovation;*
- *Improve the functioning of the internal market; and*
- *Address the public health risk of antimicrobial resistance.*

*In 2016, the European Parliament and the Council adopted the Regulation on transmissible animal diseases ("Animal Health Law").*

*Overall, the single, comprehensive new animal health law will support the EU livestock sector in its quest towards competitiveness and safe and smooth EU market of animals and of their products, leading to growth and jobs in this important sector:*

- *The huge number of legal acts are streamlined into a single law*
- *Simpler and clearer rules enable authorities and those having to follow the rules to focus on key priorities: preventing and eradicating disease*
- *Responsibilities are clarified for farmers, vets and others dealing with animals*
- *The new rules allow greater use of new technologies for animal health activities - surveillance of pathogens, electronic identification and registration of animals*
- *Better early detection & control of animal diseases, including emerging diseases linked to climate change, will help to reduce the occurrence and effects of animal epidemics*

- *There will be more flexibility to adjust rules to local circumstances, and to emerging issues such as climate and social change*
- *It sets out a better legal basis for monitoring animal pathogens resistant to antimicrobial agents supplementing existing rules and two other proposals currently being negotiated in the European Parliament and Council, on veterinary medicines and on medicated feed*

*The animal health law is part of a package of measures proposed by the Commission in May 2013 to strengthen the enforcement of health and safety standards for the whole agri-food chain. It is the biggest and the first of those to get the approval of the co-legislators. The animal health law is also a key output of the Animal Health Strategy 2007-2013, "Prevention is better than cure".*

*Several delegated and implementing acts will be adopted by the Commission until April 2019 to make the new rules applicable. The Commission will duly consult experts, Member States and other interested parties, EU stakeholders (e.g. in the Animal Health Advisory Committee) during the drafting of these delegated and implementing acts, in the spirit of better regulation."*

The following tables give a general overview on typical and frequently used compounds in veterinary applications. The review is neither complete nor claims any form of full representativeness or specific applicability for the EU. The information was compiled to facilitate the development of appropriate analytical protocols for the scope of this study. It has to be stressed that brand names appearing in this article are examples only. No endorsement is intended, nor is criticism implied of similar products not mentioned.

Some of the compounds listed may be banned or restricted in the EU or single Member States and have been considered only for analytical methodology development purposes.

**Table 2 – Overview on selected active pharmaceutical ingredients, compounds and veterinary medicinal products used in poultry, swine, dairy and sheep industries**

| Compound class | Examples | Comments |
|---|---|---|
| | **Poultry Production** | |
| **Antibiotics** | Bacitracin (e.g., BMD, Pennitracin MD, Albac), Ionophores, Chlortetracycline (e.g., Chloratet, Aureomycin), Lincomycin (e.g., Lincomx), Oxytetracycline (e.g., Terramycin), Penicillin, Tylosin (e.g., Tylan), Virginiamycin (e.g., Stafac, V-Max) | Antibiotics are used in poultry production not only for therapeutic purposes; some producers also administer sub-therapeutic dosages for growth promoting purposes, and residues can be detected in eggs and poultry meat if proper withdrawal protocols are not followed. Furthermore, zoonotic bacteria may acquire resistance to antibiotics as a result of administration of sub-therapeutic dosages (Diaz-Sanchez *et al.*, 2015). |
| **Coccidiostats** | Amprolium (e.g., Amprol, Corid), Bambermycin (e.g., Flavomycin, GAINPRO), Decoquinate (e.g., Deccox), Diclazuril (e.g., Clinacox), Halofuginone hydrobromide (e.g., Stenorol), Lasalocid (e.g., Avatec), Monensin (e.g., Coban), Narasin (e.g., Monteban), Nicarbazin (e.g., Nicarb 25%), Salinomycin (e.g., Bio-Cox, Sacox), Semduramicin (e.g., Aviax), Sulfadimethoxine and ormetoprim 5:3 (e.g., Rofenaid) | Coccidiosis is a common parasitic disease of poultry and important from an economic point of view in poultry industry (Kant *et al.*, 2013) It is the result of an infestation of coccidia in the intestines. The agents used for the prevention and control of coccidia infections are termed as anticoccidial drugs. The agents which destroy the coccidial population are termed as coccidiocidal and agents which prevent the replication and growth of coccidial population are known as coccidiostatic. |
| **Medications for controlling intestinal worms** | Fenbendazole—for turkeys only (e.g., Safe-Guard), Hygromycin B—for chickens only (e.g., Hygromix-8) | There are several types of parasitic worm that can infest poultry, including roundworm, tapeworm, cecal worms, and capillary worms. There are only a few products that can be added to conventional poultry feed to control internal parasites. No products are approved for use with egg-laying hens. |
| **Products for Controlling External Parasites** | Permethrin-based medications (e.g., Prozap Garden and Poultry Dust), Tetrachlorvinphos-based medications (e.g., Rabon), Carbaryl-based medications (e.g., Sevin - voluntarily withdrawn for use with poultry) | Typical external parasites of poultry include mites, lice, fleas, and ticks |
| **Products for Controlling Darkling Beetles** | Imidacloprid (Brand name: CREDO)<br>Cyfluthrin (Bfrand name: TEMPO) | Darkling beetles are a common problem in poultry facilities. The adults are black with hardened front wings and antennae that start under a ridge near the eyes. The larvae (referred to as mealworms) are worm-like and slightly hardened for burrowing. Both the larvae and beetles eat decaying leaves, sticks, grass, dead insects, faeces, and grains. |
| **Products for Fly Control** | Cyromazine (e.g., Flyzine, Larvadex, and Solitude IGR) | |

| Compound class | Examples | Comments |
|---|---|---|
| | **Swine industry** | |
| **Antibiotics** | Amoxicillin, Ampicillina, Apramycin, Arsenilic acid, Bacitracin, Bambermycins, Chlortetracycline, Efrotomycin, Erythromycin, Gentamycin, Lincomycin, Neomycin, Oleandomycin, Oxytetracycline, Penicillin, Spectinomycin, Streptomycin, Tetracycline, Tiamulin, Tylosin, Virginiamycin | Antibiotics are typically used in hogs to treat various infections or increased growth and feed efficiency (Source Compendium, 1997, Source: Compiled from FDA Approved Animal Drug List (Green Book), 1998a, and Feed Additive) |
| **Chemotherapeutics used for hogs** | Arsanilate sodium, Arsanilic acid, Carbadox, Roxarsone, Sulfaethoxypyridazine, Sulfachlorpyidazine, Sulfamethazine, Sulfathiazole | Unlike antibiotics, which aim specifically on bacteria, chemotherapeutics kill body cells. |
| | **Dairy industry** | |
| **Antibiotics** | Amoxicillin, Ampicillin, Bacitracin, Ceftiofur, Cephapirin, Chlortetracycline, Cloxacillin, Dihydrosteptomycin, Erythromycin, Furamazone, Gentimycin, Hetacillin, Lasalocida, Monensinc, Neomycin, Novobiocin, Oxytetracycline, Penicillin, Pirlimycin, Streptomycin, Tetracycline, Tilmicosin, Tylosin | |
| **Sulfonamides** | Sulfabromomethazine, Sulfachloropyridazine, Sulfadimethoxine, Sulfaethoxypyridazine, Sulfamethazine, Sulfamethoxine | |
| **Steroid Products** | Estradiol, Estradiol/Progesterone, Estradiol/Testosterone, Estradiol/Trenbolone, Melengestrol, Trenbolone, Zeranol | |
| | **The sheep industry** | |
| **Antibiotics** | Chlortetracycline, Erythromycin, Neomycin, Oxytetracycline, Penicillin, Penicillin/Streptomycin | |

**Table 3 – Examples of selected active pharmaceutical ingredients, compounds and veterinary medicinal products used for Minor Species**

| Species | Compound/Active ingredient | Claims |
|---|---|---|
| **Reindeer** | Ivermectin | Grubs |
| **Duck** | Chlortetracycline | Growth, feed efficiency, various infections |
| | Novobiocin | Various infections |
| **Goat** | Decoquinate | Coccidiosis |
| | Monensin | Coccidiosis |
| | Neomycin | Enteritis |
| | Penicillin/ streptomycin | Various infections |
| | Phenothiazine | Worms |
| | Thiabendazole | Worms |
| **Pheasant** | Amprolium | Coccidiosis |
| | Bacitracin | Growth, feed efficiency, various infections |
| | Penicillin | Growth, feed efficiency |
| | Thiabendazole | Worms |
| **Quail** | Bacitracin | Growth, feed efficiency, various infections |
| | Monensin | Coccidiosis |
| | Penicillin | Growth, feed efficiency |
| **Rabbits** | Penicillin/ streptomycin | Various infections |
| | Sulfaquinoxaline | Coccidiosis |

**Table 4 – Examples of selected active pharmaceutical ingredients, compounds and veterinary medicinal products used in aquaculture**

| Drug | Active Ingredient | Indication | Species |
|---|---|---|---|
| **Finquel, MS-222** | Tricaine methanesulfonate | Sedation/anesthesia | Fish (Ictaluridae, Salmonidae, Esocidae, Percidae), other aquatic poikilotherms |
| **Formalin-F; Paracid-F; Parasite-S** | Formalin | Control protozoa and monogenetic trematodes (Icthyopthirius, Chilodonella, Costia, Scyphidia, Epistylis, Trichodina spp. and Cleidodiscus, Gyrodactylus, Dactylogyrus spp.) | Salmonids, catfish, largemouth bass, bluegill |
| | | Control fungi of the family Saprolegniaceae | Salmodi and esocid eggs |
| **Parasite-S** | Formalin | Control protozoan parasites (Bodo spp., Epistylis spp., and Zoothamnium spp.) | Panaeid shrimp |
| **Romet-30** | Sulfadimethoxine and ormetoprim | Control furunculosis (Aeromonas salmonicida) | Salmonids |
| | | Control enteric septicemia (Edwardsiella ictaluri) | Catfish |
| **Terramycin** | Oxytetracycline monoalkyl trimethyl ammonium | Mark skeletal tissue | Pacific salmon |
| | | Control ulcer disease, furunculosis, bacterial hemorrhagic septicemia, and pseudomonas disease (Hemophilus piscium, Aeromonas salmonicida, Aeromonoas liquefaciens, Pseudomonas) | Salmonids |
| | | Control bacterial hemorrhagic septicemia and pseudomonas disease | Catfish |
| | | Control gaffkemia (Aerococcus viridans) | Lobster |

## 1.6 Manure storage in the EU

In the context of compiling agro-environmental indicators (AEI), DG EUROSTAT assessed the management of manure in the EU with a focus on trends in manure storage facilities in agricultural holdings (Table 5). The indicator is primarily of relevance for the agri-environmental indicator AEI 18 - Ammonia emissions and nutrient leaching losses from animal manures.

**Table 5 - Holdings with manure storage facilities, EU-27 and NO[6]**

| | Holdings with manure storage facilities | Holdings with storage facilities for solid dung | | Holdings with storage facilities for liquid manure | | Holdings with storage facilities for slurry | |
|---|---|---|---|---|---|---|---|
| | # | # | % | # | % | # | % |
| EU-27 | 1 977 530 | 1 591 830 | 80% | 959 290 | 49% | 610 910 | 31% |
| BE | 35 210 | 26 110 | 74% | 16 910 | 48% | 17 130 | 49% |
| BG | 530 | 430 | 81% | 80 | 15% | 50 | 9% |
| CZ (1) | 11 690 | 13 640 | 117% | 15 040 | 129% | 1 180 | 10% |
| DK (3) | 29 060 | 21 060 | 72% | 7 880 | 27% | 16 040 | 55% |
| DE (1) | 261 440 | 211 320 | 81% | 162 310 | 62% | 149 140 | 57% |
| EE | 1 760 | 1 510 | 86% | 220 | 13% | 90 | 5% |
| IE | 88 860 | 54 560 | 61% | 0 | 0% | 67 100 | 76% |
| EL | 10 000 | 9 070 | 91% | 570 | 6% | 860 | 9% |
| ES (3) | 81 960 | 27 150 | 33% | 36 810 | 45% | 23 230 | 28% |
| FR | 146 270 | 107 200 | 73% | 61 470 | 42% | 56 140 | 38% |
| IT (2) | 126 100 | 112 800 | 89% | 15 600 | 12% | 42 870 | 34% |
| CY | 200 | 100 | 50% | 100 | 50% | 50 | 25% |
| LV (4) | 11 650 | 4 630 | 40% | 390 | 3% | 8 970 | 77% |
| LT | 2 830 | 1 140 | 40% | 100 | 4% | 2 090 | 74% |
| LU (3) | 1 590 | 1 240 | 78% | 1 030 | 65% | 1 030 | 65% |
| HU | 44 490 | 39 140 | 88% | 4 840 | 11% | 4 090 | 9% |
| MT | 580 | 520 | 90% | 50 | 9% | 80 | 14% |
| NL (3) | 39 160 | 24 690 | 63% | 7 430 | 19% | 32 330 | 83% |
| AT | 115 630 | 103 340 | 89% | 92 180 | 80% | 28 890 | 25% |
| PL (1) | 701 900 | 598 820 | 85% | 442 010 | 63% | 68 630 | 10% |
| PT | 22 650 | 9 960 | 44% | 10 590 | 47% | 10 640 | 47% |
| RO | 3 010 | : | : | 3 010 | 100% | 120 | 4% |
| SI | 57 330 | 54 400 | 95% | 26 230 | 46% | 12 080 | 21% |
| SK (1) | 36 070 | 59 480 | 165% | 12 070 | 33% | : | : |
| FI (4) | 30 930 | 22 610 | 73% | 14 570 | 47% | 11 560 | 37% |
| SE (3) | 43 640 | 39 150 | 90% | 8 240 | 19% | 26 070 | 60% |
| UK (1) | 72 990 | 47 760 | 65% | 19 560 | 27% | 30 450 | 42% |
| NO (3) | 41 030 | 14 890 | 36% | 2 760 | 7% | 28 650 | 70% |

\# Number of holdings

% Share of holdings with manure storage facilities

Notes are explained in the section 'Data sources and availability'.

Special values:

0 Less than half the final digit shown and greater than real zero

: Data not available

[6] Source: Eurostat, http://ec.europa.eu/eurostat/statistics-explained/index.php/File:Holdings_with_manure_storage_facilities,_EU-27_and_NO,_2003.jpg

It is measured by the following indicators:

- Share of holdings with livestock which have manure storage facilities in total holdings with livestock.
- Share of holdings with different manure storage facilities.

According to the collected information the number of holdings with manure storage facilities in EU-27 increased from 2.0 to 2.2 million between 2003 and 2010. In 2010, 33% of the holdings with livestock in EU-28 had storage facilities for manure.

At country level there were however large differences: while in Austria, Slovakia, Sweden and Switzerland almost all holdings with livestock had manure storage facilities, in Bulgaria and Cyprus almost none of the holdings with livestock had manure storage facilities.

While in EU-28 only 14% of the holdings with manure storage facilities for solid dung used a cover to protect emissions to air and water in 2010, this was the case for 87% of the holdings which had manure storage facilities for liquid manure and for 69% of the holdings with manure storage facilities for slurry.

Data on manure storage and related information on animal housing and manure application techniques are available for all EU-28 Member States from a special one-off survey carried out in 2010, namely the Survey on agricultural production methods (SAPM).

# 2 Towards an EU-wide pilot-campaign

As indicated by the information collected in Chapter 1 of this report, understanding the role of agricultural application of manure in the propagation of anti-microbial resistances (AMR), the interspecies exchange or antibiotic-resistant genes as well as the role of veterinary antimicrobial agents is a priority field of research. There is still a significant data gap on the drainage from lands that have been irrigated with treated wastewater or that have been fertilised directly with animal manure or derived biosolids (e.g. after digestion). Veterinary medicinal products (VMPs), many of which administered as antimicrobial agents (AMA) are excreted by the treated animals in the form of unchanged parent substances and metabolized compounds. The excrements from stabled animals in Europe and North America are collected and stored mainly as liquid or solid manure before they are used as fertilizers on arable land and grassland. Biocides, which are used for the disinfection of stables, end up in the stored animal excrements. Via manure application in agriculture, the veterinary medicines and biocides are released into the environment and consequently affect soil and water quality. Questions on the spatial occurrence and fate of these compounds, as well as of their metabolization and degradation compounds remain open and only sporadic studies with selected applications exist. An EU-wide analyses of the environmental impact has not been undertaken and existing case studies are of limited comparability due to the different methodological approaches chosen.

To address these questions properly, the collection of EU-wide data sets are of utmost importance, e.g. for the subsequent development of modelling scenarios (Pistocchi *et al.* 2010, 2012). Gawlik *et al.* (2012) as well as Loos *et al.* (2009, 2010) successfully developed and tested an appropriate approach to deliver such data sets for compounds of emerging concern.

The advances made by the JRC Exploratory research Programme lead to the development of a robust and reliable sampling device allowing the onsite-extraction and stabilisation of compounds of emerging concern such as the aforementioned AMAs and VMPs (Mariani *et al.*, 2017). In order to develop an appropriate testing protocol, which combines the features of the MARIANI-Box as well as elucidates the transfer and fate pathways of active pharmaceutical ingredients used in livestock applications a first exploratory pilot campaign was conducted in November 2017.

## 2.1 Objectives of the exploratory pilot sampling

When addressing the risk of contamination of surface and groundwater adjacent to lands fertilised with animal manure or biosolids, both physico-chemical characteristics of AMA/VMP and of the matrix (manure) of interest must be accounted for. Polluted runoff, caused by rainfalls or snowmelt or even irrigation, moves over and through the ground and carries natural and human-made pollutants that can potentially reach surface water and the underground source of drinking water.

An appropriate test site for the development of a protocol needed hence to consider the following aspects:

- Access to manured lands and information on current manure application practices.
- Manure characterisation (i.e.: kind of manure (solid or liquid), manure storage temperature and time, manure dry matter content, timing of application, etc.).
- Availability of information on manure application techniques, weather data, farming and livestock.
- Access to water samples in surface ditches and/or drainage tile channels surrounding the manured soil.
- Access to the underground water wells (if available).

- Development of extraction and analytical method for non-processed and processed manure samples.

The primary objective of the first exploration was hence to define a realistic sampling scenario, which could be repeated at several occasions in other EU sites. It needed to be tested to which extent the sampling equipment can be operated under field conditions and whether it would be possible to collect additional information on manure properties, the agricultural context and the receiving water bodies.

In addition, it was attempted to question whether a (semi)quantitative relationship between the veterinary medicinal application and occurrence of the targeted compounds can be established.

The third aspect of this first exploration was to lay down a basis for the development of an analytical approach for a multi-compound method possibly addressing also a non-target approach.

## 2.2 Sampling location

### 2.2.1 Site characteristics and information

The testing site chosen was suggested by the Agricultural University of Nitra (AUN), which maintains an extensive manure research facility aiming inter alia at reuse of processed manure for applications others than direct fertilisation. Thus, the University's extensive research programs on the direct and indirect use of manure from different origin (cattle, pig, poultry and sheep) prior to or after digestion investigates to which extend the substrate can be used as growing media for algae aiming at fuel production.

The field sites examined in this exploratory investigation are in the Danube River Basin in vicinity to the Nitra River, thus offering interesting macro-regional aspects (Fig. 6, Tab. 6). The geographical position of Nitra (1h drive from Bratislava airport), facilitates dispatch operations. Furthermore, the test sites offer also access to groundwater wells and surface water bodies receiving agricultural runoff, thus allowing for a complete assessment on AMA/AMR/VMP propagation.


**Figure 6 - Satellite map of Oponice settlement and sampling sites**

The University offers also know-how on farm management practises and on use of veterinary drugs, thus facilitating the interpretation of results. The activity was indeed embedded into running field experiments. Manure application takes place usually in autumn and the collection of runoff in that period is favoured by seasonal precipitations. A subsequent additional 3 sampling in spring can be performed to ensure a seasonal comparison.

The Oponice site operated by the Slovak University of Agriculture has stock of approx. 700 dairy cattles whose consumption of food and pharmaceuticals is closely monitored. It has a solid production of crops such as wheat, sugar beet, corn and Medicago L. and investigates also the reuse of manure, e.g. through biogasification. The site covers an area of more than 500 Ha and the manure produced from its own cattle stock is also used as fertilizers on their fields. Table 6 gives some more information on the agricultural parcels used in this investigation.

**Table 6 – Information about agricultural parcels used for sampling. For geographical position of the single parcels (Numbered 1 to 7) refer to Figure 6**

| Parcel N° | Name | Crop | Harvest yield | Used fertilizer | Amount of fertilizer used | Date of application |
|---|---|---|---|---|---|---|
| **1** | Pod hradskou | Winter wheat | 4.71 t/ha | Liquid manure (Urine) | 30 $m^3$/ha | 12/08/2018 |
| | | | | Saltpetre | 150 kg/ha | 16/02/2017 |
| | | | | DASA (Ammonium nitrate + Ammonium sulphate) | 150 kg/ha | 02/03/2017 |

| Parcel N° | Name | Crop | Harvest yield | Used fertilizer | Amount of fertilizer used | Date of application |
|---|---|---|---|---|---|---|
| **2** | Lúka | Seed corn | 5.4 t/ha | Liquid manure (Urine) | 25 $m^3$/ha | 10/10/2016 |
| | | | | N-P-K (15:20:30) | 200 kg/ha | 03/05/2017 |
| | | | | Saltpetre | 150 kg/ha | 23/05/2017 |
| **3** | Parcelová časť | Seed corn | 5.4 t/ha | Manure (Cattle) | 30 t/ha | 22/09/2016 |
| | | | | N-P-K (15:20:30) | 200 kg/ha | 07/05/2017 |
| | | | | Saltpetre | 150 kg/ha | 23/05/2017 |
| **4** | Úzka | Medicago L. | 33.6 t/ha (green) | Liquid manure (Urine) | 20 $m^3$/ha | 15/08/2016 |
| **5** | Pod hradom | Winter wheat | 6.5 t/ha | Liquid manure (Urine) | 30 $m^3$/ha | 15/08/2016 |
| | | | | Saltpetre | 150 kg/ha | 17/02/2017 |
| | | | | DASA (Ammonium nitrate + Ammonium sulphate) | 150 kg/ha | 02/03/2017 |
| | | | | NITROHUM (nitrogen content of 390 kg/t of fertiliser) | 150 kg/ha | 18/03/2016 |
| **6** | Nad hradskou | Sugar beet | Not available | Manure | 40 t/ha | 01/09/2016 |
| | | | | Saltpetre | 150 kg/ha | 23/03/2017 |
| **7** | Za depom | Winter wheat (seeded) | Not available | Liquid manure (urine) | 30 $m^3$/ha | 03/08/2017 |

### 2.2.2 Impressions from the landscape

The following photographs were taken in occasion of a preparatory excursion in Mar. 2017 and document the rural characteristics of the side.

Water storage for farmland application. These water towers are typical for the area. Groundwater is pumped to the towers prior to use. Local water abstraction and consumption is documented and a tariff system for farmland use is in place. Access to this information is possible.

**Figure 7 – Water storage tower at the site**

The landscape is mainly flat with slight hills and elevations. The entire area is characterised by land use mainly for feeding crop production.

**Figure 8 – Landscape at the sampling site**

Artificial lakes like the ones shown are used for fish farming and exposed to run off. They interact also with natural buffer strips to regulate the water levels in the area and help to regulate nutrient loads.

**Figure 9 – Artificial lake at the Oponice site**

These channels are art of the typical drainage systems in the area by which water is drained on or in the soil to enhance agricultural production of crops. It may involve any combination of stormwater control, erosion control, and watertable control.

**Figure 10 – Drainage channel with water**

Larger drainage water agglomeration in vicinity to receiving water body connect the agricultural run-off with the local water bodies in the Nitra river basin.

**Figure 11 –Larger drainage channel**

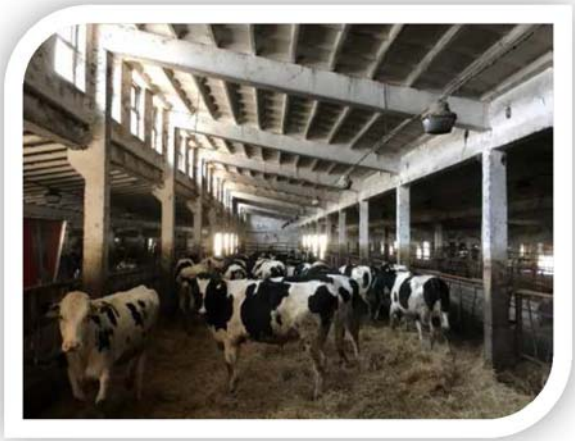
Biogas production from manure is a common practice in the area. The digested manure, which is stored in such facilities, is used as fertilizer once the digestion process is terminated. It has a significantly higher solid content than untreated manure. If used undigested, the pig manure contains ca. 5% dry matter, thus being similar to sewage sludge.

Both type of manure applications could be sampled in the study.

**Figure 12 – Storage for processed manure**

Pig manure is mainly used first in bio-gasification plants. The University research installation processes all pig manure from its research station.

**Figure 13 –Experimental bio-gasification plant**



Manure from cattle is used directly in the research plant. Here the stables of the University. The regions investigates also the further use of manure, e.g. as nutrient input to algae biofuel production thus increasing resource recovery.

**Figure 14 – Cow stables at the research facility**

### 2.2.3 Supplementary information on veterinary drugs employed in the area

The administration of veterinary medicinal products is highly regulated, in particular for livestock for food production. At the research station, the VMPs administered are documented in a logbook, which is updated continuously (Fig. 15).

While there is not direct traceability of the APIs used and the occurrence of the compounds in the manure-exposed waters, the logbook entries deliver very pertinent and useful information in the development of an analytical method.

The logbook in question documented date of application, the brand name of the product administered, reason for prescription, date and dose as well as the identification number of the animal receiving it and the signature of the authorized veterinarian.

Based on this information, as list of active pharmaceutical ingredients, which are likely to be detected in the manure-exposed waters was compiled (Table 7). The list was subsequently completed with a series of (commercially) available standards for likely APIs and compounds (see next chapter).

It is to be annotated that there is no direct traceability between the use of an API and its occurrence in the investigated samples. This applies also to the occurrence of non-authorized substances.

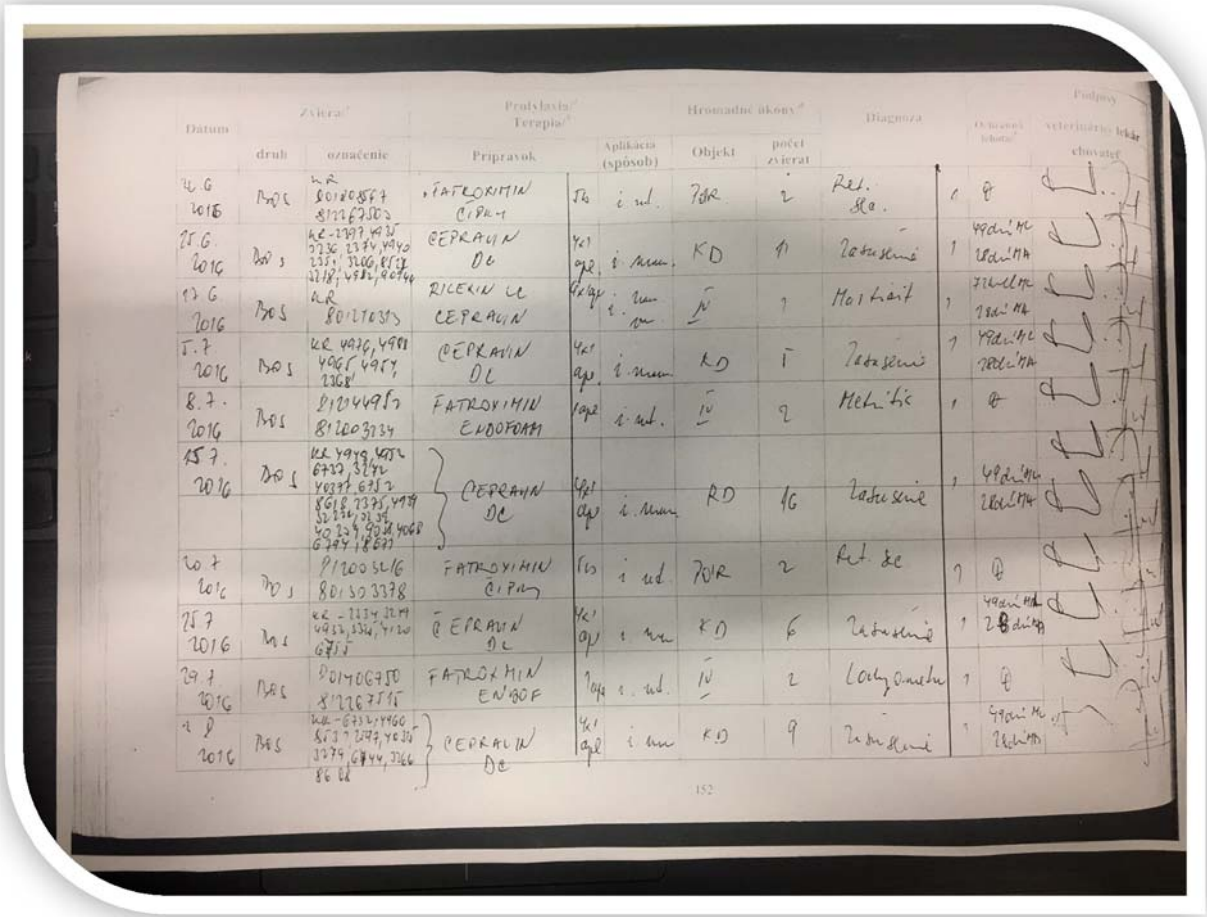
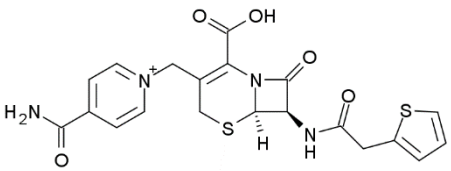
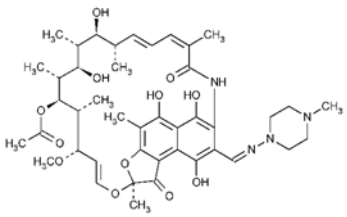
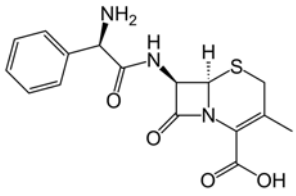
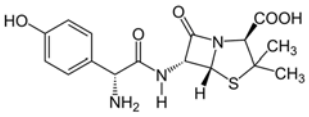
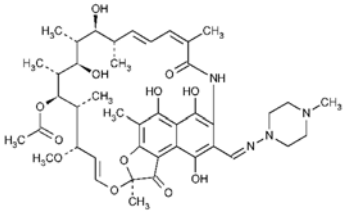
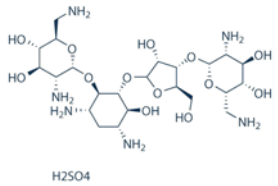
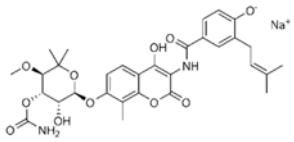
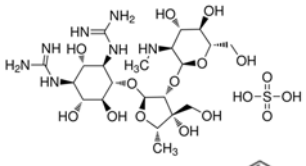
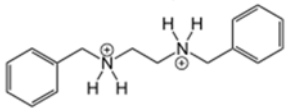
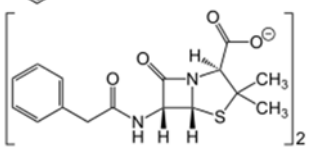
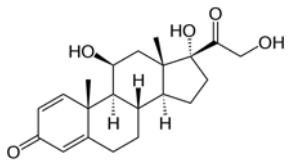
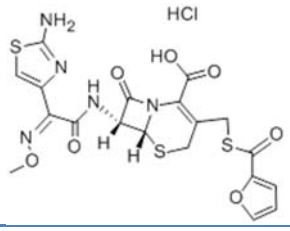
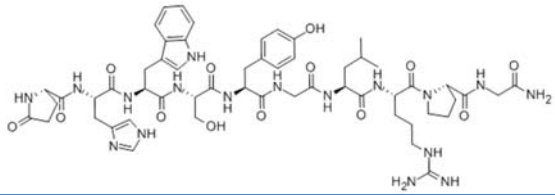
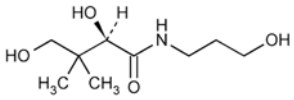
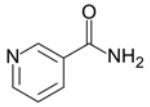
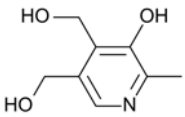
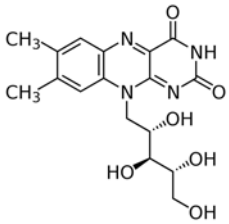
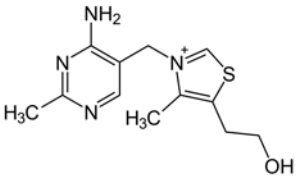
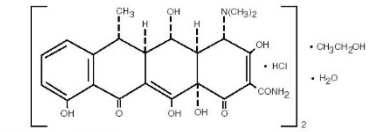


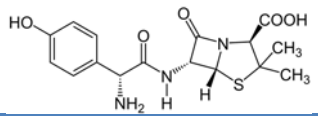
**Figure 15 - Extract of the logbook documenting application of VMPs at the research station**

**Table 7 – List of likely active pharmaceutical ingredients (APIs) in the cattle manure and receiving waters**

| Commercial name | Structural formula | Active ingredient |
|---|---|---|
| **Cepravin DC** |  | Cephalonium AB |
| **Fatroximin** |  | Rifaximin used as Anti-inflammatories Anti-mastitic Antibiotic |

| Commercial name | Structural formula | Active ingredient |
|---|---|---|
| **Rilexine 200** |  | Cefalexin. 1st generation cephalosporin AB (The cephalosporins are a class of β-lactam antibiotics) |
| **Duphamox:** |  | Amoxicillin trihydrate |
| **FATROXMIN foam:** |  | Rifaximin |
| **RISPOVAL IBR MARKER LIVE** | | Vaccine against Infectious Bovine Rhinotracheitis |
| **BOVIGAL IBR** | | Vaccine against Infectious Bovine Rhinotracheitis |
| **TETRA DELTA** |  <br>  <br>  <br>  <br>  | Active substances:<br>• Neomycin (as Neomycin Sulphate)<br>• Novobiocin (as Novobiocin Sodium)<br>• Dihydrostreptomycin (as Dihydrostreptomycin Sulphate)<br>• Procaine Benzylpenicillin<br>• Prednisolone |

| Commercial name | Structural formula | Active ingredient |
|---|---|---|
| |  | |
| **NAXCEL HD** |  | Safe and effective antibiotic against bacterial Pneumonia |
| **ACEGON** |  | Gonadorelin (as acetate) per animal |
| **DUPHALYTE** |  <br>  <br>  <br>  <br>  | Active Ingredients:<br>• Dexpanthenol<br>• Nicotinamide<br>• Pyridoxine Hydrochloride<br>• Riboflavin<br>• Sodium Phosphate, Thiamine Hydrochloride |
| **DOXYGAL PLV** |  <br>Doxycycline Hyclate<br>$(C_{22}H_{24}N_2O_8 \cdot HCl)_2 \cdot C_2H_6O \cdot H_2O$ M.W. 1025.89 | Doxycyclini hyclas 50.0 mg.<br>Doxycycline hyclate is a broad-spectrum antibiotic synthetically derived from oxytetracycline. |

| Commercial name | Structural formula | Active ingredient |
|---|---|---|
| **BETAMOX LA** |  | Amoxicillin |

## 2.1 Sampling

The exploratory sampling was carried out in November 8 to 10, 2017 in the Oponice /Nitra area. Sampling was conducted jointly by JRC Staff (H. Skejø, G. Mariani) and AUN Staff (N. Głowacka, J. Gaduš). Impressions from the sampling activities are shown in Figures 16 to 19. Weather conditions for the day of sampling were as follows:

**Table 8 - Weather conditions on day of sampling[7]**

| Temperature | Parametric value |
|---|---|
| **Mean Temperature** | 8 °C |
| **Max Temperature** | 9 °C |
| **Min Temperature** | 7 °C |
| **Degree Days** | |
| **Heating Degree Days** | 18 |
| **Moisture** | |
| **Dew Point** | 6 °C |
| **Average Humidity** | 84 |
| **Maximum Humidity** | 89 |
| **Minimum Humidity** | 77 |
| **Precipitation** | |
| **Precipitation** | 0.0 mm |
| **Sea Level Pressure** | |
| **Sea Level Pressure** | 1 022.38 hPa |
| **Wind** | |
| **Wind Speed** | 4 km/h |
| **Max Wind Speed** | 14 km/h |
| **Max Gust Speed** | MM |
| **Visibility** | 4.0 kilometers |
| **Events** | None |

[7] Source: Averaged Metar Reports. https://www.wunderground.com/history/wmo/11855/2017/11/9/DailyHistory.html?req_city=Nitra&req_state=NI&req_statename=Slovakia&reqdb.zip=00000&reqdb.magic=1&reqdb.wmo=11855 (last accessed 04/01/2018)

Along with a nutrient management plan, many farmers use manure storage structures and barnyard runoff controls to improve manure management and protect water quality. Storage allows manure to be safely stockpiled until conditions are environmentally safe for spreading.

**Figure 16 – Manure deposit at the Oponice Plant**

Water that does not soak into the ground, whether from rain, snowmelt, a hose, or leaking pipes, is called runoff. Runoff picks up contaminants, such as nutrients, pathogens, and bacteria from manure and can transport them to the nearest water resource (lake, pond, wetland, stream, or river).

**Figure 17 – Sampling of surface water samples adjacent to field site**

Manure runoff from cropland and pastures or discharging animal feeding operations and concentrated animal feeding operations not only reaches surface water bodies but often also groundwater.

**Figure 18 – Sampling at groundwater well**

Manure has value. That value may result from improvements in soil quality, increases in yield, and replacement of commercial nutrient required for crop production.

Therefore the use of organic manure is one of the alternative ways for enhancing production and improves the soil health.

**Figure 19 – Soil sampling**

## 2.2 Samples registry

In total 12 water samples from field run-off/drainage channels and from ground water wells (7 filter samples [run through approx. 1 l]) were taken using the in-house developed Mariani Box. In addition 5 large scale samples for exploratory investigations of AMR were taken. The sample set was completed by 4 soil samples from the test fields as well as 2 solid and 1 liquid manure samples. A five replicate sample from the digestion plant was taken prior to the campaign.

**Table 9 –List of samples taken during the campaign**

| Sample name | Sampling date | Sample type |
|---|---|---|
| **Nitra - Oponice Farm, Field no7, location A** | O9/11/2017 | Water (MB Filter) |
| **Nitra - Oponice Farm, Field no7, location B** | O9/11/2017 | Water (MB Filter) |
| **Nitra - Oponice Farm, Ground Water from Well Field 2 location C** | O9/11/2017 | Water (MB Filter) |
| **Nitra - Oponice Farm, Run off water from field 2&3 location D** | O9/11/2017 | Water (MB Filter) |
| **Nitra - Oponice Farm, Stream Oponice location E , 50 m from Nitra River** | O9/11/2017 | Water (MB Filter) |
| **Nitra - Oponice Farm, Stream Oponice location F , 25 m from Nitra River** | O9/11/2017 | Water (MB Filter) |
| **Nitra - Oponice Farm, Nitra River near pumphouse , location G** | O9/11/2017 | Water (MB Filter) |
| **Nitra - Oponice Farm, Nitra River near pumphouse , location G** | O9/11/2017 | Water (MB Filter) |
| **Nitra - Oponice Farm, Field no7, location B** | O9/11/2017 | Large volume for AMR tests 5L |
| **Nitra - Oponice Farm, Ground Water from Well Field 2 location C** | O9/11/2017 | Large volume for AMR tests 5L |
| **Nitra - Oponice Farm, Run off water from field 2 & 3 location D** | O9/11/2017 | Large volume for AMR tests 5L |
| **Nitra - Oponice Farm, Stream Oponice location E, 50 m from Nitra River** | O9/11/2017 | Large volume for AMR tests 5L |
| **Nitra - Oponice Farm, soil sample Field 1** | O9/11/2017 | Soil (plastic bag) |

| Sample name | Sampling date | Sample type |
|---|---|---|
| **Nitra - Oponice Farm, soil sample Field 2** | O9/11/2017 | Soil (plastic bag) |
| **Nitra - Oponice Farm, soil sample Field 3** | O9/11/2017 | Soil (plastic bag) |
| **Nitra - Oponice Farm, soil sample Field 7** | O9/11/2017 | Soil (plastic bag) |
| **Nitra - Oponice Farm, solid cattle manure 1** | O9/11/2017 | Manure (plastic bag) |
| **Nitra - Oponice Farm, solid cattle manure 2** | O9/11/2017 | Manure (plastic bag) |
| **Nitra - Oponice Farm, liquid cattle manure** | O9/11/2017 | liquid manure (glass bottle) |
| **Nitra - Oponice Farm, digested pig manure** | September 2017 | Digested manure |

# 3 Towards a multi-compound analytical methodology

While the experiences gained during the exploratory sampling are useful to set-up a sampling protocol for an EU-wide approach, the samples obtained are key elements in the development of an appropriate and versatile multi-compound analytical methodology. The development of such a method is obviously challenging and needs to consider a series of limiting boundary conditions, such as:

1. The variety of chemical compounds to be addressed: as shown in the previous chapters, it is difficult to fully anticipate all occurring active pharmaceutical ingredients occurring in (processed) manure and subsequently in fields and waters exposed to it. In addition, modern animal husbandry uses also a series of pesticides for sanitation and precaution purposes. Many of these compounds are relevant, too, and need to be considered from an eco-toxicological perspective.
2. The fundamentally different properties of the sample matrices: manure and processed manure may vary significantly in their matrix properties, which in return influence the extraction behaviour of the analytical compounds of interest. The same applies to the soil properties or water quality.
3. The broad range of concentrations: in general the closer the matrix, from which a sample is obtained, to the animal, the higher the concentration of the compounds of interest will be. Thus, we can expect concentrations that are orders of magnitude higher in unprocessed or poorly processed manure compared to for instance surface or groundwater samples.
4. The limited resources available: the most time consuming step in trace organic analyses is the final evaluation of the acquired spectra. In addition the amount of compounds to be addressed is very large. To cope with this analytical challenge a so-called non-targeted approach which will lead to a "digital freezing" of the chemical information in the samples will be used in conjunction with a targeted analyses of likely compounds of interest.
5. The logistic and organisational challenges: a high degree of logistical coordination is needed to ensure that data are reproducible and quality documented with an appropriate level of accuracy and precision.

Considering these restraints it was decided to aim the development of a combined methodology using both, a targeted and non-targeted approach, which will be refined in the forthcoming steps.

## 3.1 Preliminary compound selection and initial choice

Table 10 reports the compounds considered initially in the present study. Some of them were indicated as "*priority veterinary medicine active ingredients recommended for further study to assess toxicological risks resulting from human exposure pathways*" (Boxall, 2012). Others relate more to the classical context of EU legislation and soil management aspects.

Starting from this first set of substances, a sketch for an appropriate sampling preparation protocol and subsequent analyses using an LC-MS/MS approach was developed.

The technique used was already tested successfully in similar occasions, e.g. in the analyses of effluents from waste water treatment plants (Loos *et al.*, 2012; Jarosova *et al.*, 2014), the stability assessment of compounds of emerging concern in environmental water samples (Mariani *et al.*, 2017) and the investigation of illicit drug residues and other polar compounds in reclaimed waters used in water reuse applications (Tavazzi *et al.*, 2017).

**Table 10 – Initial selection of study compounds to start the analytical method development for a multi-matrix method combining a targeted and non-targeted analytical approach**

| Analyte | Comment |
|---|---|
| **Heavy metals* (Cadmium, Copper Nickel, Lead, Zinc, Mercury, Chromium** | Council Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture |
| **Nitrogen*** | |
| **Phosphorous*** | |
| **Potassium*** | |
| **Dry matter*** | |
| **Ketoconazole** | Imidazole antifungal drugs |
| **Miconazole** | |
| **Fluconazole** | |
| **Climbazol** | |
| **Levamisole hydrochloride** | Imidazothiazole anthelmintic |
| **Oxolinic acid** | Quinolone antimicrobial agent |
| **Sulfadiazin** | Sulfonamide antimicrobial agent |
| **Toltrazuril** | Triazinetrione derivative; anti protozoal agent |
| **Diazinon** | Organothiophosphate insecticide |
| **Florfenicol** | Broad-spectrum, primarily bacteriostatic, antibiotic |
| **Bronopol** | Antimicrobial agent |
| **Albendazole** | Benzimidazole anti protozoal agent |
| **Monensin sodium** | Polyether antimicrobial agent |
| **Sarafloxacin** | Quinolone antimicrobial agent |
| **Ofloxacin** | second-generation fluoroquinolone |
| **Enrofloxacin** | Fluoroquinolone antimicrobial agent |
| **Marbofloxacin** | |
| **Oxytetracycline** | Tetracycline antibiotic, |
| **Chlorotetracycline** | |
| **Amoxicillin** | β-lactam antimicrobial agent |
| **Sulfadimethoxine** | Sulfonamide antimicrobial agent |
| **Sulfathiazole** | |
| **Sulfamethoxazole** | |
| **Sulfamethazine** | |
| **Roxithromycin** | Macrolides antimicrobial agent |
| **Erythromycin** | |
| **Clarythromycin** | |

## 3.2 Sample preparation

To initiate the development of the analytical procedure it was decided to perform the tests on non-processed (i.e.: cattle urine) and processed manure (i.e.: digestate) (Table 9).

### 3.2.1 Non-processed manure (cattle urine)

Cattle urine sample was allowed to equilibrate at room temperature, then vigorously hand-shaken and a 0.5 ml aliquot transferred into a 1.5 ml Eppendorf PP vial. The opportune amount of internal standard mixture was added and the sample was vortex mixed for 30 seconds.

An aliquot of 0.5 ml of acetonitrile was then added for protein precipitation.

The mixture was vortex mixed again for 30 seconds and then centrifuged at 10 000 rpm for 10 minutes.

An aliquot of 100 µl of the supernatant was finally transferred to an auto-sampler vial for LC-MS analysis.

### 3.2.2 Processed manure

For chromatographic purposes, a digestate sample was filtered through 5um and 1 µm glass fibre disks, consecutively.

10 ml filtered aliquot was then withdrawn and diluted with 90 ml of MilliQ water to obtain a final volume of 100 ml. A Polypropylene bottle was used as sample container.

The opportune amount of Internal Standard mixture was added and the sample was then extracted using OASIS HLB cartridges according to the procedure reported In Table 11.

**Table 11 - Solid-phase extraction protocol applied**

| SPE Step | Solvent | Volume (ml) | Flow (ml/min) |
|---|---|---|---|
| **Conditioning** | Ethyl acetate | 5 | 15 |
| | Methanol | 5 | 15 |
| | Water | 5 | 15 |
| **Loading** | 100 ml | | 5 |
| **Wash** | 10% methanol | 5 | |
| **Drying for 30 minutes under nitrogen flow** | | | |
| **Elution** | Ethyl acetate | 6 | 2 |
| | Methanol | 6 | 2 |

The ethyl acetate fraction was evaporated to dryness under nitrogen, reconstituted to 1 ml with reconstitution solution and analysed.

The methanolic fraction was reduced under nitrogen to 100 µl volume, transferred into an autosampler vial and analysed.

## 3.3 Instrumental methods

### 3.3.1 LC-MS/MS method

#### *3.3.1.1 UHPLC conditions*

The experimental conditions for polar compounds UHPLC-MSMS analysis are reported In Table 12.

**Table 12 - UHPLC experimental conditions**

| Parameter | Type/Values |
|---|---|
| **Pumps:** | Binary Solvent Manager, Model UPB, Waters (Milford, MA, USA). |
| **Autosampler:** | Sample Manager, Model UPA, Waters (Milford, MA, USA). |
| **Detector:** | QTRAP 5500, Applied Biosystems MDS SCIEX, (Foster City, CA, U.S.A) equipped with Turbo V™ ion source. |
| **Flow rate:** | 0.5 ml/min |
| **Injection volume:** | 10 µ |
| **Analytical column:** | CSH C18 (Thermo), 2.1 x 100 mm, 1.7 µm |
| **Mobile phase:** | A: 0.1% HCOOH; B: MeOH:AcN 50/50, % v/v |
| **Reconstituting solution** | 0.1% HCOOH: MeOH:AcN 95:2.5:2.5, % v/v |

The chromatography was performed in gradient mode according to the scheme reported in the Table 13.

**Table 13 - UHPLC gradient scheme**

| Time (min) | Mobile phase (A%) | Mobile Phase B (%) |
|---|---|---|
| **0** | 95 | 5 |
| **1** | 95 | 5 |
| **5** | 5 | 95 |
| **6** | 5 | 95 |
| **6.1** | 95 | 5 |
| **8** | 95 | 5 |

#### *3.3.1.2 QTRAP 5500 MS/MS operative conditions*

An ABSciex QTRAP5500 mass spectrometer equipped with Turbo V™ ion source was used for polar compounds analysis. The instrument was previously tuned and calibrated in electrospray mode using PPG's. Prior to analysis all the specific parameters were optimized infusing a 1 µg/mL standard solution of analytes and I.S.s.

The eluate from the column was introduced directly into the ion source. The rapid desolvatation and vaporization of the droplets minimizes thermal decomposition and preserves their molecular identity.

The data were collected using the software program Analyst 1.6.2

All calculations were based on chromatographic peak area ratios for the MRM precursor-product ion transitions for analytes versus I.S.s.

**Table 14 - General operating conditions for QTRAP 5500 MS/MS**

| Parameter | Value |
|---|---|
| **Scan Type:** | Scheduled MRM |
| **Polarity:** | Polarity Switching: Positive/Negative |
| **Ion Source:** | Turbo Spray |
| **Resolution Q1:** | Unit |
| **Resolution Q3:** | Unit |
| **MR Pause:** | 5.0000 msec |
| **Curtain gas (CUR):** | 25.00 |
| **Collision Gas (CAD):** | Medium |
| **Temperature (TEM):** | 550.00 |
| **IonSpray Voltage (IS):** | ± 4500.00 |
| **Ion Source Gas 1 (GS1)** | 55 |
| **Ion Source Gas 2 (GS2)** | 45 |
| **Target Scan Time** | 0.1 sec |
| **MRM detection window** | 80 sec |

MS/MS parameters of the multi-compound method are reported in Table 15. It is to be annotated that this table includes also other analytes than the ones reported in Table 10. The enlarged compound list considers specific target compounds for the antimicrobial agents monitoring. This gives the possibility of a wider characterisation of collected samples, in case of positive findings.

## Table 15 - MS-MS parameters

| Q1 (Mass) | Q3 (Mass) | Time (min) | ID | DP (Volts) | EP (Volts) | CE (Volts) |
|---|---|---|---|---|---|---|
| **255** | 197 | 4.35 | 2,4,5-T | -70 | -10 | -22 |
| **255** | 161 | 4.35 | 2,4,5-T 1 | -70 | -10 | -41 |
| **219** | 161 | 4.07 | 2,4-D | -130 | -10 | -24 |
| **219** | 125 | 4.07 | 2,4-D 1 | -130 | -10 | -38 |
| **225** | 167 | 4.07 | 2,4-D 13C6 | -68 | -10 | -19 |
| **162** | 78 | 2.75 | Acesulfame K | -120 | -10 | -46 |
| **162** | 82 | 2.75 | Acesulfame K | -120 | -10 | -27 |
| **166** | 86 | 2.75 | Acesulfame K-D4 | -151 | -10 | -20 |
| **166** | 78 | 2.75 | Acesulfame K-D4 | -151 | -10 | -42 |
| **360** | 274 | 4.13 | Bezafibrate | -100 | -10 | -24 |
| **360** | 154 | 4.13 | Bezafibrate | -100 | -10 | -39 |
| **364** | 278 | 4.13 | Bezafibrate D4 | -165 | -10 | -24 |
| **294** | 250 | 4.51 | Diclofenac | -42 | -10 | -16 |
| **294** | 214 | 4.51 | Diclofenac | -42 | -10 | -29 |
| **300** | 256 | 4.51 | Diclofenac 13C6 | -173 | -10 | -15 |
| **269** | 145 | 0 | E1 | -100 | -10 | -53 |
| **269** | 143 | 0 | E1 1 | -100 | -10 | -74 |
| **272** | 146 | 0 | E1 13C3 | -150 | -10 | -88 |
| **272** | 148 | 0 | E1 13C3 1 | -150 | -10 | -50 |
| **271** | 145 | 0 | E2 | -83 | -10 | -60 |
| **271** | 143 | 0 | E2 1 | -83 | -10 | -78 |
| **275** | 147 | 0 | E2 d4 | -100 | -10 | -55 |
| **275** | 187 | 0 | E2 d4 1 | -100 | -10 | -50 |
| **295** | 145 | 0 | EE2 | -100 | -10 | -70 |
| **295** | 143 | 0 | EE2 1 | -100 | -10 | -50 |
| **299** | 145 | 0 | EE2 D4 | -100 | -10 | -60 |
| **299** | 187 | 0 | EE2 d4 1 | -100 | -10 | -45 |
| **249** | 121 | 4.74 | Gemfibrozil | -100 | -10 | -30 |
| **249** | 106 | 4.74 | Gemfibrozil | -100 | -10 | -60 |
| **255** | 121 | 4.74 | Gemfibrozil d6 | -100 | -10 | -20 |
| **205** | 161 | 0 | Ibuprofen | -132 | -10 | -10 |
| **205** | 159 | 0 | Ibuprofen | -132 | -10 | -10 |
| **208** | 163 | 0 | Iburpofen 13C3 | -81 | -10 | -11 |
| **199** | 141 | 3.97 | MCPA | -147 | -10 | -21 |
| **199** | 105 | 3.97 | MCPA | -147 | -10 | -40 |
| **229** | 169 | 3.97 | Naproxen | -100 | -10 | -47 |
| **229** | 185 | 3.97 | Naproxen | -100 | -10 | -10 |
| **233** | 169 | 3.97 | Naproxen 13C3 | -42 | -10 | -46 |
| **395** | 359 | 2.44 | Sucralose | -145 | -10 | -17 |

| Q1 (Mass) | Q3 (Mass) | Time (min) | ID | DP (Volts) | EP (Volts) | CE (Volts) |
|---|---|---|---|---|---|---|
| **397** | 361 | 2.44 | Sucralose 1 | -145 | -10 | -17 |
| **395** | 35 | 2.44 | Sucralose 2 | -145 | -10 | -16 |
| **401** | 365 | 2.44 | Sucralose d6 | -160 | -10 | -16 |
| **294.1** | 198.1 | 2.98 | N-Acetyl-SMZ | -100 | -10 | -24 |
| **294.1** | 133.8 | 2.98 | N-Acetyl-SMZ | -100 | -10 | -31 |
| **265.3** | 35 | 0 | Pentachlorophenol | -120 | -10 | -96 |
| **265.3** | 80 | 0 | Pentachlorophenol 1 | -120 | -10 | -31 |
| **640.8** | 78.9 | 0 | HBCD | -260 | -10 | -93 |
| **640.8** | 81.1 | 0 | HBCD 1 | -260 | -10 | -66 |
| **213** | 141 | 4.21 | Mecoprop | -100 | -10 | -30 |
| **213** | 71 | 4.21 | Mecoprop 1 | -100 | -10 | -15 |
| **271** | 207 | 0 | Pentachlorophenol 13C6 | -180 | -10 | -50 |
| **271** | 236 | 0 | Pentachlorophenol 13C6 1 | -180 | -10 | -40 |
| **213** | 169 | 4.05 | PFBA | -96 | -10 | -13 |
| **217** | 172 | 4.05 | PFBA 13C4 | -99 | -10 | -13 |
| **299** | 80 | 4.81 | PFBS | -260 | -10 | -66 |
| **299** | 99 | 4.81 | PFBS | -260 | -10 | -39 |
| **313** | 269 | 5.06 | PFHxA | -107 | -10 | -12 |
| **313** | 119 | 5.06 | PFHxA | -107 | -10 | -28 |
| **315** | 270 | 5.06 | PFHxA 13C2 | -60 | -10 | -13 |
| **399** | 80 | 0 | PFHxS | -260 | -10 | -93 |
| **399** | 99 | 0 | PFHxS | -260 | -10 | -66 |
| **363** | 319 | 5.5 | PFHpA | -116 | -10 | -14 |
| **363** | 169 | 5.5 | PFHpA | -116 | -10 | -24 |
| **413** | 369 | 5.86 | PFOA | -122 | -10 | -16 |
| **413** | 169 | 5.86 | PFOA | -122 | -10 | -26 |
| **417** | 372 | 5.86 | PFOA 13C4 | -119 | -10 | -15 |
| **499** | 80 | 6.11 | PFOS | -260 | -10 | -97 |
| **499** | 99 | 6.11 | PFOS | -260 | -10 | -83 |
| **503** | 80 | 6.11 | PFOS 13C4 | -276 | -10 | -104 |
| **463** | 419 | 6.17 | PFNA | -122 | -10 | -19 |
| **463** | 219 | 6.17 | PFNA | -122 | -10 | -25 |
| **463** | 169 | 6.17 | PFNA | -122 | -10 | -27 |
| **468** | 423 | 6.17 | PFNA 13C5 | -57 | -10 | -16 |
| **202** | 144 | 3.97 | MCPA D3 | -100 | -10 | -30 |
| **202** | 108 | 3.97 | MCPA D3 1 | -100 | -10 | -45 |
| **648.9** | 78.9 | 0 | HBCD lab | -80 | -10 | -30 |
| **211** | 139 | 0 | 4-t-OP 13C6 | -80 | -10 | -30 |
| **211** | 123 | 0 | 4-t-OP 13C61 | -250 | -10 | -5 |
| **424** | 424 | 4.59 | Toltrazuril | -100 | -10 | -5 |

| Q1 (Mass) | Q3 (Mass) | Time (min) | ID | DP (Volts) | EP (Volts) | CE (Volts) |
|---|---|---|---|---|---|---|
| **356** | 185 | 2.75 | Florfenicol | -120 | -10 | -30 |
| **356** | 336 | 2.75 | Florfenicol 1 | -120 | -10 | -13 |
| **199** | 155 | 4.18 | Bronopol | -100 | -10 | -20 |
| **199** | 181 | 4.18 | Bronopol 1 | -100 | -10 | -15 |
| **199** | 80 | 4.18 | Bronopol 2 | -100 | -10 | -50 |
| **427.2** | 427.2 | 4.59 | Toltrazuril D3 | -100 | -10 | -10 |
| **271** | 180 | 2.85 | 10,11-dihydro-10,11-dihydroxy-carbamazepine | 80 | 10 | 47 |
| **271** | 210 | 2.85 | 10,11-dihydro-10,11-dihydroxy-carbamazepine 1 | 80 | 10 | 19 |
| **271** | 253 | 2.85 | 10,11-dihydro-10,11-dihydroxy-carbamazepine 2 | 80 | 10 | 10 |
| **223** | 126 | 2.85 | Acetamiprid | 80 | 10 | 29 |
| **223** | 73 | 2.85 | Acetamiprid 1 | 80 | 10 | 76 |
| **225** | 128 | 2.85 | Acetamiprid 2 | 80 | 10 | 29 |
| **225** | 75 | 2.85 | Acetamiprid 3 | 80 | 10 | 74 |
| **226** | 126 | 2.85 | Acetamiprid-d3 | 80 | 10 | 27 |
| **226** | 73 | 2.85 | Acetamiprid-d3 1 | 80 | 10 | 80 |
| **226** | 190 | 2.85 | Acetamiprid-d3 2 | 80 | 10 | 19 |
| **270** | 238 | 4.34 | Alachlor | 120 | 10 | 16 |
| **270** | 162 | 4.34 | Alachlor | 120 | 10 | 28 |
| **216** | 174 | 3.62 | Atrazine | 258 | 10 | 25 |
| **216** | 104 | 3.62 | Atrazine | 258 | 10 | 40 |
| **219** | 177 | 3.62 | Atrazine 13C3 | 100 | 10 | 25 |
| **749.6** | 591.4 | 0 | Azythromycin | 200 | 10 | 40 |
| **749.6** | 573.3 | 0 | Azythromycin 1 | 200 | 10 | 47 |
| **120** | 65 | 2.24 | Benzotriazole | 209 | 10 | 29 |
| **120** | 92 | 2.24 | Benzotriazole 1 | 209 | 10 | 24 |
| **124** | 69 | 2.24 | Benzotriazole d4 | 56 | 10 | 35 |
| **237** | 194 | 3.54 | Carbamazepine | 250 | 10 | 28 |
| **237** | 165 | 3.54 | Carbamazepine | 250 | 10 | 60 |
| **247** | 204 | 3.54 | Carbamazepine d10 | 234 | 10 | 31 |
| **222** | 77 | 2.7 | Chloridazon | 204 | 10 | 52 |
| **222** | 65 | 2.7 | Chloridazon 1 | 204 | 10 | 53 |
| **222** | 92 | 2.7 | Chloridazon 2 | 204 | 10 | 34 |
| **748.5** | 590.5 | 3.31 | Clarythromycin | 100 | 10 | 28 |
| **748.5** | 558.5 | 3.31 | Clarythromycin 1 | 100 | 10 | 31 |
| **250** | 132 | 2.62 | Clothianidin | 50 | 10 | 26 |
| **250** | 169 | 2.62 | Clothianidin 1 | 50 | 10 | 16 |
| **252** | 134 | 2.62 | Clothianidin 2 | 50 | 10 | 24 |
| **253** | 172 | 2.62 | Clothianidin-d3 1 | 50 | 10 | 18 |
| **253** | 132 | 2.62 | Clothianidin-d3 1 | 50 | 10 | 23 |

| Q1 (Mass) | Q3 (Mass) | Time (min) | ID | DP (Volts) | EP (Volts) | CE (Volts) |
|---|---|---|---|---|---|---|
| **254** | 198 | 3.77 | Cybutrine 1 | 261 | 10 | 26 |
| **263** | 74 | 3.77 | Cybutrine d9 1 | 269 | 10 | 61 |
| **254** | 74 | 3.77 | Cybutryne 2 | 261 | 10 | 30 |
| **263** | 199 | 3.77 | Cybutryune d9 | 269 | 10 | 27 |
| **233** | 72 | 3.78 | Diuron | 169 | 10 | 25 |
| **233** | 133 | 3.78 | Diuron 1 | 169 | 10 | 53 |
| **240** | 78 | 3.78 | Diuron-d6 | 156 | 10 | 24 |
| **240** | 135 | 3.78 | Diuron-d6 | 156 | 10 | 57 |
| **240** | 161 | 3.78 | Diuron-d6 | 156 | 10 | 39 |
| **734.5** | 576 | 2.99 | Erythromycin | 80 | 10 | 26 |
| **734.5** | 558 | 2.99 | Erythromycin 1 | 80 | 10 | 25 |
| **736** | 578 | 2.99 | Erythromycin 13C2 | 130 | 10 | 26 |
| **736** | 560 | 2.99 | Erythromycin 13C2 1 | 130 | 10 | 26 |
| **736** | 160 | 2.99 | Erythromycin 13C2 2 | 160 | 10 | 36 |
| **734.5** | 83 | 2.99 | Erythromycin 2 | 80 | 10 | 95 |
| **734.5** | 158.2 | 2.99 | Erythromycin 3 | 80 | 10 | 40 |
| **256** | 209 | 2.7 | Imidacloprid | 60 | 10 | 21 |
| **256** | 175 | 2.7 | Imidacloprid 1 | 60 | 10 | 27 |
| **260** | 213 | 2.7 | Imidacloprid-d4 | 60 | 10 | 26 |
| **260** | 179 | 2.7 | Imidacloprid-d4 1 | 60 | 10 | 29 |
| **207** | 72 | 3.73 | Isoproturon | 230 | 10 | 25 |
| **207** | 165 | 3.73 | Isoproturon | 230 | 10 | 20 |
| **210** | 75 | 3.73 | Isoproturon-D3 | 199 | 10 | 25 |
| **210** | 168 | 3.73 | Isoproturon-D3 | 199 | 10 | 21 |
| **226** | 169 | 4.05 | Methiocarb | 30 | 10 | 12 |
| **226** | 121 | 4.05 | Methiocarb 1 | 30 | 10 | 25 |
| **229** | 169 | 4.05 | Methiocarb d3 | 110 | 10 | 71 |
| **229** | 121 | 4.05 | Methiocarb d3 1 | 110 | 10 | 76 |
| **284** | 252 | 4.38 | Metolachlor | 200 | 10 | 22 |
| **284** | 176 | 4.38 | Metolachlor | 200 | 10 | 35 |
| **290** | 258 | 4.38 | Metolachlor d6 | 196 | 10 | 20 |
| **268** | 91 | 0 | Metopropol | 261 | 10 | 68 |
| **268** | 103 | 0 | Metopropol | 261 | 10 | 57 |
| **275** | 122 | 0 | Metopropol d7 1 | 274 | 10 | 26 |
| **345** | 220 | 5 | Oxadiazon | 90 | 10 | 28 |
| **345** | 303 | 5 | Oxadiazon 1 | 90 | 10 | 21 |
| **202** | 104 | 3.23 | Simazine | 253 | 10 | 34 |
| **202** | 132 | 3.23 | Simazine 1 | 253 | 10 | 26 |
| **205** | 70 | 3.3 | Simazine 13C3 | 218 | 10 | 45 |
| **205** | 106 | 3.3 | Simazine 13C3 1 | 218 | 10 | 35 |

| Q1 (Mass) | Q3 (Mass) | Time (min) | ID | DP (Volts) | EP (Volts) | CE (Volts) |
|---|---|---|---|---|---|---|
| **279** | 92 | 2.35 | Sulfamethazine | 232 | 10 | 41 |
| **279** | 124 | 2.35 | Sulfamethazine 1 | 232 | 10 | 32 |
| **285** | 70 | 2.35 | Sulfamethazine 13C6 | 165 | 10 | 70 |
| **254** | 156 | 2.7 | Sulfamethoxazole | 150 | 10 | 22 |
| **254** | 92 | 2.7 | Sulfamethoxazole | 150 | 10 | 38 |
| **260** | 98 | 2.7 | Sulfamethoxazole 13C6 | 70 | 10 | 36 |
| **242** | 186 | 3.54 | Terbutryn | 255 | 10 | 25 |
| **242** | 91 | 3.54 | Terbutryn | 255 | 10 | 36 |
| **247** | 191 | 3.54 | Terbutryn d5 | 228 | 10 | 27 |
| **247** | 91 | 3.54 | Terbutryn d5 1 | 228 | 10 | 36 |
| **230** | 174 | 0 | Terbutylazine | 219 | 10 | 26 |
| **230** | 132 | 0 | Terbutylazine | 219 | 10 | 35 |
| **253** | 126 | 3.08 | Thiacloprid | 100 | 10 | 27 |
| **253** | 90 | 3.08 | Thiacloprid 1 | 100 | 10 | 55 |
| **255** | 128 | 3.08 | Thiacloprid 2 | 77 | 10 | 28 |
| **255** | 90 | 3.08 | Thiacloprid 3 | 77 | 10 | 53 |
| **257** | 126 | 3.08 | Thiacloprid-d4 | 100 | 10 | 28 |
| **257** | 73 | 3.08 | Thiacloprid-d4 1 | 100 | 10 | 83 |
| **257** | 90 | 3.08 | Thiacloprid-d4 2 | 100 | 10 | 54 |
| **292** | 132 | 0 | Thiamethoxam | 60 | 10 | 35 |
| **292** | 211 | 0 | Thiamethoxam 1 | 60 | 10 | 18 |
| **295** | 214 | 0 | Thiamethoxam-d3 | 70 | 10 | 19 |
| **295** | 132 | 0 | Thiamethoxam-d3 1 | 70 | 10 | 30 |
| **291** | 123 | 1.48 | Trimethoprim | 293 | 10 | 34 |
| **291** | 230 | 1.48 | Trimethoprim 1 | 293 | 10 | 33 |
| **294** | 126 | 1.48 | Trimethoprim 13C3 | 221 | 10 | 33 |
| **294** | 233 | 1.48 | Trimethoprim 13C3 1 | 221 | 10 | 32 |
| **265.3** | 248 | 4.45 | Aclonifen | 120 | 10 | 21 |
| **265.3** | 194.1 | 4.45 | Aclonifen 1 | 120 | 10 | 25 |
| **343** | 311 | 0 | Bifenox | 40 | 10 | 13 |
| **172.3** | 137.2 | 0.64 | Gabapentin | 60 | 10 | 21 |
| **172.3** | 154.2 | 0.64 | Gabapentin 1 | 60 | 10 | 16 |
| **189.3** | 56 | 2.47 | Phenazone | 120 | 10 | 45 |
| **189.3** | 77 | 2.47 | Phenazone 1 | 120 | 10 | 51 |
| **309.3** | 273.3 | 5 | Quinoxyfen | 100 | 10 | 38 |
| **309.3** | 197 | 5 | Quinoxyfen 1 | 100 | 10 | 44 |
| **359** | 99 | 4.53 | Chlorfenvinphos | 100 | 10 | 46 |
| **359** | 170 | 4.53 | Chlorfenvinphos 1 | 100 | 10 | 55 |
| **350.6** | 96.8 | 0 | Clorpyrifos | 80 | 10 | 45 |
| **350.6** | 197.8 | 0 | Chlorpyrifos 1 | 80 | 10 | 29 |

| Q1 (Mass) | Q3 (Mass) | Time (min) | ID | DP (Volts) | EP (Volts) | CE (Volts) |
|---|---|---|---|---|---|---|
| **221.9** | 109 | 3.24 | Dichlorvos | 100 | 10 | 25 |
| **221.9** | 95 | 3.24 | Dichlorvos 1 | 100 | 10 | 50 |
| **269.8** | 253 | 4.45 | Aclonifen D5 | 120 | 10 | 22 |
| **269.8** | 186 | 4.45 | Aclonifen D5 1 | 120 | 10 | 40 |
| **283.2** | 251.1 | 4.34 | Alachlor D13 | 120 | 10 | 15 |
| **283.2** | 175.3 | 4.34 | Alachlor D13 1 | 120 | 10 | 26 |
| **346.3** | 314 | 0 | Bifenox D3 | 80 | 10 | 12 |
| **369** | 205 | 4.53 | Chlorfenvinphos D10 | 100 | 10 | 30 |
| **369** | 133 | 4.53 | Chlorfenvinphos D10 1 | 100 | 10 | 25 |
| **360.1** | 199 | 0 | Chlorpyrifos D10 | 100 | 10 | 29 |
| **360.1** | 107 | 0 | Chlorpyrifos D10 1 | 100 | 10 | 80 |
| **227** | 115 | 3.24 | Dichlorvos D6 | 100 | 10 | 26 |
| **227** | 83 | 3.24 | Dichlorvos D6 1 | 100 | 10 | 37 |
| **313** | 276.2 | 5 | Quinoxyfen D4 | 80 | 10 | 35 |
| **313** | 163.2 | 5 | Quinoxyfen D4 1 | 80 | 10 | 60 |
| **222** | 204 | 2.85 | Quinmerac | 50 | 10 | 23 |
| **222** | 140 | 2.85 | Quinmerac 1 | 50 | 10 | 50 |
| **198** | 140 | 0 | Caffeine 13C3 | 150 | 10 | 27 |
| **198** | 112 | 0 | Caffeine 13C31 | 150 | 10 | 33 |
| **192** | 91 | 0 | DEET | 244 | 10 | 41 |
| **192** | 119 | 0 | DEET | 244 | 10 | 24 |
| **198** | 91 | 0 | DEET d6 | 80 | 10 | 42 |
| **134** | 77 | 0 | 5-methyl-1H-benzotriazole | 260 | 10 | 34 |
| **134** | 106 | 0 | 5-methyl-1H-benzotriazole 1 | 260 | 10 | 23 |
| **332** | 231 | 0 | Ciprofloxacin | 290 | 10 | 37 |
| **332** | 314 | 0 | Ciprofloxacin | 290 | 10 | 25 |
| **336** | 235 | 0 | Ciprofloxacin 13C3 | 239 | 10 | 51 |
| **336** | 291 | 0 | Ciprofloxacin 13C3 1 | 239 | 10 | 25 |
| **362** | 261 | 2.01 | Ofloxacin | 120 | 10 | 39 |
| **362** | 318 | 2.01 | Ofloxacin | 120 | 10 | 27 |
| **461** | 444 | 0 | Oxytetracyclin | 120 | 10 | 80 |
| **461** | 426.3 | 0 | Oxytetracyclin | 80 | 10 | 25 |
| **461** | 201.1 | 0 | Oxytetracyclin 1 | 80 | 10 | 51 |
| **461** | 127.2 | 0 | Oxytetracyclin 2 | 80 | 10 | 104 |
| **479.2** | 444 | 2.14 | Chlorotetracyclin | 80 | 10 | 23 |
| **479.2** | 462.3 | 2.14 | Chlorotetracyclin 1 | 80 | 10 | 23 |
| **479.2** | 139 | 2.14 | Chlorotetracyclin 2 | 80 | 10 | 137 |
| **305** | 159 | 4.53 | Diazinon | 100 | 10 | 30 |
| **305** | 97 | 4.53 | Diazinon 1 | 100 | 10 | 55 |
| **251** | 156 | 1.56 | Sulfadiazine | 60 | 10 | 19 |

| Q1 (Mass) | Q3 (Mass) | Time (min) | ID | DP (Volts) | EP (Volts) | CE (Volts) |
|---|---|---|---|---|---|---|
| **251** | 99 | 1.56 | Sulfadiazine 1 | 60 | 10 | 27 |
| **251** | 108 | 1.56 | Sulfadiazine 2 | 60 | 10 | 34 |
| **531.3** | 81 | 3.21 | Ketoconazole | 120 | 10 | 144 |
| **531.3** | 489.3 | 3.21 | Ketoconazole 1 | 120 | 10 | 50 |
| **417.2** | 159 | 3.77 | Miconazole | 160 | 10 | 40 |
| **417.2** | 161 | 3.77 | Miconazole 1 | 160 | 10 | 43 |
| **417.2** | 89 | 3.77 | Miconazole 2 | 160 | 10 | 116 |
| **307.3** | 238.2 | 2.62 | Fluconazole | 70 | 10 | 24 |
| **307.3** | 220.3 | 2.62 | Fluconazole 2 | 70 | 10 | 28 |
| **293** | 69 | 3.2 | Climbazole | 90 | 10 | 27 |
| **293** | 99 | 3.2 | Climbazole 2 | 90 | 10 | 63 |
| **205.2** | 89 | 0.56 | Levamisole | 100 | 10 | 85 |
| **205.2** | 178 | 0.56 | Levamisole 1 | 100 | 10 | 30 |
| **262.3** | 244.1 | 3.09 | Oxolinic acid | 90 | 10 | 28 |
| **262.3** | 216.3 | 3.09 | Oxolinic acid 1 | 100 | 10 | 39 |
| **266.3** | 234.3 | 3.31 | Albendazole | 120 | 10 | 30 |
| **266.3** | 191 | 3.31 | Albendazole 1 | 120 | 10 | 46 |
| **266.3** | 159 | 3.31 | Albendazole 2 | 120 | 10 | 55 |
| **693.4** | 479 | 5.52 | Monensin | 100 | 10 | 70 |
| **693.4** | 461 | 5.52 | Monensin 1 | 100 | 10 | 70 |
| **693.4** | 675.6 | 5.52 | Monensin 2 | 100 | 10 | 5 |
| **269.3** | 234.2 | 3.31 | Albendazole D3 | 100 | 10 | 27 |
| **269.3** | 191.2 | 3.31 | Albendazole D3 1 | 100 | 10 | 45 |
| **269.3** | 159.3 | 3.31 | Albendazole D3 2 | 100 | 10 | 51 |
| **267.2** | 249.3 | 3.09 | Oxolinic acid D5 | 50 | 10 | 30 |
| **267.2** | 161 | 3.09 | Oxolinic acid D5 1 | 50 | 10 | 47 |
| **297** | 201.2 | 3.2 | Climbazole D4 | 80 | 10 | 42 |
| **297** | 102.8 | 3.2 | Climbazole D4 1 | 80 | 10 | 64 |
| **535.3** | 493 | 3.21 | Ketoconazole D4 | 100 | 10 | 45 |
| **535.3** | 81 | 3.21 | Ketoconazole D4 1 | 100 | 10 | 120 |
| **311** | 242.3 | 2.62 | Fluconazole D4 | 60 | 10 | 25 |
| **315.3** | 170 | 4.53 | Diazinon D10 | 70 | 10 | 35 |
| **315.3** | 154.2 | 4.53 | Diazinon D10 1 | 100 | 10 | 5 |
| **386.1** | 368.3 | 2.25 | Sarafloxacin | 120 | 10 | 33 |
| **386.1** | 299.1 | 2.25 | Sarafloxacin 1 | 120 | 10 | 37 |
| **386.1** | 342.1 | 2.25 | Sarafloxacin 2 | 120 | 10 | 28 |
| **360.3** | 316.3 | 2.17 | Enrofloxacin | 120 | 10 | 25 |
| **360.3** | 342.3 | 2.17 | Enrofloxacin 1 | 120 | 10 | 31 |
| **360.3** | 245.1 | 2.17 | Enrofloxacin 2 | 120 | 10 | 38 |
| **363.2** | 72.1 | 1.88 | Marbofloxacin | 90 | 10 | 26 |

| Q1 (Mass) | Q3 (Mass) | Time (min) | ID | DP (Volts) | EP (Volts) | CE (Volts) |
|---|---|---|---|---|---|---|
| **363.2** | 319.8 | 1.88 | Marbofloxacin 1 | 90 | 10 | 22 |
| **363.2** | 345.3 | 1.88 | Marbofloxacin 2 | 90 | 10 | 26 |
| **366.3** | 349.3 | 0 | Amoxicillin | 70 | 10 | 10 |
| **366.3** | 113.8 | 0 | Amoxicillin 1 | 70 | 10 | 30 |
| **366.3** | 134.2 | 0 | Amoxicillin 2 | 70 | 10 | 45 |
| **838** | 116.3 | 3.26 | Roxitrhomycin | 70 | 10 | 80 |
| **838** | 158.4 | 3.26 | Roxithromycin 1 | 70 | 10 | 41 |
| **838** | 679.33 | 3.26 | Roxithromycin 2 | 70 | 10 | 30 |
| **582.3** | 236.3 | 0 | Streptomycin | 30 | 10 | 40 |
| **582.3** | 246.2 | 0 | Streptomycin 1 | 30 | 10 | 48 |
| **582.3** | 540.4 | 0 | Streptomycin 2 | 30 | 10 | 38 |
| **311.3** | 156.2 | 3.08 | Sulfadimethoxine | 80 | 10 | 25 |
| **311.3** | 92 | 3.08 | Sulfadimethoxine 1 | 80 | 10 | 45 |
| **311.3** | 108 | 3.08 | Sulfadimethoxine 2 | 80 | 10 | 40 |
| **311.3** | 245 | 3.08 | Sulfadimethoxine 3 | 80 | 10 | 25 |
| **256** | 92 | 1.93 | Sulfathiazole | 60 | 10 | 40 |
| **256** | 108.2 | 1.93 | Sulfathiazole 1 | 60 | 10 | 40 |
| **256** | 156 | 1.93 | Sulfathiazoel 2 | 60 | 10 | 24 |
| **325.2** | 264.9 | 0 | Norfloxacin D5 | 80 | 10 | 10 |
| **325.2** | 233.2 | 0 | Norfloxacin D5 1 | 80 | 10 | 40 |
| **325.2** | 307.1 | 0 | Norfloxacin D5 2 | 80 | 10 | 30 |

**Q1: Parent ion $[m/z]^+$; Q3: Product ion $[m/z]^+$; RT: retention time (min); ID: analyte name; DP: Declustering Potential; EP: Entrance Potential; CE: Collision Energy; CXP: Collision Cell Entrance Potential.**

### 3.3.2 Inorganic analytical determinations

In order to better characterise the digestate samples as well as to assess the possibility to outsource routine analytical determinations, two digestate samples were sent to a commercial laboratory that operate its methods under ISO 9001 and ISO 17025, the latter only for certain measurements. Table 16 lists the methods and measurement principles used by the sub-contracted laboratory. The identity of the laboratory is not revealed in this report for data protection reasons. Table 16 also indicates the list of equivalent CEN methods where available, however the methods are operated under Italian national regulation. For each test, 500 ml of aliquots where used.

**Table 16 – Test standards used by commercial laboratory characterising the digestate samples**

| Parameter | Test method used | Test principle and summary |
|---|---|---|
| **Dry matter content at 105°C** | CNR IRSA 2 Q 64 Vol 2 1984 | After filtration at 0.45 um samples are dried to constant mass in an oven at 105 °C until a constant weigh is reached. *The method is equivalent to CEN EN 14346 (2006)* |
| **Loss on ignition 600°C** | CNR IRSA 2 Q 64 Vol 2 1984 | A dried test sample is heated in a furnace to constant mass at 600°C. The difference in mass before and after the ignition process is used to calculate the loss of ignition. The determination is performed on a dried sample or directly on the un-dried sample including a drying step or by |

| Parameter | Test method used | Test principle and summary |
|---|---|---|
| | | referring to dry matter.<br>*The method is equivalent to CEN EN 15169 (2007)* |
| **Total organic carbon** | DM 13/09/1999 GU 248 21/10/1999 Met. VII.2 | Under standard conditions, organic carbon is oxidized to carbon dioxide, using potassium dichromate solution in presence of sulphuric acid. Excess of potassium dichromate is then titrated with an iron (II) sulfate solution. Complete titration is appreciated by adding a proper redox indicator or by potentiometry, using a platinum electrode. Heating the mixture at 160°C ensures the reaction between organic carbon and dichromate being quantitative.<br>*No equivalent EU Standard* |
| **Cr VI** | APAT CNR IRSA 3010A Man. 29:2003 | Chromate is extracted from the sample with water at room temperature. The chromate concentration in the extract is measured by colorimetry with a spectrophotometer using 1,5-diphenylcarbazide.<br>When chromate reduces the 1,5-diphenylcarbazide a magenta coloured complex of 1,5-diphenylcarbazone and chromium is formed which can be measured colorimetrically at 540 nm. *The method is equivalent to CEN EN 16318 (2016)* |
| **Cd** | EN 13657:2004 + EPA 6010C:2007 | EN 13657 specifies methods of digestion with aqua regia. Solutions produced by the methods are suitable for analysis e.g. by atomic absorption spectrometry (FLAAS, HGAAS, CVAAS, GFAAS), inductively coupled plasma emission spectrometry (ICP-OES) and inductive coupled plasma mass spectrometry (ICP-MS). The method is applicable to the digestion of waste for example for the following elements: Al, Sb, As, B, Ba, Be, Cd, Ca, Cr, Co, Cu, Fe, Pb, Mg, Mn, Hg, Mo, Ni, P, K, Se, Ag, S, Na, Sr, Sn, Te, Ti, Tl, V, Zn.<br><br>EPA 6010C: 2007 is the EPA method for inductively coupled plasma-atomic emission spectrometry (ICP-AES) used to determine trace elements in solution. Digests of sludge, treated biowaste or soil with nitric acid or aqua regia are analysed by inductively coupled plasma optical emission spectrometry (ICP-OES) using sequential or simultaneous optical systems and axial or radial viewing of the plasma. The instrument measures characteristic emission spectra by optical spectrometry. Analyte species originating in the digest solution are nebulised and the resulting aerosol is transported to the plasma torch. Element-specific emission spectra are produced by a radio-frequency inductively coupled plasma. The spectra are dispersed by a grating spectrometer, and the intensities of the emission lines are monitored by photosensitive devices.<br><br>*The method is equivalent to CEN EN 16170 (2016)* |
| **Ni** | EN 13657:2004 + EPA 6010C:2007 | |
| **Pb** | EN 13657:2004 + EPA 6010C:2007 | |
| **Cu** | EN 13657:2004 + EPA 6010C:2007 | |
| **Zn** | EN 13657:2004 + EPA 6010C:2007 | |
| **Hg** | EN 13657:2004 + APAT CNR IRSA 3200A2 Man. 29:2003 | |
| **K** | EN 13657:2004 + EPA 6010C:2007 | |
| **$N_{Kjeldahl}$** | D.M. 13/09/1999 GU n° 248 21/10/1999 Met XIV.3 | The dried and homogenised material is digested in a suitable Kjeldahltube with sulfuric acid. To rise the temperature potassium sulfate is added and titanium dioxid/copper sulfate is used as a catalyst. After adding sodium hydroxide to the digestion solution the produced ammonium from all nitrogen species is evaporated by distillation as ammonia. This is condensed in a conical flask with boric acid solution. The amount is titrated against indicator with sulfuric acid.<br>The method is equivalent to CEN EN 16169 (2002) |
| **P** | D.M. 13/09/1999 GU n° 248 21/10/1999 Met XV.1 | The sample is treated with sulphuric acid, hydrogen peroxide and hydrofluoric acid. Spectrophotometric determination of phosphorous, using ascorbic acid procedure.<br>*No equivalent EU Standard* |

## 3.4 First experimental and analytical results

### 3.4.1 Non-processed manure

Results of triplicate cattle urine analysis according to 5.1 are reported in Table 17.

**Table 17 – Preliminary results of cattle urine analysis**

| Analyte | CHEMICAL CLASS | Mean Conc (ng/l) | St. Dev. | CV% |
|---|---|---|---|---|
| **Albendazole** | Benzimidazole dewarmer | 45 504.6 | 23 039.7 | 50.6 |
| **Enrofloxacin** | Fluoroquinolone antibiotic | 689.2 | 123.9 | 18.0 |
| **Erythromycin** | Macrolide | 2 651.9 | 251.9 | 9.5 |
| **Clarithrimycin** | Macrolide | 5.2 | 1.9 | 36.0 |
| **Monensin** | Polyether antibiotic | 1 011.1 | 262.7 | 26.0 |
| **Oxytetracycline** | Tetracycline antibiotic | 11 669.9 | 1 728.7 | 14.8 |
| **Chlorotetracycline** | | 699.8 | 505.3 | 72.2 |

## 3.4.2 Processed manure

Sample preparation for processed manure was characterised in two following steps:

- an evaluation of the suitability of chosen SPE polymeric phase (i.e.: HLB) for the extraction of target analytes form MilliQ water and then
- a spiking experiment using the real matrix (i.e.: digestate sample) for the evaluation of reproducibility in real analytical conditions.

### *3.4.2.1 Evaluation of suitability of HLB polymeric phase for analytes extraction*

The evaluation of the suitability of chosen polymeric phase for the extraction of target analytes was based on a triplicate extraction of each 100 ml MilliQ water spiked samples according to the procedure reported at 3.2.

The spiking level of 1 μg/l for all selected analytes. Table 18 summarises the recovery data obtained.

**Table 18 - Recovery of selected analytes for MilliQ water spiked samples.**

| Analyte | CHEMICAL CLASS | Mean REC in AcOEt | Mean REC in MeOH | Total rec of sequential elution |
|---|---|---|---|---|
| **Miconazole** | Imidazole antifungal drug | 19.6 | 1.7 | 21.3 |
| **Climbazole** | | 140.4 | | 140.4 |
| **Ketoconazole** | | 42.6 | 5.7 | 48.3 |
| **Fluconazole** | | 104.5 | 0.6 | 105.0 |
| **Albendazole** | Benzimidazole dewarmer | na | na | na |
| **Levamisole** | Imidazole* dewarmer | na | na | na |
| **Bronopol** | 2-bromo-2-nitro-propanediol antimicrobial agent | na | na | na |
| **Amoxicillin** | b-lactam | na | na | na |
| **Florfenicol** | Fluoroacetamide antibiotic | 184.3 | 3.4 | 187.7 |
| **Ofloxacin** | Fluoroquinolone antibiotic | 5.3 | 6.3 | 11.6 |
| **Marbofloxacin** | | 2.6 | 4.9 | 7.5 |
| **Enrofloxacin** | | 4.8 | 3.2 | 8.0 |
| **Sarafloxacin** | | 1.8 | 24.5 | 26.3 |

| Analyte | CHEMICAL CLASS | Mean REC in AcOEt | Mean REC in MeOH | Total rec of sequential elution |
|---|---|---|---|---|
| **Roxitrhomycin** | Macrolide | | 1.9 | 1.9 |
| **Erythromycin** | | | 1.8 | 1.8 |
| **Clarythromycin** | | | | 0.0 |
| **Diazinon** | Organophosphate insecticide | 130.1 | 3.9 | 134.1 |
| **Monensin** | Polyether antibiotic | 67.2 | 6.9 | 74.1 |
| **Oxolinic acid** | Quinolone antibiotic | 138.4 | 4.9 | 143.3 |
| **Sulfadiazine** | Sulfonamide antibiotic | 89.6 | 2.2 | 91.8 |
| **Sulfathiazole** | | 3.9 | 53.1 | 57.0 |
| **Sulfamethoxazole** | | 106.3 | 2.5 | 108.8 |
| **Sulfamethazine** | | 132.0 | 1.8 | 133.8 |
| **Sulfadimethoxine** | | 84.3 | 6.3 | 90.6 |
| **Oxytetracyclin** | Tetracycline antibiotic | 65.3 | 11.1 | 76.3 |
| **Chlorotetracyclin** | | 52.2 | 69.6 | 121.7 |
| **Toltrazuril** | Triazintrione | 26.7 | 1.2 | 27.9 |
| **Simazine** | Triazinic herbicide | 94.2 | 3.1 | 97.3 |
| **Trimethoprim** | Synthetic derivative of trimethoxybenzyl-pyrimidine with antibacterial and antiprotozoal properties | 65.7 | 29.9 | 95.6 |

No recovery resulted for albendazole and levamisole and further improvements are needed.

Low recoveries were obtained for fluoroquinolone and macrolide antibiotic residues. This finding did not surprise considering that the pH of spiked samples was not adjusted to acidic.

### *3.4.2.2 Extraction reproducibility in real matrix*

Results of extraction reproducibility in real matrix are summarised in Table 19. Digestate samples were spiked at 10 µg/l.

The experiment consisted in the triplicate extraction of spiked digestate sample, opportunely diluted to 100 ml with MilliQ water, according to the procedure described in 3.2.

**Table 19 - Results of extraction reproducibility in real matrix**

| Analyte | CHEMICAL CLASS | Mean Conc (ng/l) | REC % (vs nominal conc.) | Extraction Reproducibility % (n=3) |
|---|---|---|---|---|
| **Miconazole** | Imidazole antifungal drug | 1872.2 | 18.7 | 22.5 |
| **Climbazole** | | 11416.7 | 114.2 | 14.9 |
| **Ketoconazole** | | 4704.6 | 42.8 | 19.4 |
| **Fluconazole** | | 5656.5 | 56.6 | 9.9 |

| Analyte | CHEMICAL CLASS | Mean Conc (ng/l) | REC % (vs nominal conc.) | Extraction Reproducibility % (n=3) |
|---|---|---|---|---|
| **Albendazole** | Benzimidazole dewarmer | na | na | na |
| **Levamisole** | Imidazole* dewarmer | 4 049.0 | 40.5 | 4.1 |
| **Bronopol** | 2-bromo-2-nitro-propanediol antimicrobial agent | na | na | na |
| **Amoxicillin** | b-lactam | na | na | na |
| **Florfenicol** | Fluoroacetamide antibiotic | 8 244.7 | 82.4 | 3.6 |
| **Ofloxacin** | Fluoroquinolone antibiotic | 3 051.6 | 30.5 | 41.7 |
| **Marbofloxacin** | | 2 514.5 | 21.0 | 64.3 |
| **Enrofloxacin** | | 3 292.7 | 27.4 | 35.9 |
| **Sarafloxacin 1** | | 2 050.6 | 20.5 | 14.5 |
| **Roxithromycin** | Macrolide | 632.9 | 6.3 | 40.5 |
| **Erythromycin** | | 7 807.6 | 73.7 | 11.7 |
| **Clarithrimycin** | | 707.7 | 3.5 | 69 |
| **Diazinon** | Organophosphate insecticide | 16 098.3 | 123.8 | 2.7 |
| **Monensin** | Polyether antibiotic | 7 371.8 | 73.7 | 66.3 |
| **Oxolinic acid** | Quinolone antibiotic | 21 311.1 | 236.8 | 4.4 |
| **Sulfadiazine** | Sulfonamide antibiotic | 1 179.0 | 11.8 | 14.8 |
| **Sulfathiazole** | | 2 738.4 | 21.1 | 22 |
| **Sulfamethoxazole** | | 7 954.2 | 38.5 | 16.5 |
| **Sulfamethazine** | | 3 941.7 | 19.2 | 2.1 |
| **Sulfadimethoxine** | | 2 893.3 | 26.3 | 47.3 |
| **Oxytetracycline** | Tetracycline antibiotic | na | na | na |
| **Chlorotetracycline** | | na | na | na |
| **Toltrazuril** | Triazintrione | 16 597.9 | 166.0 | 9.8 |
| **Simazine** | Triazinic herbicide | 5 275.3 | 48.4 | 18.3 |
| **Trimethoprim** | Synthetic derivative of trimethoxybenzyl-pyrimidine with antibacterial and antiprotozoal properties | 6 519.0 | 74.8 | 24.6 |

### *3.4.2.3 Results of processed manure analysis*

Results of positive finding in the analysis of processed manure are reported in Table 19.

**Table 20 - Results of processed manure analysis**

| Processed manure (Digestate) | | Mean Conc (ng/l) | St. Dev. | CV% |
|---|---|---|---|---|
| Analyte | CHEMICAL CLASS | | | |
| Miconazole | Imidazole antifungal drug | 109.03 | 36.2 | 33.2 |
| Climbazole | | 13.25 | 6.4 | 48.6 |
| Fluconazole | | 16.07 | 2.0 | 12.5 |
| Diazinon | Organophosphate insecticide | 93.29 | 13.2 | 14.1 |
| Monensin | Polyether antibiotic | 785.15 | 210.9 | 26.9 |
| Quinoxyfen | Fungicide (Dichloro - fluorophenoxyquinoline) | 634.4 | 79.9 | 12.6 |

Test results obtained by the commercial laboratory are summarised in Table 21. Original certificates of analyses are available upon request.

**Table 21 – Results of inorganic characterisation of two independent digestate samples**

| Parameter | Results (Sample 1 and 2) | Unit | Reported uncertainty in rel.% |
|---|---|---|---|
| **Dry matter content at 105°C** | 2.1<br>2.1 | Wgt.% | 12 |
| **Loss on ignition 600°C** | 0.6<br>0.7 | Wgt.% | 8 |
| **Total organic carbon** | 5 834<br>5 963 | mg/l | 12.5 |
| **Cr VI** | <0.5<br><0.5 | mg/l | 20 |
| **Cd** | <0.04<br><0.03 | mg/l | 11 |
| **Ni** | <0.04<br><0.03 | mg/l | 15 |
| **Pb** | 0.12<br>0.06 | mg/l | 17 |
| **Cu** | 0.50<br>0.44 | mg/l | 14 |
| **Zn** | 3.40<br>2.88 | mg/l | 17 |
| **Hg** | <0.01<br><0.01 | mg/l | 19 |
| **K** | 17.1<br>15.8 | mg/l | 16 |
| **$N_{Kjeldahl}$** | 392<br>297 | mg/l | 15 |
| **P** | 119<br>165 | mg/l | 13.5 |

*Sample 1 – Identifier: 0062_MA_17072_00_00 MANURE 2 SLOVAKIA*

*Sample 2 – Identifier: 0062_MA_17073_00_00 MANURE 3 SLOVAKIA*

# 4 Preliminary conclusions and next steps

The primary objective of this first exploration was the development of a realistic sampling scenario in view of a larger EU-wide campaign. Indeed, the testing site operated by the Agricultural University of Nitra (AUN), its availability in terms of geographical position and infrastructures demonstrated to be a suitable example of an experimental site for future official EU-wide manure monitoring campaign.

In addition, availability of information on manure application techniques, weather data, farming and livestock were shared timely. This constitutes an essential element in streamlining subsequent data interpretation and the planning of further experimental activities.

Furthermore, the testing site granted access to surface ditches and groundwater wells for water sample collection. These aspects could not be fully exploited in this first reporting.

First operative findings and field test proved the full applicability of the MARIANI-Box even under extreme field and sampling conditions. It could indeed be shown that it is possible to collect additional information on manure properties, the agricultural context and the receiving water bodies combining traditional sampling with the SPE features of the box. To which extent information on anti-microbial resistances can be obtained is still to be discovered while analytical determinations are still ongoing. It can be anticipated, that the handling of large scale sampling volumes of several litres will be challenging, if not impossible in such a campaign. A "microbiological" adaptation of the Mariani-Box, which would allow for similar, microbiological imprinting or field-based extraction is an idea to be explored further.

In addition, it was attempted to answer the question whether a (semi)quantitative relationship between the veterinary medicinal application and the occurrence of the targeted compounds can be established. First preliminary result, indicate indeed a very close relationship between active pharmaceutical ingredients (API) applied to specific and individual animals and, on the other side, a very significant concentration in the applied manure. In order to establish quantitative estimates, e.g. defining percentages of applied APIs, which finally reach (aquatic) ecosystems, the analytical methods needs to be refined further and eventually validated according to the requirements of ISO 17025.

The third aspect of this first exploration was to lay down a basis for the development of an analytical approach for a multi-compound method possibly addressing also a non-target approach. The analytical procedures reported in the present report demonstrate to be a consistent starting point in terms of

- Suitability of chosen polymeric phase for analytes' extraction and quantification.
- Reproducibility both in the analysis of non-treated and treated manure samples.

Further procedural improvements are obviously needed but these preliminary analytical evidences do constitute a considerable basis for future analytical method validations.

An unexpected side aspect was the opportunity to retrieve also analytical information contributing to better evaluate the agronomic value and fertilizing characteristics of the (processed) manure. Provided that a sufficiently good data density covering the major techniques listed in the introduction part of the paper can be obtained, the envisaged EU-wide campaign will retrieve a unique data set answering this question.

Part 2 of this report will address analytical aspects of the water samples. An instruction manual for the box in an EU-wide campaign is shown in the annex of this report. A call for participation will be published in the forthcoming weeks.

The exploratory investigations undertaken in this first campaign proofed an EU-wide exercise on VMPs in processed manure and exposed waters viable and feasible.

# References


BIO Intelligence Service (2013), *Study on the environmental risks of medicinal products*, Final Report prepared for Executive Agency for Health and Consumers. Last access 08/01/2018 at https://ec.europa.eu/health//sites/health/files/files/environment/study_environment.pdf

Boxall, A. (2012), New and Emerging Water Pollutants arising from Agriculture, OECD, Paris, 2012, 49 pages. (accessible online. Last access 05/01/2017 at: https://www.oecd.org/tad/sustainable-agriculture/49848768.pdf et al.

Diaz-Sanchez, S., Moscoso, S., Solís de los Santos, F., Andino, A., Hanning, I. (2015). Antibiotic Use in Poultry: A Driving Force for Organic Poultry Production. Food Protection Trends, Vol 35, No. 6, p. 440–447.

EMA (2010) Trends in the sales of veterinary antimicrobial agents in nine European countries: Reporting period: 2005- 2009. Last access 08/01/2018 at: www.ema.europa.eu/docs/en_GB/document_library/Report/2011/09/WC500112309.pdf

EMA (2014). European Surveillance of Veterinary Antimicrobial Consumption, 2016. '*Sales of veterinary antimicrobial agents in 29 European countries in 2014*'. (EMA/61769/2016). Last access 08/01/2018 at: http://www.ema.europa.eu/docs/en_GB/document_library/Report/2016/10/WC500214217.pdf

Flotats, X., Foged, H. L., Bonmati Blasi, A., Palatsi, J., Magri, A., Schelde, K. M. (2011). *Manure processing technologies*. Technical Report No. II concerning "Manure Processing Activities in Europe" to the European Commission, Directorate-General Environment. 184 pp.

Foged, H. L., Flotats, X., Bonmati Blasi, A., Palatsi, J., Magri, A., Schelde, K. M. (2011a). *Inventory of manure processing activities in Europe.* Technical Report No. I concerning "Manure Processing Activities in Europe" to the European Commission, Directorate-General Environment. 138 pp.

Foged, H. L., Flotats, X., Bonmati Blasi, A., Schelde, K. M., Palatsi, J., Magri, A., Juznik, Z. (2011b). *Assessment of economic feasibility and environmental performance of manure processing technologies.* Technical Report No. IV to the European Commission, Directorate-General Environment. Unpublished draft. 130 pp.

Gawlik, B.M., Loos, R., Bidoglio, G., Fauler, G., Guo, X., Lankmayr, E., Linsinger Th. (2012). Testing sample stabiklity in short-term isochronous stability studies for EU-wide monitoring surveys of polar organic contaminants in water. Trends in Analytical Chemistry, 36, 36-46.

Głowacka, N., Gaduš, J., Slobodnik, J. (2017). Biomass of microalgae as a source of renewable energy. Acta Regionalia et Environmentalica 1, 24-29.

Jarosova, B., Ersekova, Hilscherova, K., Loos, R., Gawlik, B. M., Giesy, J. P., Blaha, L. (2014). Europe-wide survey of estrogenicity in wastewater treatment plant effluents: the need for the effect-based monitoring. Environ. Sci. Pollut. Res. 21, 10970-10982.

Kant, V., Singh, P., Verma, P.K., Bais, I., Parmar, M. S., Gopal, A., Gupta V. (2013). Anticoccidial Drugs Used in the Poultry: An Overview. Science International, 1: 261-265.

Kools, S.A.E., Moltmann, J.F., Knacker, T. (2008) Estimating the use of veterinary pharmaceuticals in the European Union. Regulatory Toxicology and Pharmacology, 50, 59-65.

Loos, R., Gawlik, B. M., Locoro, G., Rimaviciute, E., Contini, S., Bidoglio, G. (2009). EU-wide survey on polar organic persistent pollutants in European river waters. Environmental Pollution 157, 561-568.

Loos, R., Locoro, G., Comero, S., Contini, S., Schwesig, D., Werres, F., Balsaa, P., Gans, O., Weiss, S., Blaha, L., Bolchi, M., Gawlik, B. M. (2010). Pan-European Survey on the occurrence of selected polar organic persistent pollutants in groundwater. Water Research 44, 4115-4126.

Loos, R., Carvalho, R., Comero, S., Antonio, D., Ghiani, M., Lettieri, T., Locoro, G., Parachini, B., Tavazzi, S., Gawlik, B.M., Blaha, L., Jarosova, B., Voorspoels, S., Schwesig, D., Haglund, P., Fick, J., Gans, O. (2012). EU-wide monitoring survey on wastewater treatment plant effluents. Office for Offcial Publiocations, Luxembourg, EUR 25563 EN, doi:10.2788/60704.

Mariani, G., Tavazzi, S., Comero, S., Buttiglieri, G., Parachini, B., Skejø, H., Alcalde-Sanz, L., Gawlik, B. M. Short-term isochronous stability study of contaminants of emerging concern in environmental water samples. Office for Official Publications Luxembourg, EUR 25425 EN, doi:102760/488206.

Pistocchi, A., Sarigiannis, D. A., Vizcaino, P. (2010). Spatially explicit multimedia fate models for pollutants in Europe: State of the art and perspectives. Science of the Total Environment 408, 3817-3830.

Pistocchi, A., Marino, D., Pontes, S., Gawlik, B. M. (2012). Continental scale inverse modelling of common organic water contaminants in European rivers. Environmental Pollution 162, 159-167.

Tavazzi, S., Mariani, G., Skejø, H., Lobo-Vincente, J., Chassaigne H., Vandecasteele, I., Comero S., Gawlik, B. M. (2017). iDRIP-What goes down the drainpipes? – Illicit drugs, pollutants and other chemicals in recycled water and wastewater. Office for Official Publications, Luxembourg, EUR 28573 EN, doi:10.2760/70591.

Thanner, S., Drissner, D., Walsh, F. (2016). Antimicrobial Resistance in Agriculture, mBIO 7, 1-7.

Wohde, M., Berkner, S., Junker, T., Konradi, S., Schwarz, L., During, R. (2016). Occurrence and transformation of veterinary pharmaceuticals and biocides in manure: a literature review. Environmental Science Europe 28:33

## List of abbreviations and definitions

| | |
|---|---|
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| 2,4,5-T | 2,4,5-Trichlorophenoxyacetic acid |
| 4-t-OP | 4-Tert-octylphenol |
| AMA | anti-microbial agent |
| API | active pharmaceutical ingredient |
| CEC | compounds of emerging concern |
| DEET | N, N-Diethyl-meta-toluamide |
| D.M. | dry matter |
| E1 | Estrone |
| E2 | Estradiol |
| EE2 | Ethinylestradiol |
| EQS | Environmental Quality Standard |
| EC | European Commission |
| EMA | European Medicines Agency |
| EU | European Union |
| HBCD | Hexabromocyclododecane |
| I.S. | Internal Standard |
| ISO | International Standardization Organization |
| IUPAC | International Union for Pure and Applied Chemistry |
| JRC | Joint Research Centre |
| MCPA | 2-methyl-4-chlorophenoxyacetic acid |
| MeOH | methanol |
| ml | millilitre |
| MRM | multiple reaction monitoring |
| MS | mass spectrometer |
| µg | microgram |
| ND | Nitrates Directive |
| ng | nanogram |
| NVZ | Nitrates Vulnerable Zones |
| PFBA | Perfluorobutanoic acid |
| PFBS | nonafluoro-1-butanesulfonic acid |
| PFHxA | perfluorohexanoic acid |
| PFHxS | Perfluorohexane sulfonate |
| PFNA | Perfluorononanoic acid |
| PFOA | Perfluorooctanoic acid |
| PFOS | Perfluorooctanesulfonic acid |
| PP | polypropylene |
| PPG | polypropylene glycol |
| SMZ | sulfamethazine |
| VMP | veterinary medicinal product |

All country names are abbreviate using the ISO “Alpha 2 codes”

Chemical elements are expressed using IUPAC Nomenclature rules

## List of figures

Figure 1 - Entry pathways of CECs into the agricultural environment..... 5
Figure 2 – Overview on the transformation processes to be considered for CECs in agricultural soils (Modified from Boxall, 2012) ..... 6
Figure 3 – Components of an optimal processing of pig slurry (modified from EC 2010).. 7
Figure 4 – Overview of manure processing technologies..... 10
Figure 5 –Relevant entry pathways of VMP via manure into the environment ..... 11
Figure 6 - Satellite map of Oponice settlement and sampling sites..... 22
Figure 7 – Water storage tower at the site ..... 24
Figure 8 – Landscape at the sampling site..... 24
Figure 9 – Artificial lake at the Oponice site..... 24
Figure 10 – Drainage channel with water ..... 25
Figure 11 –Larger drainage channel ..... 25
Figure 12 – Storage for processed manure..... 25
Figure 13 –Experimental bio-gasification plant..... 26
Figure 14 – Cow stables at the research facility ..... 26
Figure 15 - Extract of the logbook documenting application of VMPs at the research station..... 27
Figure 16 – Manure deposit at the Oponice Plant..... 31
Figure 17 – Sampling of surface water samples adjacent to field site ..... 31
Figure 18 – Sampling at groundwater well ..... 31
Figure 19 – Soil sampling ..... 32

## List of tables

Table 1 – Overview on technologies used for livestock manure treatment in the Member States (data from Foged *et al.*, 2011a) ..... 9

Table 2 – Overview on selected active pharmaceutical ingredients, compounds and veterinary medicinal products used in poultry, swine, dairy and sheep industries ..... 14

Table 3 – Examples of selected active pharmaceutical ingredients, compounds and veterinary medicinal products used for Minor Species ..... 16

Table 4 – Examples of selected active pharmaceutical ingredients, compounds and veterinary medicinal products used in aquaculture ..... 17

Table 5 - Holdings with manure storage facilities, EU-27 and NO ..... 18

Table 6 – Information about agricultural parcels used for sampling. For geographical position of the single parcels (Numbered 1 to 7) refer to Figure 6 ..... 22

Table 7 – List of likely active pharmaceutical ingredients (APIs) in the cattle manure and receiving waters ..... 27

Table 8 - Weather conditions on day of sampling ..... 30

Table 9 –List of samples taken during the campaign ..... 32

Table 10 – Initial selection of study compounds to start the analytical method development for a multi-matrix method combining a targeted and non-targeted analytical approach ..... 35

Table 11 - Solid-phase extraction protocol applied ..... 36

Table 12 - UHPLC experimental conditions ..... 37

Table 13 - UHPLC gradient scheme ..... 37

Table 14 - General operating conditions for QTRAP 5500 MS/MS ..... 38

Table 15 - MS-MS parameters ..... 39

Table 16 – Test standards used by commercial laboratory characterising the digestate samples ..... 46

Table 17 – Preliminary results of cattle urine analysis ..... 48

Table 18 - Recovery of selected analytes for MilliQ water spiked samples. ..... 48

Table 19 - Results of extraction reproducibility in real matrix ..... 49

Table 20 - Results of processed manure analysis ..... 51

Table 21 – Results of inorganic characterisation of two independent digestate samples 51

# Annexes

## Annex 1. MARIANI Box - Instructions for use

---

**INSTRUCTIONS**

**FOR USE**

*Dear Colleagues,*

*You received one parcel containing the Mariani Box sampling device and a cooling bag with cooling bricks.*

*Inside the Mariani Box you can find:*

- *DVD containing instruction video,*
- *Instruction sheet (present document),*
- *2 sampling bill sheets*
- *3 filter holders containing activated disk for extraction (DO NOT OPEN)*
- *1 battery charger,*
- *2 x 3mt PTFE tubes*
- *Male-male Luer-Lock connector.*
- *Power plug adapter*

*We suggest freezing immediately the cooling bricks in order to have them frozen when necessary.*

*In order to avoid possible cross contamination between samples, we recommend proceeding first with extraction of less contaminated sample (i.e.: effluent), followed by the extraction of influent sample, which likely would exhibits higher level of concentration of contaminants.*

### Description of sampling device

*The Mariani box consists in the following parts:*

*1. Holder SPE disk*

*2. Flow meter*

*3. Counter*

*4. Pump (Flow rate 0.1-0.2 l/min)*

*5. Battery*

*6. Water sampling line*

*7. Waste line*

*Each holder contains HLB disk ready for sampling. Use one holder for each sample.*

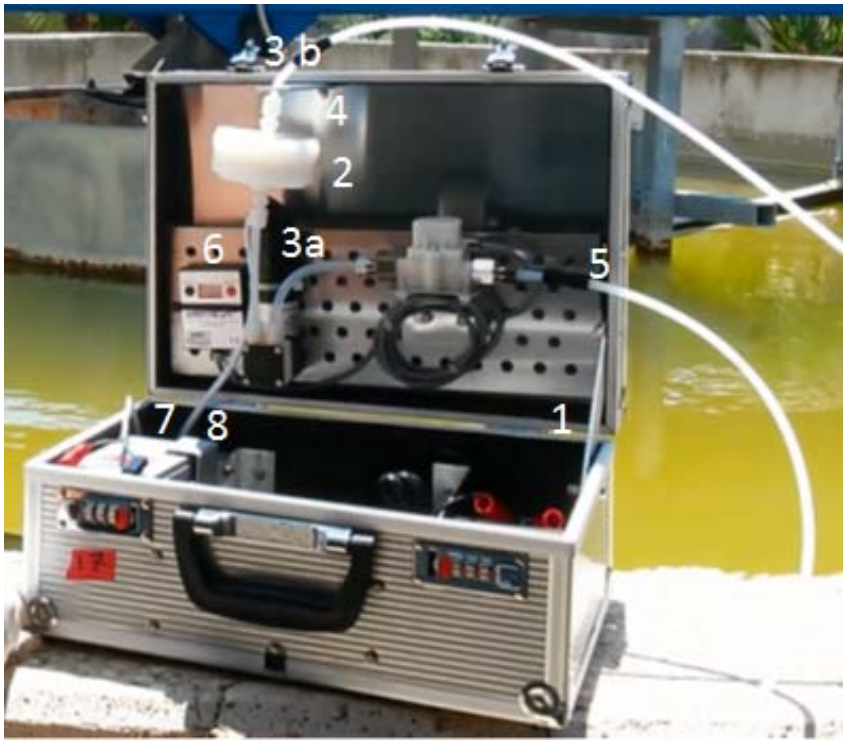
*Connect the holder to the pump according to sides' definitions (↑upper side; ↓bottom side).*

*Luer-Lock openings located on upper and bottom side of the holder will host PFTE tubes for sampling.*

*Please fill in the sampling bill with requested information before starting sampling and register site, date and time of sampling on the holder, too.*

## Operations

*From this section onwards, please refer to the more detailed picture reported below:*




*Once on site, open the box and blocks the opening by fixing the metal shaft (1).*

- *Connect the waste line: unscrew the Luer-Lock cap from the bottom side of the holder and connect it to position 3a, screwing gently; remove Luer-Lock caps from the upper side of the holder and insert the 3 mt PTFE tube. Tighten screwing gently both connections (respectively 3a and 3b) and fix the waste line (3b) with the metal shaft (4).*
- *Connect the sampling line: screw the second 3 mt PTFE tube into the Luer-Lock connection placed in 5.*
- *Register the starting reading of the counter (6);*
- *Connect the battery cable (red positive and black negative (7))*
- *Turn on the device pushing the red switch (8).*

*At this point the system start to pump the water sample from the sampling point (influent/effluent).*

*The water will pass through the sampling line (5), the flow meter, the pump and finally in the holder, where HLB disk is located.*

*When 0.5 l volume is sampled (read the sample volume on the counter (6)), keep out of water the water sampling line (5) and wait for all water in the tubes and in holder being expelled.*

- *Turn off the device when the waste line is completely empty (8).*
- *Register the final reading of the counter (6);*

*Holder for blank sample, must be on site during sampling, without any processing.*

*After extraction, the holder must be removed from the sampling device and the Luer-Lock connections must be closed with their original caps. Samples must be stored at 4-6°C and in cooling bag with frozen cooling bricks.*

*At the end of sampling, please coil the PTFE tubes and put together all spare parts in their dedicated spaces inside the box.*

## Cleaning

*After each sampling, cleaning PTFE tubes and pump will avoid cross-contamination.*

*The same cleaning procedure must be performed after last sample extraction.*

*Using the male-male Luer-Lock connection provided, placed in place of the holder, pass through the system about 1 liter of clean water (i.e.: distilled, MilliQ water, etc.). Please use a clean container for the water.*

*ONLY if distilled and or MilliQ water were not available use tap water.*

## Sample storage

*Environmental water samples extracted on SPE disks can be stored at in the fridge at 4°C until pick-up by JRC service.*

**GETTING IN TOUCH WITH THE EU**

**In person**

All over the European Union there are hundreds of Europe Direct information centres. You can find the address of the centre nearest you at: http://europea.eu/contact

**On the phone or by email**

Europe Direct is a service that answers your questions about the European Union. You can contact this service:

- by freephone: 00 800 6 7 8 9 10 11 (certain operators may charge for these calls),

- at the following standard number: +32 22999696, or

- by electronic mail via: http://europa.eu/contact

**FINDING INFORMATION ABOUT THE EU**

**Online**

Information about the European Union in all the official languages of the EU is available on the Europa website at: http://europa.eu

**EU publications**
You can download or order free and priced EU publications from EU Bookshop at: http://bookshop.europa.eu. Multiple copies of free publications may be obtained by contacting Europe Direct or your local information centre (see http://europa.eu/contact).

KJ-NA-29065-EN-N

## JRC Mission

As the science and knowledge service of the European Commission, the Joint Research Centre's mission is to support EU policies with independent evidence throughout the whole policy cycle.

**EU Science Hub**
ec.europa.eu/jrc

@EU_ScienceHub

EU Science Hub - Joint Research Centre

Joint Research Centre

EU Science Hub

Publications Office

doi:10.2760/835417

ISBN 978-92-79-77754-7